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Quantitative studies of the binding of wheat germ agglutinin (WGA) to chitin-oligosaccharides and partially *N*-acetylated chitosans suggest inequivalence of binding sites

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Abstract

We have investigated the interactions between wheat germ agglutinin (WGA) and highly de-*N*-acetylated chitosans of low molecular weight (prepared by nitrous acid degradation and subsequent reduction) by ^{1}H and ^{19}F NMR spectroscopy. Three isolectins were separated from an unfractionated WGA preparation. No significant differences among isolectins were found with respect to binding properties towards GlcNAc or towards chitosans. We observed a specific interaction between WGA and *N*-acetylated sugar residues in highly de-*N*-acetylated chitosans of low molecular weight, as observed by line-broadening of the *N*-acetyl ^{1}H NMR resonance. The interaction was not inhibited by fully de-*N*-acetylated chitosan of low molecular weight, although evidence of weak interaction between WGA and de-*N*-acetylated residues was found. We estimated a dissociation constant, K_D, of 0.15–0.20 mM for the complex between WGA and a single *N*-acetylated residue in the chitosan chain (i.e. an *N*-acetylated residue surrounded primarily by de-*N*-acetylated residues). Competitive binding experiments with tri-*N*-fluoroacetylglucosamine (F_3-GlcNAc) were performed by ^{19}F NMR spectroscopy. Surprisingly, the *N*-acetylated residues of highly de-*N*-acetylated chitosans were much less effective competitors for binding to WGA than expected from K_D-values, while GlcNAc and $(GlcNAc)_3$ showed the expected competition with F_3-GlcNAc for binding to WGA. These data suggest that internal *N*-acetylated sugar residues in chitosans bind primarily to other sites of WGA than chitin-oligosaccharides. Support of this suggestion is found in recent reports on the structure of WGA. © 1999 Elsevier Science Ltd. All rights reserved

Keywords: Chitin; Chitosan; Wheat germ agglutinin (WGA); Binding studies; ^{1}H NMR spectroscopy; ^{19}F NMR spectroscopy

1. Introduction

Chitosans are soluble, linear, binary polysaccharides which contain 2-acetamido-2-deoxy-β-D-glucose (GlcNAc; **A**-unit) and 2-amino-2-deoxy-β-D-glucose (GlcN; **D**-unit) residues in various proportions, connected through (1 → 4) glycosidic linkages. Chitosans can be prepared commercially by de-*N*-acetylation of chitin, which is an abundant and renewable resource mainly found in the exoskeleton of crustaceans and insects. Chitin and chitosans are also common constituents of fungal cell walls, and they have been shown to elicit active defence reactions in higher plants (for review, see Hahn (1996) and Refs. therein). Although the molecular basis of such responses has not yet been fully elucidated, it has been suggested that plant lectins play an important role through recognition of foreign carbohydrate structures (Peumans and van Damme, 1995). Wheat germ agglutinin (WGA) is a plant lectin which role in nature is assumed to be linked to its specificity for GlcNAc and GlcNAc-containing oligosaccharides, although this has not been conclusively demonstrated (Raikhel et al., 1993).

Wheat germ agglutinin is a well-characterised lectin (for review, see Goldstein and Poretz (1986) and Refs. therein) in a highly conserved family of chitin-binding lectins from cereals (*Gramineae*), sources including rye, barley, rice and wheat (Raikhel et al., 1993). WGA can be isolated as a mixture of three molecular species (isolectins) which differ in their amino acid compositions (Wright and Raikhel, 1989), electrophoretic mobility (Allen et al., 1973; Rice and Etzler, 1975) and affinity for some NeuNAc-containing oligosaccharides (Kronis and Carver, 1982). All three WGA isolectins consist of two identical 171-residue polypeptides (protomers) that associate to form active dimers of 36 kDa above pH 4 (Monsigny et al., 1979). Their overall dimer structure and subunit interactions are very similar (Wright

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and Olafsdottir, 1986; Wright and Raikhel, 1989; Harata et al., 1995). The WGA polypeptide structure is composed of four homologous domains of 42–43 amino acids (Wright, 1980; Wright and Raikhel, 1989; Wright, 1984), where each domain possesses sugar-binding capability. Thus, potentially there are eight binding sites per dimeric WGA (Wright et al., 1991). Additional binding strength comes from the co-operative interaction between opposing domains from different protomers in the active dimeric lectin. However, previous crystallographic studies on several different oligosaccharide complexes (Wright, 1984, 1990) as well as solution binding studies (Nagata and Burger, 1974; Privat et al., 1974b; Kronis and Carver, 1985) have suggested four binding sites per dimer. On the other hand, recent experimental data on the X-ray structure of WGA crosslinked with a bivalent sialoglycopeptide fragment demonstrated binding to sites of WGA that was not observed previously with oligosaccharides (Wright, 1992). Clearly, the sugar binding properties of WGA might be more complex than inferred from earlier studies with simple oligosaccharides.

The binding properties of WGA towards partially *N*-acetylated chitosans have not previously been studied quantitatively, although it has been shown that WGA interacts with internal GlcNAc residues in polysaccharides (Goldstein et al., 1975; Lotan et al., 1975). Senstad and Mattiasson (1988) used a partially *N*-acetylated chitosan in an affinity precipitation scheme for purification of WGA. However, all these studies were based on observation of precipitation of polyvalent ligands, and quantitative data were not obtained.

We have used one-dimensional ^{1}H and ^{19}F NMR spectroscopy to study quantitatively the interaction between WGA and highly de-*N*-acetylated chitosans of low molecular weight, which were prepared by nitrous acid degradation of high-molecular weight chitosans. As it has previously been shown that the sequential arrangement of **A**-units and **D**-units in water-soluble, partially *N*-acetylated chitosans are governed by Bernoullian statistics (Vårum et al., 1991a, b), **A**-units in highly de-*N*-acetylated chitosans may be considered as single, i.e. they occur predominantly with **D**-units as nearest neighbours. We report that single **A**-units in highly de-*N*-acetylated chitosans bind specifically to WGA, and that the binding is not inhibited by fully de-*N*-acetylated chitosan. However, data from competitive binding studies with a fluorlabelled monosaccharide ligand (tri-*N*-fluoroacetylglucosamine) were not consistent with K_D-values obtained by direct binding studies, suggesting that highly de-*N*-acetylated chitosans primarily do not bind to the same sites on WGA as simple chitin-oligosaccharides do.

2. Experimental

2.1. Materials

Wheat germ agglutinin was purchased from Sigma (USA, Lot no. 56H4054). GlcN (Koch Laboratories), GlcNAc (Sigma, Lot no. 41H0515), $(GlcN)_3$ and $(GlcNAc)_3$ (Seikagaku Corporation, Lot nos. 9409220 and 9508040, respectively) were purchased. Chitosans were prepared by homogeneous de-*N*-acetylation of chitin (Sannan et al., 1976) and were depolymerised with nitrous acid and subsequently reduced with $NaBH_4$ as previously described (Vårum et al., 1994). Three different de-*N*-acetylated chitosans were prepared with fractions of *N*-acetylated groups (F_A) of 0.00 (<0.001), 0.04 and 0.13, with number average degrees of polymerisation, $\overline{DP_n}$, of 17–20. Such low-molecular weight chitosans were used to reduce viscosity of the solutions yielding narrower NMR lines. F_A values were determined by ^{1}H NMR spectroscopy (Vårum et al., 1991a). The $\overline{DP_n}$ was determined by estimating the fractions of non-reducing ends based on ^{13}C NMR assignments reported by Domard and Cartier (1989). ^{13}C NMR spectra were also used to confirm the random distribution of **A**- and **D**-units along the chitosan chain, according to Vårum et al. (1991b). For example, in the ^{13}C NMR spectrum of the chitosan with $F_A = 0.04$, no signals representative of the diade –**AA**– could be found, i.e. the large majority of **A**-units occur with **D**-units as nearest neighbours (data not shown).

2.2. Separation of isolectins

Separation of isolectins from the commercial WGA was performed by ion-exchange chromatography as described by Rice and Etzler (1975), with minor modifications. We used a prepacked 1-ml cation-exchange column from Pharmacia (HiTrap-SP, Code no. 17-1151-01). The column was equilibrated with 0.1 M sodium acetate (NaAc) buffer in 0.02 M NaCl (pH 3.8). Twenty mg WGA were applied to the column in 20 ml of 0.1 M NaAc buffer in 0.02 M NaCl. The column was then eluted with the same buffer at a flow rate of 1 ml/min until the baseline of the UV-detector (absorbance at 295 nm) was reached (elution volume of 40 ml). A linear gradient from 0.02 to 0.05 M NaCl in 0.1 M NaAc (30 ml) was then applied to the column, followed by elution with 0.05 M NaCl/0.1 M NaAc. The fractionated WGA was collected (WGA I, II and III) and dialysed against water at pH > 5.5, and lyophilised.

2.3. NMR procedures

WGA samples were prepared by dissolving 0.05–0.1 mM of the lectin in D_2O containing 0.1 M NaCl, followed by stirring for 2 h and filtration through 0.45 μm low protein-binding filter (Millipore Millex-HV Lot. H2NM97519). The exact concentration of WGA samples was determined by measuring the absorbance at 277 nm and using the reported extinction coefficients of 1.70 and 1.60 cm^2/mg for WGA I and WGA II, respectively (Rice and Etzler, 1974). For WGA III and the commercial WGA mixture we used extinction coefficients of 1.60 and 1.65 cm^2/mg, respectively. All experiments were performed in 0.1 M NaCl, since an ionic strength of at least 0.05 M was necessary to obtain maximum interaction between the chitosans investigated and WGA, which are both positively charged at the conditions studied (WGA has pI~8.5 (Rice and Etzler, 1975), while the pK_a value of the amino group on chitosans is ~6.5 (Anthonsen and Smidsrød, 1995)). Uncorrected readings from the pH-meter in D_2O solutions are reported as pH*. Binding experiments between WGA and chitosans were performed at pH* 5.2–5.3, where the amino groups in the chitosan are predominantly charged and WGA occurs predominantly as the active, dimeric form. The temperature was chosen to optimise the changes that were quantified; binding studies with oligosaccharides were studied at 308 and 323 K, while binding studies with chitosan preparations were performed at 323 K. Oligosaccharides or chitosans were added stepwise to the WGA solution and NMR data were acquired at each step for determination of the dissociation constant, K_D. pH* varied less than 0.05 units from the mean value in a single titration experiment.

^{1}H NMR spectra were acquired at 300.13 or 400.13 MHz with continuous wave saturation of the residual water resonance during relaxation delay. Eight to 1000 scans were acquired, according to signal/noise requirements. Chemical shifts were measured relative to TSP (3-(trimethylsilyl)-propionate) with a chemical shift of −0.01 ppm at pH* 5.3 and ionic strength 0.1 M (Wishart et al., 1995). ^{19}F NMR spectra were acquired at 376.47 MHz using 8–200 scans according to signal/noise requirements. ^{19}F chemical shifts were externally referenced against trifluortoluene ($C_6H_5CF_3$) at −63.7 ppm (Bovey, 1988).

2.4. Data analysis

In the ^{1}H NMR experiments we observed small upfield shifts and changes in linewidths of the *N*-acetyl signal of GlcNAc and of the **A**-units in the low-acetylated chitosans upon binding to WGA. The magnitude of the shift changes were too small for accurate determination of K_D-values, and the changes in the linewidths of the *N*-acetyl resonance were used instead. The data analysis followed the procedures outlined by Dahlquist and Raftery (1968) for a similar system (binding of GlcNAc to lysozyme), and also used previously in the analysis of WGA binding to oligosaccharide ligands (Jordan et al., 1977; Kronis and Carver, 1982). We assumed a microscopic dissociation constant, K_D, defined by

$$K_D = \frac{(nP_0 - [PL])(L_0 - [PL])}{[PL]} \quad (1)$$

where n is the number of binding sites per WGA monomer, and it is assumed that binding sites are independent and identical. P_0 is the total concentration of WGA (molar concentration of polypeptide chains) and L_0 is the total ligand concentration. Assuming that the induced line-broadening, $\delta\nu$, is proportional to the bound fraction of ligands ($[PL]/L_0$), Eq. (2) is obtained, which is valid when the bound fraction of ligand is low:

$$L_0 = P_0 n \frac{\Delta\nu}{\delta\nu} - (K_D + nP_0) \quad (2)$$

where $\Delta\nu$ is the limiting line-broadening, obtained by extrapolation to $[PL]/L_0 = 1$. By plotting ligand concentration against $1/\delta\nu$, $n(\Delta\nu)$ and $(K_D + nP_0)$ can be estimated by linear regression. The value of n is not known for the chitosan ligands; however, different probable values of n ($n = 1$ or 2) did not significantly alter estimates of K_D-values. Similar equations are easily derived when chemical shift changes are monitored instead of line-broadening. The bound fraction of ligand-binding sites on WGA can be determined without prior knowledge of n according to the equation:

$$\frac{[PL]}{nP_0} = \frac{L_0}{P_0} \frac{\delta\nu}{n(\Delta\nu)} \quad (3)$$

since $n(\Delta\nu)$ is determined from the fit to Eq. (2).

3. Results

3.1. Separation and characterisation of WGA isolectins

We separated three distinct isoforms of WGA from the commercial preparation by ion-exchange chromatography. The isolectins were numbered WGA I, WGA II and WGA III by their elution order in accordance with the nomenclature used by Allen et al. (1973) and Rice and Etzler (1975). The protein recovery was nearly 100%, and the relative amounts of the three isoforms were approximately 50% WGA I, 30% WGA II and 20% WGA III.

^{1}H NMR spectra of the isolectins were distinct but similar, with most similarities shared by WGA II and WGA III (data not shown), consistent with the reported amino acid sequence (Wright and Raikhel, 1989). The most notable difference between the isolectins is that WGA II and WGA III contain two histidine residues per monomer while WGA I has none (Rice and Etzler, 1975). In spectra obtained at pH* 2.8 we observed two sharp signals at 8.7 and 8.75 ppm in WGA II and WGA III that were not present in WGA I (data not shown), which might be assigned to He protons of histidines on the basis of their chemical shifts and linewidths (Wüthrich, 1986), as well as previous reports (Kronis and Carver, 1982). At 400 MHz and 308 K we also observed two slowly exchanging signals in WGA II and WGA III at 6.4 and 6.65 ppm, the former dominating at low pH* and the latter at higher pH* (data not shown). When observing the intensities of these resonances upon addition of acid, we obtained a pK_a* value for the dimerisation of WGA II/III of 3.7–3.8 (data not shown), in agreement with previous reports (Monsigny et al., 1979).

3.2. Interactions of mono- and oligosaccharides with isolectins and unfractionated WGA

Binding of GlcNAc to WGA has been studied previously

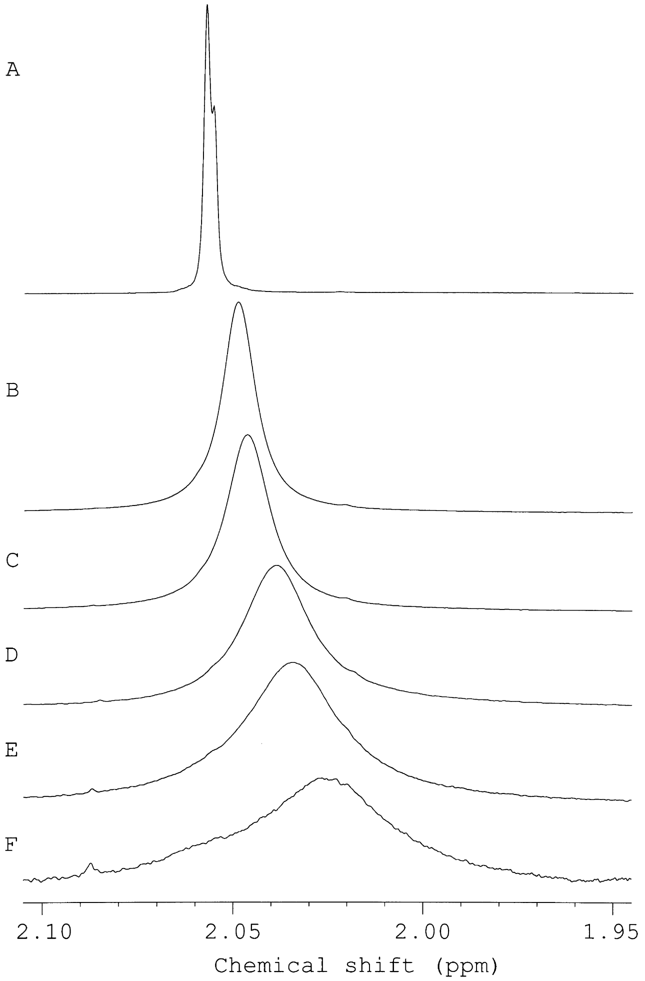

Fig. 1. 400 MHz ^{1}H NMR spectra of the *N*-acetyl region of GlcNAc in the absence (A) and presence (B–F) of 0.09 mM WGA I at pH* 5.3, 308 K and ionic strength 0.1 M; (B) 15.1 mM GlcNAc; (C) 10.1 mM GlcNAc; (D) 5.6 mM GlcNAc; (E) 3.1 mM GlcNAc; (F) 1.2 mM GlcNAc.

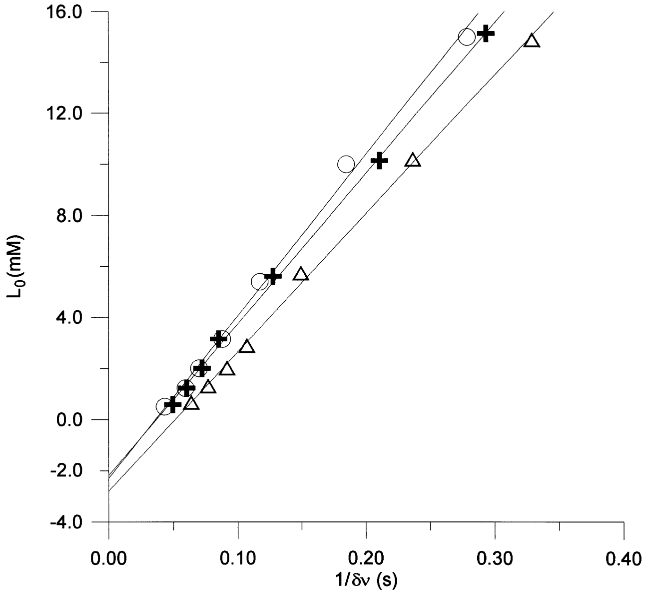

Fig. 2. Binding of GlcNAc to isolectins of WGA. Plots of GlcNAc (L_0) vs 1/$\delta\nu$ are shown for binding to WGA I (+), WGA II (○) and WGA III (△).

by ^{1}H NMR (Jordan et al., 1977, 1981) and other techniques (Nagata and Burger, 1974; Privat et al., 1974a; Bains et al., 1992), but the binding affinities of different isolectins towards GlcNAc have not been reported. We observed an upfield shift and a significant line-broadening of the *N*-acetyl resonance of GlcNAc in the presence of all isolectins of WGA at 308 K, as shown in Fig. 1 for WGA I. Dissociation constants, limiting linewidths (when all ligands are bound, $\Delta\nu$) and limiting shifts (when all ligands are bound, Δ) for the complexes formed between GlcNAc and the different isolectins (as well as the unfractionated WGA) were equal within error limits (Fig. 2 and Table 1). As α- and β-anomers were not observed separately, the values listed in Table 1 must be considered as an average state of the anomers. The K_D-value at 323 K was 1.5–2 times larger than at 308 K, i.e. weaker binding (data not shown). The bound fraction of GlcNAc molecules was 0.01–0.08 in all experiments performed, while the corresponding bound fraction of binding sites on WGA varied from 0.85 to 0.15, demonstrating the validity of approximations made in the data analysis.

Table 1
Summary of GlcNAc binding to unfractionated WGA (0.09 mM) and isolectins of WGA (0.072–0.076 mM), obtained at ionic strength 0.1 M, 308 K, pH* 5.3

	K_D (mM)	$\Delta\nu$ (Hz)[a]	Δ (ppm)[b]
Unfractionated WGA	2.2 ± 0.2	285	0.52
WGA I	2.0 ± 0.3	275	0.52
WGA II	2.1 ± 0.5	280	0.57
WGA III	2.6 ± 0.3	265	0.43

It is assumed that $n = 2$ (Nagata and Burger, 1974; Privat et al., 1974b).
[a] Limiting linewidth, the linewidth when all GlcNAc molecules are bound.
[b] Limiting shift, the chemical shift of the *N*-acetyl resonance when all GlcNAc molecules are bound.

No interaction was detected between GlcN and WGA, in accordance with previous reports (Allen et al., 1973; Goldstein et al., 1975). When (GlcN)$_3$ was added to a WGA solution of 0.09 mM, we observed very small chemical shift changes (<0.01 ppm) of specific ^{1}H signals (data not shown). However, no competition between (GlcN)$_3$ and GlcNAc for binding to WGA was detected, i.e. the binding of GlcNAc was not affected by addition of a five-times higher molar concentration of (GlcN)$_3$.

3.3. Interaction between fully de-N-acetylated chitosan ($F_A < 0.001$) and WGA

When fully de-*N*-acetylated chitosan ($F_A < 0.001$) of low molecular weight was added stepwise to 0.045 mM unfractionated WGA solution at pH* 5.3, we observed significant shifts (<0.04 ppm) of H1, H2 (Fig. 3) and H3 (data not shown) on the fully de-*N*-acetylated chitosan, while there was only insignificant changes on other protons. We did not

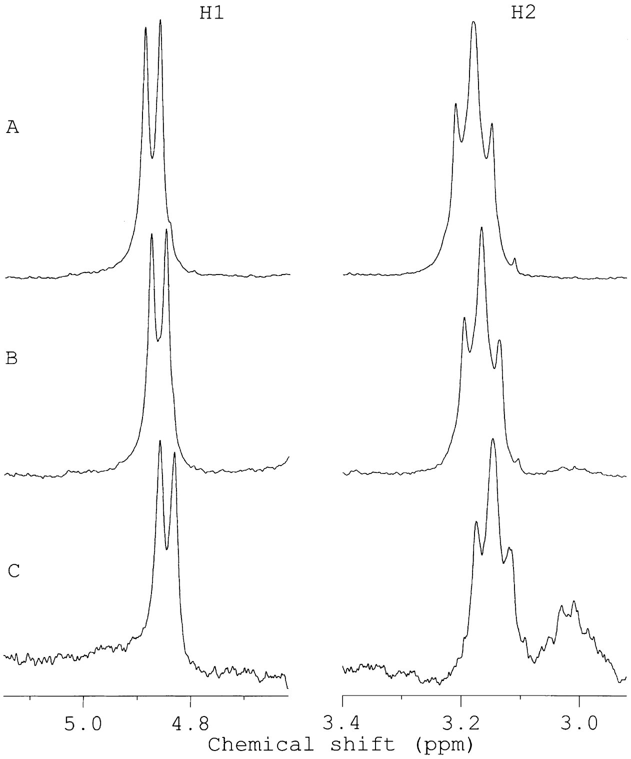

Fig. 3. 300 MHz ^{1}H NMR spectra of H1 and H2 of a fully de-*N*-acetylated chitosan ($\overline{\mathrm{DP}_\mathrm{n}}$ 17) in the absence (A) and presence of 0.09 mM WGA (B–C) at 323 K, ionic strength 0.1 M and pH* 5.3: (B) 9.4 mM **D**-units; (C) 1.24 mM **D**-units.

observe similar shift changes at pH* 3. The fully de-*N*-acetylated chitosan did not compete with GlcNAc for binding to WGA (data not shown). We were also unable to observe any competition with $(\mathrm{GlcNAc})_3$, which binds to all three subsites of the binding sites on WGA.

3.4. Binding of highly de-N-acetylated chitosans ($F_A = 0.04$ and $F_A = 0.13$) to isolectins of WGA and unfractionated WGA

The most notable effect of WGA (pH* 5.2, ionic strength 0.1 M, 323 K) on the chitosan with $F_\mathrm{A} = 0.04$ ($\overline{\mathrm{DP}_\mathrm{n}}$ 20) is a significant line-broadening of the *N*-acetyl resonance, as shown in Fig. 4 for WGA III, indicating a specific interaction between WGA and the *N*-acetyl group. We also observed shifts of H1, H2 and H3 protons on **D**-units that were similar in magnitude with or slightly larger than those observed with the fully de-*N*-acetylated chitosan (data not shown). At pH* 3 we did not observe any perturbation of the chitosan spectrum in the presence of WGA, indicating that only the dimeric WGA interacts strongly with **A**-units in the chitosan.

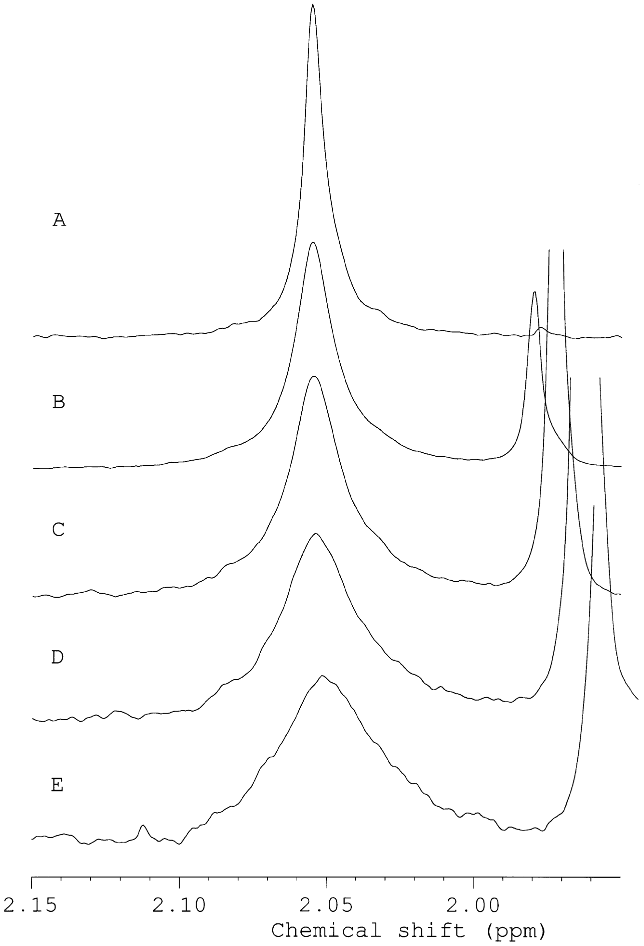

Fig. 4. The *N*-acetyl region of a chitosan with $F_\mathrm{A} = 0.04$ ($\overline{\mathrm{DP}_\mathrm{n}}$ 20) in the absence (A) and presence (B–E) of 0.038 mM WGA III at pH* 5.3, 323 K and ionic strength 0.1 M: (B) 1.00 mM **A**-units; (C) 0.40 mM **A**-units; (D) 0.20 mM **A**-units; (E) 0.10 mM **A**-units.

We performed a competitive experiment between the chitosan preparation with $F_\mathrm{A} = 0.13$ ($\overline{\mathrm{DP}_\mathrm{n}}$ 18) and the fully de-*N*-acetylated chitosan, to investigate whether the specific binding of **A**-units is inhibited by a large concentration of **D**-units in the solution. For simplicity, assuming that the chitosan with $F_\mathrm{A} = 0.13$ consists only of 20-mers, each chain contains on average 2–3 **A**-units, and the fraction of chains that contain no **A**-units is 0.06 (calculated from a random distribution of **A**- and **D**-units). In the presence of 0.045 mM WGA we observed line-broadening of the *N*-acetyl resonance (Fig. 5A,B), indicating a specific interaction with WGA as with the chitosan with $F_\mathrm{A} = 0.04$. The line-broadening did not decrease in magnitude when adding a 5-fold molar surplus of chitosan chains with $F_\mathrm{A} < 0.001$ (Fig. 5B,C). We conclude that binding of **A**-units apparently occurred independently of the presence of **D**-units, or with a dissociation constant significantly smaller than that of binding of **D**-units.

Thus, the **A**-units in highly de-*N*-acetylated chitosans may be considered as ligands for WGA, allowing us to define a K_D-value for the complex between WGA and single **A**-units (i.e. they are primarily surrounded by **D**-units) in the chitosan with $F_\mathrm{A} = 0.04$. The analysis of the binding of **A**-units to isolectins of WGA using Eq. (2) is shown in Fig. 6

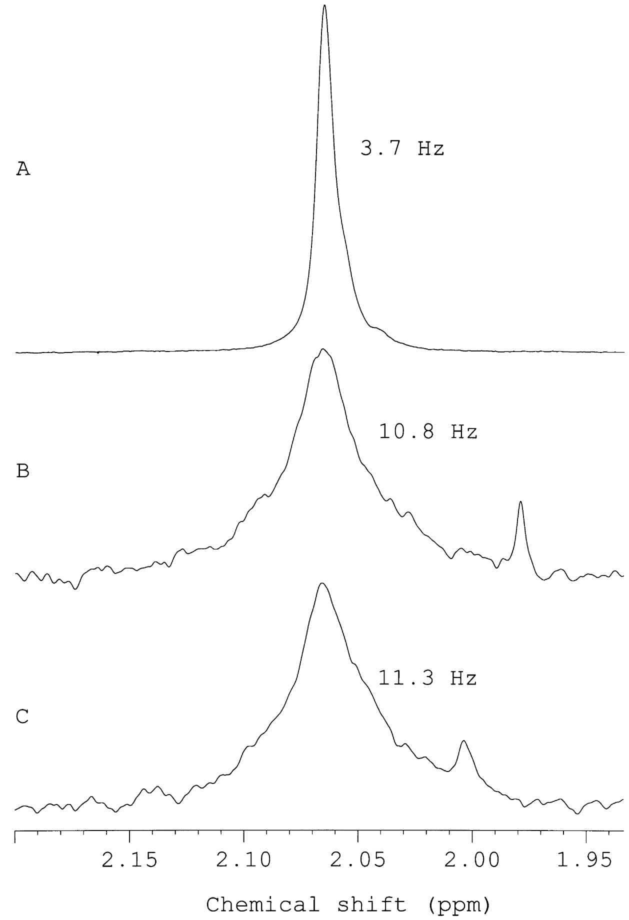

Fig. 5. 300 MHz ^{1}H NMR spectra of the *N*-acetyl region of a chitosan with $F_A = 0.13$ ($\overline{\mathrm{DP}_n}$ 18) at pH* 5.3, 323 K and ionic strength 0.1 M: (A) 1 mM of **A**-units ($F_A = 0.13$), no protein; (B) 0.30 mM **A**-units (equivalent with 0.13 mM of chitosan chains of $\overline{\mathrm{DP}_n}$ 18) and 0.045 mM WGA. (C) as (B) $+$ 0.5 mM of fully de-*N*-acetylated chitosan chains (10 mM of **D**-units).

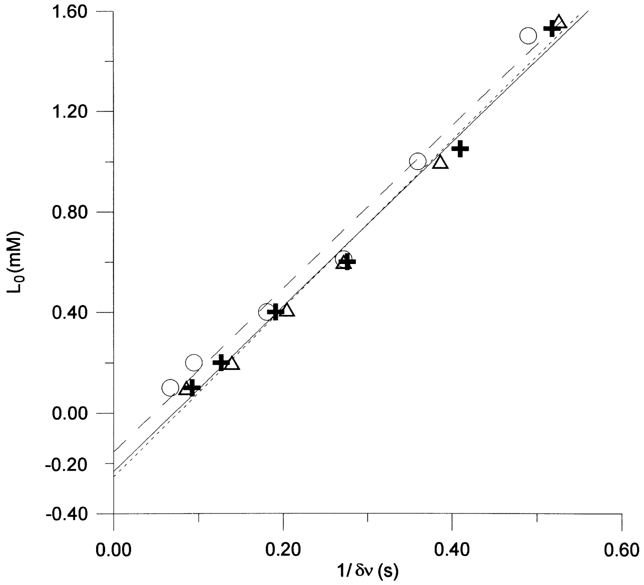

Fig. 6. Binding of **A**-units in a highly de-*N*-acetylated chitosan ($F_A = 0.04$, $\overline{\mathrm{DP}_n}$ 20) to isolectins of WGA. Plots of **A**-units (L_0) vs $1/\delta\nu$ is shown for binding to WGA I (+), WGA II (○) and WGA III (△).

and Table 2. We have no experimental data on the number of binding sites (n) per protomer for this system, but from previous reports it is probable that n equals 1 or 2. Note that no differences in the K_D-values of isolectins were found within error limits. The bound fraction of **A**-units was 0.02–0.17 in these experiments, while the corresponding bound fraction of binding sites on WGA varied from 0.95 to 0.35. Note also the slight curvature that can be observed in Fig. 6, suggesting deficiencies in the model used, which is based on independent and identical binding sites.

3.5. The interaction between chitosans with higher F_A values and WGA

It was possible to study the interaction between a chitosan with $F_A = 0.13$ ($\overline{\mathrm{DP}_n}$ 18) and WGA, however, no quantitative data analysis were performed as the assumption of single **A**-units is not valid. At F_A values of 0.20 and higher ($\overline{\mathrm{DP}_n}$ around 20) we observed precipitation in solutions with WGA and chitosans at pH* 5.2. By lowering pH* to 3 (well below the pK_a* value for dimerisation of WGA) the precipitation was reversed and clear solutions were obtained. Clearly, precipitation of chitosans with WGA is linked to binding of **A**-units to the active dimeric form of the lectin, as is binding of **A**-units in highly de-*N*-acetylated chitosans.

Table 2
Summary of binding of **A**-units in highly de-*N*-acetylated chitosans to unfractionated WGA (0.045 mM) and purified isolectins of WGA (0.036–0.038 mM), obtained at ionic strength 0.1 M, 308 K, pH* 5.3

	K_D (mM) ($n = 1$)[b]	$\Delta\nu$ (Hz)[a] ($n = 1$)[b]	K_D (mM) ($n = 2$)[c]	$\Delta\nu$ (Hz)[a] ($n = 2$)[c]
Unfractionated WGA	0.20 ± 0.07	96	0.15 ± 0.07	48
WGA I	0.20 ± 0.08	92	0.16 ± 0.08	46
WGA II	0.12 ± 0.07	86	0.08 ± 0.07	43
WGA III	0.22 ± 0.06	88	0.18 ± 0.06	44

Data is shown for $n = 1$ and $n = 2$ (n cannot be unambiguously determined).
[a] Limiting linewidth (the linewidth when all **A**-units are bound).
[b] One binding site per protomer.
[c] Two binding sites per protomer.

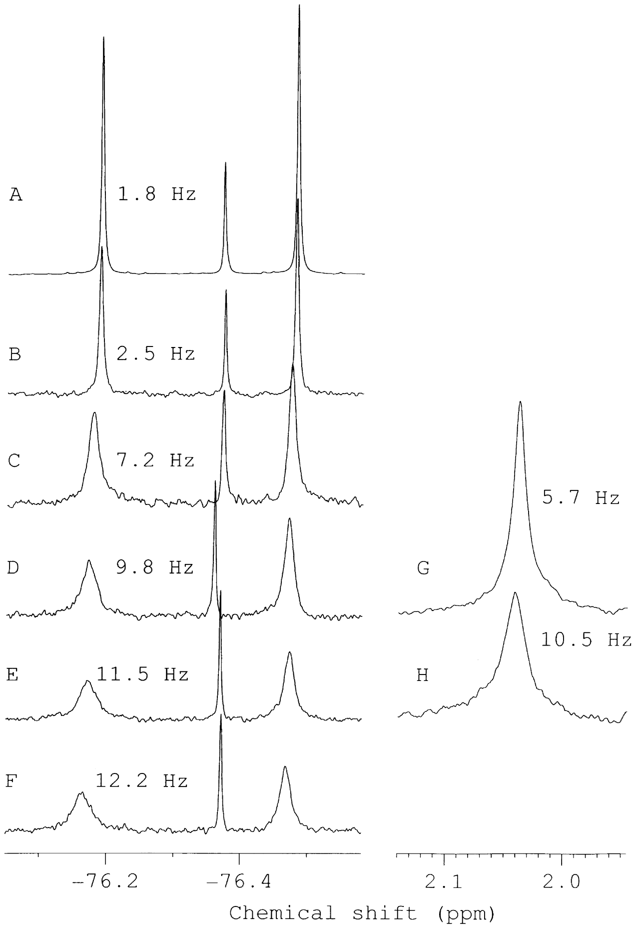

Fig. 7. 376 MHz ^{19}F NMR spectra of 0.27 mM F_3-GlcNAc in the absence (A) and presence of 0.09 mM WGA and various GlcNAc-containing ligands (B–F), and corresponding 400 MHz ^{1}H NMR spectra (G–H) at pH* 5.3, 323 K and ionic strength 0.1 M: (B) 0.33 mM $(GlcNAc)_3$; (C) 1.2 mM GlcNAc; (D) 1.16 mM **A**-units ($F_A = 0.04$); (E) 0.40 mM **A**-units ($F_A = 0.04$); (F) no inhibitor added; (G) ^{1}H NMR spectrum of the *N*-acetyl region, same sample as (D); (H) ^{1}H NMR spectrum of the *N*-acetyl region, same sample as (E).

3.6. Competitive binding of tri-N-fluoroacetylglucosamine and GlcNAc-containing ligands to WGA

Competitive binding experiments suggested that highly de-*N*-acetylated chitosans were not able to compete with GlcNAc or $(GlcNAc)_3$ for binding to WGA, although the interpretation of the ^{1}H NMR spectra were complicated by overlapping *N*-acetyl resonances from both ligands. This lead us to use tri-*N*-fluoroacetylglucosamine (F_3-GlcNAc) as a probe to investigate whether the ligands studied in this work compete for binding to the same sugar binding sites on WGA. The ^{19}F NMR spectrum of F_3-GlcNAc at 376.47 MHz showed three lines (Fig. 7A). The resonance at −76.2 ppm was assigned to the α-anomer of F_3-GlcNAc, while the resonance at −76.5 ppm was assigned to the β-anomer, as reported previously (Ashton et al., 1971). The middle line in Fig. 7A is probably an impurity of tri-*N*-fluoroacetate, as it is not affected by the presence of WGA, and it behaves similar to the ^{1}H resonance of acetate upon addition of acid or chitosan (i.e. shifts slightly downfield).

In the presence of WGA, the tri-*N*-fluoroacetyl resonances of F_3-GlcNAc shifted slightly downfield and broadened significantly (Fig. 7F). We obtained $K_D \approx$ 1.5 mM for binding of F_3-GlcNAc to WGA by adding increasing amounts of ligand to 0.09 mM WGA solution (pH* 5.2, 308 K, ionic strength 0.1 M) and by analysing the linewidths and chemical shifts according to Eq. (2) (data not shown). Next, we performed a series of competitive experiments between F_3-GlcNAc and the other GlcNAc-containing ligands by ^{19}F NMR. Relevant data are shown in Fig. 7, and can be summarised as follows: $(GlcNAc)_3$ competed very efficiently with F_3-GlcNAc for binding to WGA (Fig. 7B), while GlcNAc competed relatively weakly (Fig. 7C), as expected from the present study and previous reports on K_D-values for these ligands (Lotan and Sharon, 1973; Privat et al., 1974a; Jordan et al., 1981). Surprisingly, there was only weak competition between single **A**-units in the highly de-*N*-acetylated chitosan ($F_A = 0.04$ and $\overline{DP_n}$ 20) and F_3-GlcNAc (Fig. 7D,E). Clearly, single **A**-units in the chitosan chain were less efficient than GlcNAc for inhibiting the binding of F_3-GlcNAc, although we have measured 10 times higher affinity of WGA for single **A**-units as compared to GlcNAc (Tables 1 and 2). Note also that even though the chitosan is a poor inhibitor of the binding of F_3-GlcNAc to WGA, the ^{1}H NMR spectrum of these samples show that there is significant interaction between **A**-units and WGA (Fig. 7G,H).

4. Discussion

4.1. Use of low-molecular weight chitosans as model substances

The model chitosans used to study the interactions with WGA were all depolymerised to $\overline{DP_n}$ 17–20 using nitrous acid followed by reduction with $NaBH_4$, which means that the low-molecular weight chitosans are terminated with 2,5-anhydro-D-mannitol units. However, we consider it unlikely that the interactions between WGA and the model chitosans should not be valid for intact chitosans for the following reasons: (i) the interactions between WGA and simple sugars except GlcNAc are very limited, even *N*-acetyl-D-mannosamine does not inhibit the agglutinating activity of WGA (Allen et al., 1973); (ii) the interaction between WGA and **A**-units in de-*N*-acetylated chitosans was not inhibited by fully de-*N*-acetylated chitosans, where both chitosans contain about equal amounts of the modified end groups.

4.2. Binding properties of isolectins of WGA

Isolectins of WGA were used separately in binding studies with the monosaccharide GlcNAc and to study

WGA binding to single **A**-units in highly de-*N*-acetylated chitosans. The K_D-values were equal within error limits for each type of ligand, although the data on highly de-*N*-acetylated chitosans may be too imprecise to conclude fully (Table 2). Previously, it has been reported that WGA I and WGA II bind to sialyl-trisaccharides with different affinities (Kronis and Carver, 1982), however, the relevance of these data to our system is uncertain. Crystallographic data show that there are differences between WGA I and WGA II in their sugar-binding sites (Wright, 1990), which have been correlated with different K_D-values for sialyloligosaccharides obtained in solution (Kronis and Carver, 1982; Wright and Olafsdottir, 1986). However, similar data has not been reported for GlcNAc-oligosaccharides. Thus, as our data did not show any differences between isolectins, most of the following experiments were performed with the unfractionated WGA.

4.3. Interaction of monosaccharides with WGA

We obtained values for K_D and parameters of the bound state (Table 1) that agree well with previous reports of WGA binding to GlcNAc (Nagata and Burger, 1974; Privat et al., 1974a; Jordan et al., 1977; Bains et al., 1992). The K_D-value obtained for F_3-GlcNAc (1.5 mM) was slightly higher than the value of 1 mM reported by Jordan et al. (1981). Competitive experiments clearly showed that GlcNAc, F_3-GlcNAc and $(GlcNAc)_3$ compete for the same binding sites on WGA, as expected. No significant interactions were observed between WGA and GlcN.

4.4. Interaction of **D**-units with WGA

Neither $(GlcN)_3$ nor the fully de-*N*-acetylated chitosan ($F_A < 0.001$) competed for binding to WGA with any of the GlcNAc-containing ligands studied in this work, including GlcNAc, $(GlcNAc)_3$ and a partially *N*-acetylated chitosan ($F_A = 0.13$, $\overline{DP_n}$ 18). This is surprising, as Goldstein et al. (1975) showed that $(GlcN)_3$ is stronger as an inhibitor of precipitation of a glycoprotein than GlcNAc. However, we observed shifts of specific ^1H signals on **D**-units in the presence of WGA (Fig. 3), suggesting weak interactions. The maximum shift observed was 0.04 ppm for H2 when the concentration of added **D**-units was 1.24 mM (i.e. 0.07 mM of chitosan chains with $\overline{DP_n}$ 17) and the WGA concentration was 0.045 mM (which equals 0.09 mM of sugar-binding sites assuming $n = 2$), while H1 and H3 shifted 0.03 ppm at the same conditions (Fig. 3). No line-broadening of these resonances were observed, indicating that the chemical exchange rate is fast with respect to chemical shift differences. Assuming that the shifts observed are due to the interaction of one **D**-unit with a specific site on WGA ($n = 2$), the limiting shift would be > 0.5 ppm (0.04 ppm* 1.24/0.09), which equals 150 Hz at a spectrometer frequency of 300 MHz. Thus, the off rate, k_{off}, must be much larger than 150 Hz. Taking $k_{off} > 10$ kHz, and assuming that the on rate for such a relatively large ligand is $k_{on} \sim 10^6\ M^{-1}\ s^{-1}$, we obtained $K_D > 10$ mM, i.e. weak binding.

Interestingly, the shifts of proton signals of **D**-units upon addition of WGA are observed of the three closest observable protons to the predominantly positively charged amino group. When titrating a fully de-*N*-acetylated chitosan with H^+-ions, upfield shifts are observed of H1, H2 and H3 with increasing pH*, with relative magnitudes of shifts identical to those observed in the present study with WGA (Kristiansen et al., unpublished data), from which we obtained a pK_a* value of 6.6–6.7 (measured in D_2O solution). The maximum upfield shift observed at pH* 5.3 (Fig. 3C) is equivalent with a lowering of the pK_a* value of approximately 0.5 units. Thus, it seems probable that there is an interaction between WGA and **D**-units of chitosans which changes the protonation of the amino group, so that the uncharged amino group is slightly favoured in the presence of WGA. A similar effect at pH* 3 would not lead to detectable shifts of protons, as the fractional change of protonation is too small. Consequently, the present data does not allow the conclusion that there is no interaction at this pH* value.

As no competition of **D**-units with GlcNAc for binding to WGA was observed, interaction is probably not located to sites that are specific for GlcNAc-oligosaccharides. Furthermore, we note that the shift changes observed in the fully de-*N*-acetylated chitosan ($\overline{DP_n}$ 17) upon interaction with WGA are 5–10 times larger than those observed with $(GlcN)_3$. As binding sites on WGA are believed to contain three subsites, it seems improbable that we have observed site binding of **D**-units.

4.5. Interaction of **A**-units in highly de-*N*-acetylated chitosans with WGA

As expected, we observed a specific interaction between **A**-units in highly de-*N*-acetylated chitosans and WGA. The affinity of this ligand to WGA was approximately 10 times higher than that of GlcNAc, suggesting that neighbouring **D**-units contribute significantly to the binding. To our knowledge, the only other report of WGA binding to a partially *N*-acetylated chitosan is that by Senstad and Mattiasson (1988), who used a chitosan with $F_A = 0.25$ in affinity precipitation of WGA in solution. Although their study was only semi-quantitative, they estimated that the chitosan–WGA interaction had K_D-values in the range 0.05–0.8 mM, in agreement with the present results. The specificity of WGA towards GlcNAc on cell surfaces is well documented through precipitation studies (Goldstein et al., 1975; Lotan et al., 1975; Goldstein and Poretz, 1986, and Refs. therein), but there are no structural data on such complexes with respect to binding modes or number of binding sites. However, the inherent symmetry of the WGA molecule suggests that there is at least one binding site per protomer (two per dimer), unless there are steric hindrances to binding large ligands as chitosan at more than one site per dimer. The precipitation observed in this study with chitosans of higher F_A values and WGA would suggest at least two binding sites per dimeric WGA, as it is generally accepted that such precipitation is due to multivalency of both lectin and ligands (Weis and Drickamer, 1996).

The current work has revealed a discrepancy between the direct binding studies (Figs. 4, and 6) and the competitive studies (Fig. 7) performed with **A**-units in highly de-*N*-acetylated chitosans. The competitive experiments were not analysed quantitatively due to lack of an appropriate binding model; however, some quantitative remarks can be made: all spectra shown in Fig. 7 were acquired at 308 K, where the limiting linewidth of the *N*-acetyl resonance is approximately half that of the value at 323 K (data not shown), i.e. $n(\Delta\nu)\sim50$ Hz. Using known concentrations and a K_D-value of 0.15 mM (with $n = 2$, Table 2), the expected linewidths of the *N*-acetyl resonance in Fig. 7G and Fig. 7H are 7 and 11 Hz, respectively, assuming no competition between F_3-GlcNAc and **A**-units. The observed linewidths are 5.7 and 10.5 Hz, respectively, showing that data from ^{19}F and ^{1}H NMR spectra are consistent both with a model of weak competition between F_3-GlcNAc and single **A**-units, and with a K_D-value of ~0.15 mM for the WGA–chitosan complex.

Wright (1984) has previously suggested that the four binding sites per dimer observed in the crystal structure of WGA II complexed with $(GlcNAc)_2$ are specific for non-reducing end **A**-units. The molar concentration of **A**-units on the non-reducing end is 1/20 of the total **A**-unit concentration (random distribution of units and $\overline{DP_n}$ 20), and the weak competition observed in Fig. 7 is compatible with a model assuming that only terminal non-reducing **A**-units in chitosans are competitive inhibitors of F_3-GlcNAc binding to WGA. Clearly, the current data suggest that binding of internal single **A**-units in highly de-*N*-acetylated chitosans primarily occur to other sites than the chitin-oligosaccharide-specific sites. Note also that no shift changes were observed when an internal **A**-unit is bound to WGA (Fig. 4), as opposed to the bound state of GlcNAc, which is shifted ~0.5 ppm upfield (Fig. 1), indicating different binding sites or different bound orientations of the *N*-acetyl group.

It has previously been reported that WGA has four functional sugar-binding sites per protomer (eight per dimer by symmetry), as each of the homologous domains (named A–D) of the protomer is a self-containing sugar-binding unit (Wright, 1984, 1992). This is clearly demonstrated in nature by the existence of *hevein*, which is a GlcNAc-specific lectin of 43 amino acids from *Hevea brasiliens*, with very similar sequence to the domains of WGA protomers. Recently, Wright and Kellogg (1996) have reported on a molecular modelling study of the four possible distinct binding modes for $(GlcNAc)_2$ to WGA, suggesting that two high-affinity sites for $(GlcNAc)_2$ per protomer are located in the B/C domain interfaces, while two additional low-affinity binding sites are located in the A/D domain interfaces (a total of eight sites per dimeric WGA). Clearly, with respect to oligosaccharides there are (at least) two classes of sites. Assuming that the B/C domain sites are the primary binding sites for GlcNAc-oligosaccharides, we may only speculate that one or two of the remaining sites located in the A/D domain interface (termed low-affinity sites for $(GlcNAc)_2$) can be functional high-affinity binding sites for the chitosan ligands studied in this work, while only low-affinity binding occurs to the sites that are specific for $(GlcNAc)_2$. Such discrimination might arise due to different specificities of sites with respect to chemistry (affinity for **D**-units) or steric hindrances. Note that Wright (1984) has previously predicted that the two distinct binding sites observed in the crystal structure (one in the B/C interface and another in the A/D interface) are specific for terminal non-reducing GlcNAc residues, although there are evidence of binding to internal **A**-units both in the present study and in the literature (Goldstein et al., 1975; Lotan et al., 1975; Senstad and Mattiasson, 1988). A qualitative evaluation of the models reported by Wright and Kellogg (1996) suggests that three of the four described binding sites per monomer have steric hindrances to chain elongation at C4 of the terminal non-reducing GlcNAc-residue. This is due to steric crowding of substituents on C4 with the opposing WGA domain (from the other protomer) that contributes significantly to binding. However, the binding site located in the A-domain lacks the opposing D-domain contribution to binding, thus, there is no requirement for a terminal GlcNAc residue in this binding site. Assuming that an **A**-unit adopts the same WGA-bound orientation whether it is terminal as in $(GlcNAc)_2$, or internal in a chitosan chain, the data presented in this work would suggest binding of **A**-units in highly de-*N*-acetylated chitosans to the binding site located in the A-domain of WGA. This would imply that there are two binding sites per WGA dimer which are able to bind internal GlcNAc residues in chitosans.

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