Ligand-induced perturbations in *Urtica dioica* agglutinin

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Abstract The binding of the trisaccharide, *N,N′,N″*-triacetylchitotriose, to *Urtica dioica* agglutinin (UDA) was investigated using ^{1}H NMR spectroscopy. UDA is a small antiviral plant lectin containing two homologous 43-amino acid domains. Carbohydrate-induced perturbations occur in one domain of UDA at trisaccharide concentrations below equimolar. Residues in the second domain are shifted at higher carbohydrate concentrations. This data confirms the presence of two binding sites of non-identical affinities per UDA monomer. Qualitative analysis of the 2D NOESY spectra indicates that UDA contains two short stretches of antiparallel β-sheet. The ^{1}H resonance assignments for both antiparallel β-sheet sequences have been completed and there is one β-stretch per domain. A number of these β-sheet residues are perturbed in the presence of carbohydrate.

Key words: Lectin; *N,N′,N″*-Triacetylchitotriose; Lectin–carbohydrate interaction; NMR; *Urtica dioica*

1. Introduction

Urtica dioica agglutinin (UDA) is a chitin-binding lectin isolated from the stinging nettle rhizome [1] with insecticidal [2], antifungal [3], and selective antiviral [4] activities. UDA is capable of inhibiting HIV infectivity [4], a property usually associated with mannose-binding lectins [4]. This antiviral effect is linked to its ability to recognize and bind a specific type of carbohydrate. The present study was designed to characterize the UDA carbohydrate binding sites.

Nuclear magnetic resonance (NMR) techniques were used to examine the complex between UDA and *N,N′,N″*-triacetylchitotriose. Previous studies concluded that this trisaccharide is complementary to the binding site [5]. Because of the small size (89 amino acids) and monomeric nature of UDA, NMR has provided an excellent method for studying carbohydrate-induced perturbations of specific lectin residues. Trisaccharide-induced shifts occur selectively for one set of UDA resonances at concentrations below equimolar and for a second group of resonances at higher concentrations. These results are consistent with the presence of two binding sites of non-identical affinities per monomer of UDA. The titration data were used to estimate the binding affinities at each site. A number of the resonances perturbed in the presence of trisaccharide have been sequentially assigned. We find that most of these perturbed residues reside in one of the two short stretches of antiparallel β-sheet located in UDA.

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Abbreviations: UDA, *Urtica dioica* agglutinin; GlcNAc, *N*-acetylglucosamine.

2. Materials and methods

UDA was isolated and purified as described previously [6]. A single isolectin of UDA was obtained from reverse phase HPLC using a Vydac C-18 column (Hesperia, CA) and a 45 min linear gradient of 3.6–36% acetonitrile in 0.1% trifluoroacetic acid.

The NMR samples were prepared by dissolving lyophilized lectin in either 90% H_2O/10% D_2O or 99.996% D_2O. The samples prepared in D_2O were lyophilized several times from 99.96% D_2O prior to the final preparation. In order to maximize solubility, the pH was adjusted to 3.3–3.5 by adding small amounts of DCl. Initial studies monitoring the T_1 relaxation of UDA at 35°C as a function of concentration indicated some aggregation of the protein at concentrations above 3 mM. Therefore, protein samples containing 3 mM UDA were used for the two-dimensional studies.

The NMR experiments were carried out either on the GN-500 or Omega 500 MHz NMR spectrometers (University of California, San Francisco, UCSF) or on a Bruker AMX500 (Bruker Instruments, Fremont, CA).

One-dimensional NMR spectra at different ratios of protein to carbohydrate were obtained by titrating a solution containing UDA (~1.0 mM) with a solution containing the same concentration of UDA plus a high concentration of the trisaccharide (~15 mM). Both protein and trisaccharide samples were lyophilized from 99.96% D_2O separately prior to the final dissolution in 99.996% D_2O. The pH was maintained at 3.4. A total of 512 scans were obtained at each titration point.

A series of two-dimensional data sets were acquired including double-quantum filtered COSY (DQF-COSY) [7], exclusive COSY (E.COSY) [8], Homonuclear Hartman–Hahn (HOHAHA) [9], and NOESY [10,11]. The majority of the spectra were obtained at 35°C. Several NOESY spectra were recorded at 45°, 20° and 11°C in order to take advantage of the temperature sensitivity of the H_2O and amide proton shifts. For spectra obtained in 90% H_2O, suppression of the HDO signal was accomplished by presaturation in the COSY experiments. The DANTE [12] and *jump-and-return* [13] pulse sequences were used for the HOHAHA and NOESY experiments, respectively. Typically, the spectra were recorded with 4,096 complex points in w_2 at 512 t_1 values with 16–64 scans per t_1. The proton chemical shifts were referenced relative to TSP present in the sample.

The two-dimensional data were processed either on a Sun Microsystems Sparc2 workstation at UCSF using the processing package STRIKER (M. Day, 1992, copyright UCSF) and spectral display and analysis program, SPARKY (D.G. Kneller, 1992, copyright UCSF) or on a Silicon Graphics 4D/25 Personal Iris computer using Felix software (Biosym Technologies, San Diego, CA). Typically data was zero-filled once in w_1. Gaussian filters with line-broadening parameters of 2 Hz were applied.

The association constant K_a for the trisaccharide binding to the low affinity site was estimated from the NMR version of the Scatchard equation [14]:

$$(\Delta/\Delta o)/[L]_f = -K_a n + K_a(\Delta/\Delta o) \quad (1)$$

$[L]_f$ is the concentration of free trisaccharide, $\Delta = \delta_{Pobsd} - \delta_{Pf}$ and $\Delta_o = \delta_{Pb} - \delta_{Pf}$, where δ_{Pf} and δ_{Pb} are the free and bound UDA chemical shift respectively and δ_{Pobsd} is the observed UDA chemical shift at each titration point. The concentration of UDA with the low affinity site occupied, $[P]_{b2}$, was determined from $[P]_{b2} = (\Delta/\Delta o)P_t$ where

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P_t is the total UDA concentration which was held constant at 1.17 mM. The titration results indicate that the high affinity site is substantially occupied before changes occur in resonances from the second binding site (0.9 trisaccharide/UDA). The concentration of free ligand was adjusted assuming full occupation of the first site giving $[L]_f = [L]_{total} - [P]_{b2} - 1.17$ mM. Excluding data points obtained from spectra containing $[L]_t \leq 3$ mM; the regime expected to be most sensitive to complete occupation of the first site, the plot of $(\Delta/\Delta o)/[L]_f$ versus $(\Delta/\Delta o)$ is linear and $-K_a$ can be estimated from the slope. The intercept is $K_a n$, where 'n' is the number of binding sites.

3. Results

3.1. 1D NMR studies of UDA-N,N′,N″-triacetylchitotriose complex

The 1D NMR spectrum of UDA in D_2O was monitored in the presence of increasing amounts of *N,N′,N″*-triacetylchitotriose. Plots indicating the change in chemical shift as a function of the trisaccharide/UDA ratio for those peaks which can be unambiguously identified in the 1D spectrum and for which the sequential assignments have been made (see below) are shown in Fig. 1.

At ratios of carbohydrate to UDA below 0.9, we observe changes in a subset of aromatic, non-exchangeable, and upfield shifted proton resonances (Fig. 1a) while others remain sharp and unshifted. Resonances from Trp^{21} (C^2H, C^4H), Gly^{22} (NH) and Trp^{23} (C^4H) broaden beyond detection at these trisaccharide concentrations. The Ile^{20} $C^{\delta}H_3$ resonance is observed at −0.163 ppm in the free UDA while the position of the bound $C^{\delta}H_3$ resonance is observed upfield at −0.546 ppm (Fig. 2). These peaks are the only resonances of the group of peaks perturbed at these trisaccharide to UDA ratios that are fully resolved throughout the titration. The bound peak increases in intensity as the trisaccharide is added while that of the free diminishes. The linewidth of the free peak increases as a function of increased ligand concentration while that of the bound remains constant. At trisaccharide-UDA ratios above equimo-

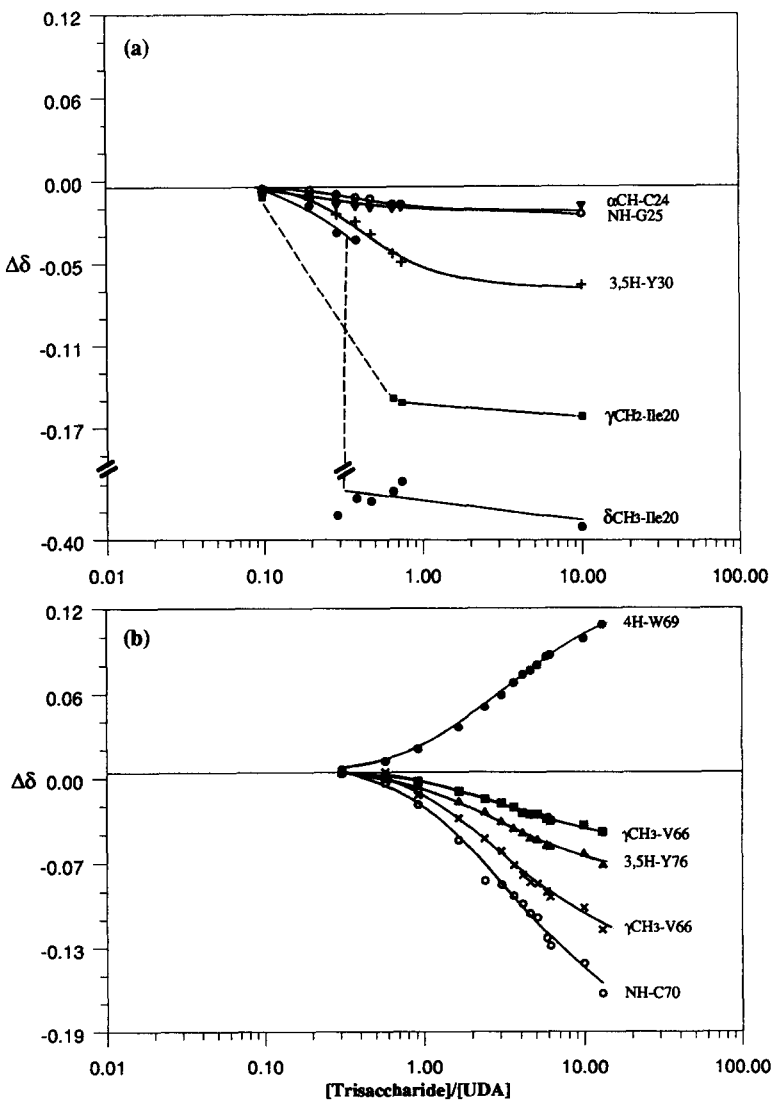

Fig. 1. Titration profiles for UDA with *N,N′,N″*-triacetylchitotriose at 35°C. $\Delta\delta$ is the chemical shift of the UDA resonance in the presence of the trisaccharide minus that in the absence of carbohydrate. Resonances are grouped according to (a) slow to intermediate and (b) fast exchange rate. The dashed lines in (a) connect resonances which are in slow exchange.

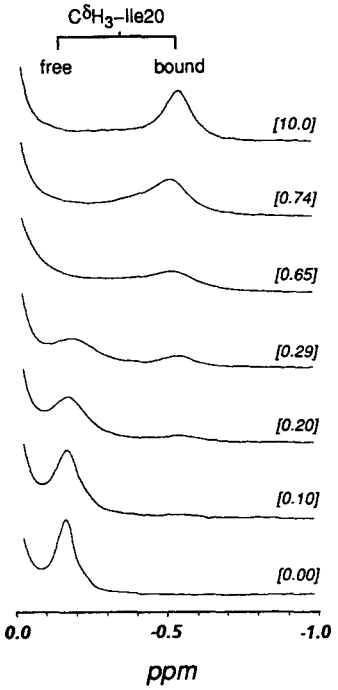

Fig. 2. ^{1}H NMR spectra of the C$^{\delta}$H$_3$ resonance of Ile^{20} illustrating the position and intensity changes for the free (−0.163 ppm) and bound (−0.546 ppm) peaks as a function of *N,N′,N″*-triacetylchitotriose concentration. The ligand concentration (mM) is indicated at the right. The spectra are resolution enhanced using exponential linebroadening of 10 Hz.

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lar the bound peaks sharpen but no further shifts are observed. These observations are consistent with slow to intermediate exchange [15].

This titration study was conducted using 1.0 mM UDA with the addition of trisaccharide in the range of 0–1 mM. From the integrated intensities of the bound protein Ile^{20} C$^{\delta}$H$_3$ peak it is evident that under these conditions each aliquot of trisaccharide added becomes bound to protein. This implies that the dissociation constant K_d is $\ll 10^{-3}$ M; too small to measure by this method. Concentrations of ligand and protein in the micromolar range are needed to provide a sensitive probe of K_d. However, we were able to estimate the off rate k_{-1} from the linewidth of the bound Ile^{20} C$^{\delta}$H$_3$ peak and the linewidths and shifts of the C^{3,5}H Tyr^{30} resonance during the titration [16]. A value of k_{-1} of 60–90 s^{-1} was obtained. Estimating an on rate k_1 of ~ 10^6 s^{-1} · M^{-1} [17], gives K_d ~ 0.1 mM for this site. The affinity is lower than that previously obtained for the tetrasaccharide (K_d = 0.0024 mM) containing one additional GlcNAc [5]. This

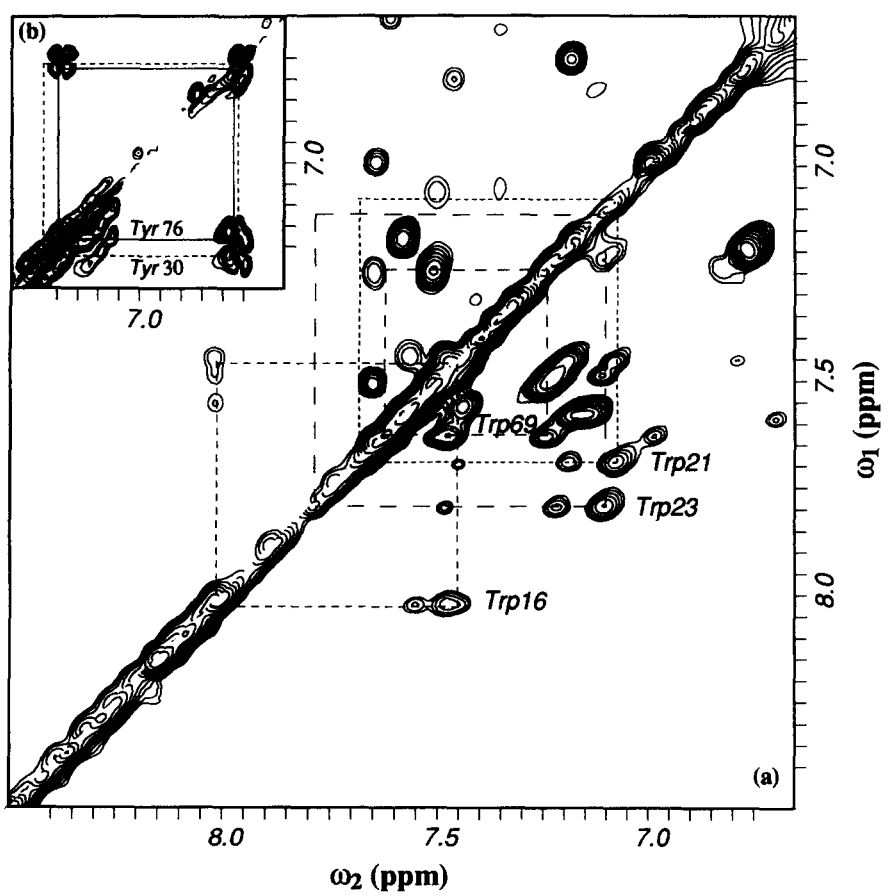

Fig. 3. The aromatic region of the HOHAHA spectrum (a) recorded for UDA in H_2O, 35°C, pH 3.4 is plotted below the diagonal. The spin systems for Trp^{16}, Trp^{21}, Trp^{23}, and Trp^{69} are indicated. The corresponding region of UDA in the presence of equimolar amounts of *N,N′,N″*-triacetylchitotriose is plotted above the diagonal. The C^{2,6}H/C^{3,5}H DQF-COSY cross peaks for Tyr^{30} and Tyr^{76} in UDA alone and in the 1 : 1 complex are shown below and above the diagonal of the insert (b), respectively.

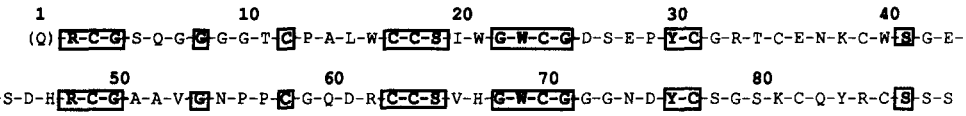

Fig. 4. Primary sequence of UDA. The N-terminal is pyroglutamate (Q). Areas of sequence identity between the two 43-amino acid domains are boxed.

is consistent with the relative activities measured for these two oligosaccharides [5].

At carbohydrate–protein ratios above 0.9, a separate set of peaks begin to shift (Fig. 1b). All of these resonances shift continuously with no linebroadening as the concentration of carbohydrate is increased until saturation. This is characteristic of fast exchange [15].

The association constant K_a for the trisaccharide binding at this site was calculated using equation 1 as described in section 2. The average value obtained from the five curves shown in Fig. 1b was $0.43 \pm 0.04\ mM^{-1}$, $n = 1$ ($K_d = 2.3$ mM). The trisaccharide has one order of magnitude lower binding affinity to the low affinity site than that previously reported [5] for the tetrasaccharide ($K_a = 6.6\ mM^{-1}$).

3.2. *2D NMR studies of UDA and the UDA-N,N′,N″-triacetylchitotriose complex*

Using standard procedures for spin system identification and sequential assignments [18], a number of residues perturbed in the presence of low and high concentration of trisaccharide have been identified.

Fig. 3a shows the aromatic region of the HOHAHA for UDA free (bottom) and in the presence of 1:1 trisaccharide (top). In the spectrum for UDA free, complete spin system networks for four of the five tryptophan indole ring protons are easily traced. Their respective C^{α} and C^{β} protons can be identified from intense NOESY cross peaks correlating the C^2H indole ring to the side chain. Three of the five Trp belong to unique tripeptide sequences (Fig. 4). The assignment of Trp^{21}-Gly^{22}-Trp^{23} allowed the subsequent sequence specific assignments for residues Ala^{14} to Gly^{25}. Likewise, resonances for residues Cys^{63} through Gly^{71} were sequentially assigned after the initial assignment of the unique tripeptide His^{67}-Gly^{68}-Trp^{69}.

As shown in Fig. 5, NOE patterns indicative of antiparallel β-sheet structure [18] are observed for Cys^{17} to Gly^{25} and Cys^{63} to Gly^{71}.

In the presence of 1:1 trisaccharide (Fig. 3a, top), indole ring resonances for Trp^{21} and Trp^{23} are no longer observed. Trp^{16}, on the other hand is completely unaffected while the ring protons of Trp^{69} have started to shift slightly.

Overlapping tyrosyl ring proton cross peaks from two of the three tyrosines are observed in free UDA (Fig. 3b; bottom). In the presence of equimolar trisaccharide (Fig. 3b, top), one of the tyrosyl ring proton crosspeaks is broadened beyond detection. In addition, a number of NOESY cross peaks are observed between this tyrosyl ring proton cross peak and resonances from Ser^{19}, Trp^{23}, and Gly^{25} (data not shown) suggesting that this tyrosine ring is in close proximity to the β-sheet region from Cys^{17} to Gly^{25}. These NOESY correlations plus the observed perturbations upon binding lead to the assignment of this tyrosine to Tyr^{30}. The other tyrosyl ring proton cross peak is shifted only at higher trisaccharide-UDA ratios (Fig. 1b). NOESY correlations between these ring protons and residues Ser^{65}, Trp^{69} and Gly^{71} from the second β-sheet region in UDA lead to its subsequent assignment to Tyr^{76}.

4. Discussion

UDA, is an 9.4 kDa lectin rich in glycines, cysteines and tryptophans. Based on internal primary sequence homology, UDA can be divided into two 43-amino acid domains [19] (Fig. 4). The qualitative analysis of the NMR NOE data indicate each UDA domain contains a short stretch of antiparallel β-sheet. The sequence from Cys^{17} to Gly^{25} forms one β-sheet stretch with a reverse turn at Ile^{20} to Gly^{22}. The second β-sheet structure is in the analogous position in the second domain of UDA from Cys^{63} to Gly^{71} looping at Val^{66} to Gly^{68}. The primary sequences of these two β-strands are similar in that the two 9-amino acid β-sheet sequences only differ by two conserved substitutions; Ile^{20} to Val^{66} and Trp^{21} to His^{67}.

The NMR data indicate clearly the presence of two binding sites per monomer of UDA with trisaccharide binding affinities differing by more than an order of magnitude. Binding at the higher affinity site is associated with changes in the resonances assigned to the β-strand in the first 43-amino acid domain and Tyr^{30}. The changes include the broadening of resonances assigned to the reverse turn loop residues Ile^{20}-Trp^{21}-Gly^{22} and Trp^{23}. The β-sheet resonances of the second domain are unaffected at ratios below equimolar. At higher ratios of ligand to protein, some of the resonances in the second β-sheet stretch from Cys^{63} to Gly^{71} as well as Tyr^{76} are perturbed. Ligand-induced shifts occur again in the reverse turn region of the β-sheet plus Trp^{69}. These data indicate that each domain of UDA contains an independent trisaccharide binding site.

From this study, we have concluded that the β-sheet stretches in UDA are intimately involved in trisaccharide binding. We are currently investigating the underlying structural origins for the difference in affinity between the two 43-amino acid domains. We are also examining the binding affinity of a series of alternative carbohydrate ligands that may be involved in

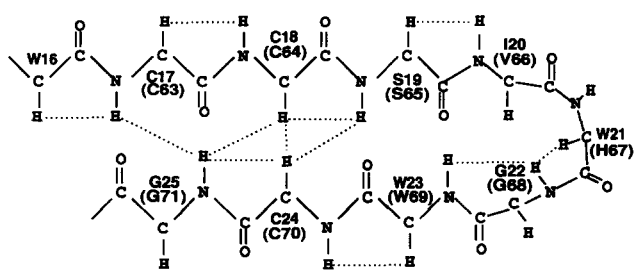

Fig. 5. Schematic diagram of the antiparallel β-sheet structure of UDA. The characteristic NOE patterns observed in the D_2O spectrum including the interstrand $C^{\alpha}H/C^{\alpha}H$ cross peak and the interchain $NH/C^{\alpha}H$ correlations are indicated by the dashed lines. Residue numbers shown in parentheses are for the second β-sheet sequence.

the antiviral effect of UDA. UDA provides a contained and accessible system from which to study the effect of subtle amino acid changes on the biological properties of a molecule.

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