

## Mutational Analysis at Asn-41 in Peanut Agglutinin

A RESIDUE CRITICAL FOR THE BINDING OF THE TUMOR-ASSOCIATED THOMSEN-FRIEDENREICH ANTIGEN\*

Received for publication, April 5, 2001, and in revised form, June 25, 2001  
Published, JBC Papers in Press, July 10, 2001, DOI 10.1074/jbc.M103040200

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**Peanut agglutinin is a clinically important lectin due to its application in the screening of mature and immature thymocytes as well as in the detection of cancerous malignancies. The basis for these applications is the remarkably strong affinity of the lectin for the tumor-associated Thomsen-Friedenreich antigen (T-antigen) and more so due to its ability to distinguish T-antigen from its cryptic forms. The crystal structure of the complex of peanut agglutinin with T-antigen reveals the basis of this specificity. Among the contacts involved in providing this specificity toward T-antigen is the water-mediated interaction between the side chain of Asn-41 and the carbonyl oxygen of the acetamido group of the second hexopyranose ring of the sugar molecule. Site-directed mutational changes were introduced at this residue with the objective of probing the role of this residue in T-antigen binding and possibly engineering an altered species with increased specificity for T-antigen. Of the three mutants tested, *i.e.* N41A, N41D, and N41Q, the last one shows improved potency for recognition of T-antigen. The affinities of the mutants can be readily explained on the basis of the crystal structure of the complex and simple modeling. In particular, the change of asparagine to glutamine could lead to a direct interaction of the side chain with the sugar while at the same time retaining the water bridge. This study strengthens the theory that in lectins the nonprimary contacts generally made through water bridges are involved in imparting exquisite specificity.**

Lectins are a class of nonenzymic carbohydrate-binding proteins, which are found ubiquitously in nature. They are mostly multivalent in their sugar binding characteristics thus functioning as cellular adhesives in a range of biological systems and are put to use in biomedical research as carbohydrate-selective probes based on the binding to surface sugars (1, 2). Plant lectins have no clearly defined function, but a number of them have been implicated in defense-related roles (3). Although a global utilization for them has not been identified, a number of lectins have shown great potential in clinical applications. Peanut agglutinin in particular has displayed immense utility for the screening of mature and immature thymocytes prior to bone marrow transplantation (4) as well as in

the detection of cancerous malignancies (5). The status of PNA<sup>1</sup> as a valuable clinical tool has been recognized largely based on its strong affinity for the tumor-associated T-antigenic disaccharide (Gal $\beta$ 1 $\rightarrow$ 3GalNAc, Thomsen-Friedenreich antigen), which is of non-oncofetal origin. The other anti-T probes, amaranthin and jacalin, are not as useful and convenient because they are unable to discriminate between the more abundant cryptic T- and Tn-antigens (GalNAc $\alpha$ -O-Ser/Thr), which are sialylated derivatives of the T- and Tn-antigens, respectively (6–8).

A member of the legume lectin family, PNA is a homotetramer with an unusual open quaternary structure (6, 9). This 110-kDa lectin binds to carbohydrates by means of conserved residues used by other members of this family as well as a few that are specific to itself. These amino acids are distributed within the binding pocket in four distinct loops designated A, B, C, and D found in all legume lectins (10). Within this family, PNA belongs to the group of Gal/GalNAc-specific lectins classified on the basis of its monosaccharide specificity wherein three-dimensional structures of four have been solved, namely *Erythrina corallodendron* lectin (11), *Griffonia simplicifolia* lectin (12), *Glycine max* lectin (13), and peanut agglutinin (9, 14). The molecular basis of carbohydrate specificity for PNA was delineated at atomic resolution initially as a complex with lactose, which is involved in nine hydrogen bonds with the residues emanating from the four loops (9, 14). The galactose residue of lactose is tethered to the protein through hydrogen bonds and water bridges as well as a stacking interaction with a tyrosyl residue from loop C. The PNA-T-antigen crystal structure (15) solved subsequently revealed the similarity in the mode of binding of T-antigen and lactose. Notwithstanding the similarities, the 20-fold higher affinity for T-antigen is contributed by the liganding contacts mediated by two additional water bridges. These water molecules, although present in the binding site of the PNA-lactose complex, are not engaged in any bonding interactions between the protein and the sugar (15). Among these, one molecule links Ile-101, while the other links both Asn-41 and Leu-212 of PNA with the carbonyl oxygen of the acetamido group of the  $\beta$ 1 $\rightarrow$ 3-linked GalNAc residue of the T-antigenic disaccharide. The Leu-212 residue was probed by site-directed mutagenesis for its role in the binding of T-antigen at a time when the structure of the complex of PNA and T-antigen had not been solved. This was attempted based on its proximity to the *N*-acetyl group of T-antigen as visualized from the PNA-lactose structure and was thereafter proven to be critical in the binding of this sugar (16). Subsequently the

\* This work was supported by a grant from the Department of Biotechnology, Government of India (to A. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>1</sup> The abbreviations used are: PNA, peanut agglutinin; rPNA, recombinant PNA; T-antigen, Thomsen-Friedenreich antigen (Gal $\beta$ 1 $\rightarrow$ 3GalNAc); WT, wild-type.

solution of the complex of PNA with T-antigen confirmed the importance of this residue and revealed the participation of the same water molecule in linking both Leu-212 and Asn-41 to T-antigen (15, 17). We extend this study further on the role of Asn-41 in defining the anti-T specificity of PNA more so in relation to the role of the water-mediated hydrogen bond in imparting the lectin its exquisite specificity for the tumor-associated antigen.

EXPERIMENTAL PROCEDURES

**Materials**—Restriction and modifying enzymes were purchased from Amersham Pharmacia Biotech and New England Biolabs. Neuraminidase was procured from Sigma. The *Escherichia coli* strain used for expression and mutagenesis was XL1Blue. The expression construct equivalents of pBSH-PN expressing the N41A, N41D, and N41Q mutant versions of PNA are designated as pN41A-PN, pN41D-PN, and pN41Q-PN, respectively. Sugars used in hemagglutination inhibition and BIAcore2000™ (Pharmacia Biosensor AB, Uppsala, Sweden) biosensor assays were procured from Sigma, Dextra Laboratories Ltd. (Reading, United Kingdom), or Aldrich. Biotinylated T-antigen conjugate for surface plasmon resonance studies was purchased from Syntosome (Munich, Germany). The conjugate consists of poly[N-(2-hydroxy-

ethyl)-acrylamide] polymer to which ω-aminoalkyl-α-glycoside of T-antigenic disaccharide (Galβ1→3GalNAcα1-O-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-NH<sub>2</sub>) and biotin are conjugated (18), the final composition of the conjugate being polyacrylamide:T-antigen:biotin 75:20:5.

**Concentration Determination**—Concentration of rPNA and its various mutants were determined by the absorbance of their solutions, *i.e.* A<sup>1%</sup><sub>280</sub> = 7.7 (6, 8). Concentrations of sugars were determined by weight.

**Site-directed Mutagenesis**—Selected mutational changes at Asn-41 were generated through polymerase chain reaction splicing by overlap extension (19). The mutagenic oligonucleotides used to generate the mutated PNA species are shown in Table I. After the desired mutation was established in the PNA gene they were transferred into the same expression context as that of the WT PNA gene in pBSH-PN. Transformants were then screened for the desired recombinants by restriction analysis, first to check for the presence of insert and subsequently to check for the presence of the preengineered restriction site change directly linked to the specific mutational change selected for (Table I). Sequencing of the entire polymerase chain reaction-generated area was performed for all identified mutant clones to ensure the presence of the desired change and the absence of any second site changes (20, 21).

**Expression and Purification of WT and Mutant PNA**—Growth of cultures, expression of the PNA species, and their isolation was performed as described previously (22)

**Hemagglutination / Inhibition Assays**—These assays were performed with human O<sup>+</sup> red blood cells as previously described (22). For inhibition assays, the protein was preincubated with the sugar being tested for 1 h at room temperature prior to the addition of red blood cells at a final concentration of 1%. The amount of protein used in the assays was 4–8 times the previously established value for the minimum hemagglutination concentration. The minimum inhibitory concentration for a particular sugar was recorded at the first well showing complete inhibition of hemagglutination.

**BIAcore Biosensor Assays**—Surface plasmon resonance analysis was performed using a BIAcore 2000™ (Pharmacia Biosensor AB) biosensor system. The T-antigen as a biotinylated derivative containing a polyacrylamide spacer arm between the biotin and the sugar moiety was immobilized on a streptavidin chip. Nearly 400 response units of T-antigen (0.1 mg/ml in 50 mM phosphate-buffered saline) were coupled to the chip at a flow rate of 1 μl/min for 50 min. All measurements were done using the running buffer (20 mM phosphate-buffered saline). All protein samples were dialyzed extensively in the same buffer prior to injection. For determination of on rates, recombinant PNA or the mu-

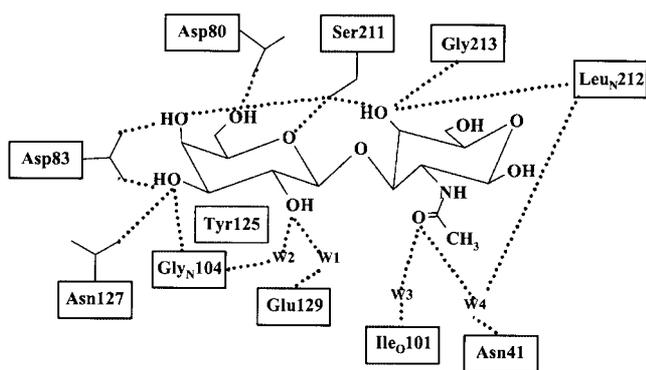


FIG. 1. Schematic representation of protein-carbohydrate interactions in the PNA-T-antigen complex.

TABLE I  
Oligonucleotides used in the generation of Asn-41 mutant PNAs

Oligonucleotide	Sequence	Comments
N41Afor	5'-ACCTCAACAAGGTAGCTAGCGTCGGCCCGT-3'	Sense strand primer for creation of N41A change in conjunction with a new <i>NheI</i> site
N41Arev	5'-ACACGGCCGACGCTAGCTACCTTGTGAGGTTTCG-3'	Antisense strand primer overlapping with N41Afor
N41Dfor	5'-ACCTCAACAAGGTAGACAGCGTCGGCCCGT-3'	Sense strand primer for creation of N41D change in conjunction with a new <i>AccI</i> site
N41Drev	5'-ACACGGCCGACGCTGTCTACCTTGTGAGGTTTCG-3'	Antisense strand primer overlapping with N41Drev
N41Qfor	5'-ACCTCAACAAGGTACAAGCGTCGGCCCGT-3'	Sense strand primer for creation of N41Q change in conjunction with a new <i>RsaI</i> site
N41Qrev	5'-ACACGGCCGACGCTTTGTACCTTGTGAGGTTTCG-3'	Antisense strand primer overlapping with N41Qrev

TABLE II  
Hemagglutination assay of PNA and its mutants

Values represent hemagglutinating activity of wild-type and mutant PNAs using desialylated human O<sup>+</sup> red blood cells. Neither rPNA nor any of the other mutants showed inhibition with 100 mM *N*-acetylgalactosamine.

PNA species	MHC <sup>a</sup>	Gal	MeαGal	MeβGal	Lactose	LacNAc	T-antigen
	μg/ml				mm		
rPNA	0.3	4.5	1.0	1.5	1.5	6	0.12
N41A	0.3	4.5	2.0	1.5	1.5	6	12.0
N41D	0.3	4.5	1.0	1.5	1.5	6	0.48
N41Q	0.3	4.5	1.0	1.5	1.5	6	0.03

<sup>a</sup> MHC, minimum hemagglutination concentration.

tant species were perfused over the sensor chip containing biotinylated T-antigen (0.5–0.03  $\mu\text{M}$ ) at a flow rate of 5  $\mu\text{l}/\text{min}$  until equilibration was attained. Subsequently the off rates were determined by passing a solution of lactose (5 mM) in the same buffer at a flow rate of 50  $\mu\text{l}/\text{min}$ . The surface was regenerated by a 5-s pulse of NaOH (1 mM) flowing at 50  $\mu\text{l}/\text{min}$ .

**Data Analysis**—Association ( $k_1$ ) and dissociation ( $k_{-1}$ ) rate constants were obtained by nonlinear fitting of the primary sensorgram data using the BIA evaluation software, Version 3.0. The dissociation rate constant is derived using the equation:

$$R_t = R_{t_0} e^{-k_{-1}(t-t_0)} \quad (\text{Eq. 1})$$

where  $R_t$  is the response at time  $t$ ,  $R_{t_0}$  is the amplitude of the initial response, and  $k_{-1}$  is the dissociation rate constant. The association rate constant  $k_1$  can be derived using Eq. 2 from the measured  $k_{-1}$  values.

$$R_t - R_{\text{max}}(1 - e^{-(k_1 C + k_{-1})(t-t_0)}) \quad (\text{Eq. 2})$$

where  $R_t$  is the response at time  $t$ ,  $R_{\text{max}}$  is the maximum response,  $C$  is the concentration of the analyte in the solution,  $k_{\text{on}}$  is the association

TABLE III  
Kinetics for the binding of rPNA and its mutants to immobilized T-antigen

Values in parentheses indicate standard errors.

PNA species	$k_{\text{on}}$	$k_{\text{off}}$	$K_a \times 10^5$
	$M^{-1} s^{-1}$	$s^{-1}$	$M^{-1}$
rPNA	5.9 ( $\pm$ 0.15)	0.03 ( $\pm$ 0.0007)	1.96
N41A	No binding	No binding	No binding
N41D	3.5 ( $\pm$ 0.11)	0.05 ( $\pm$ 0.001)	0.70
N41Q	6.3 ( $\pm$ 0.012)	0.02 ( $\pm$ 0.0008)	3.15

FIG. 2. Sensorgrams representing the concentration-dependent binding of rPNA (A), N41D (B), N41Q (C), and N41A (D) to immobilized T-antigen. The concentrations of rPNA (A), N41D (B), and N41Q (C) used were, from top to bottom, 500, 250, 125, 60, and 30 nM, while for the N41A mutant sensorgram, 500 nM of the protein alone is shown. RU, response units.

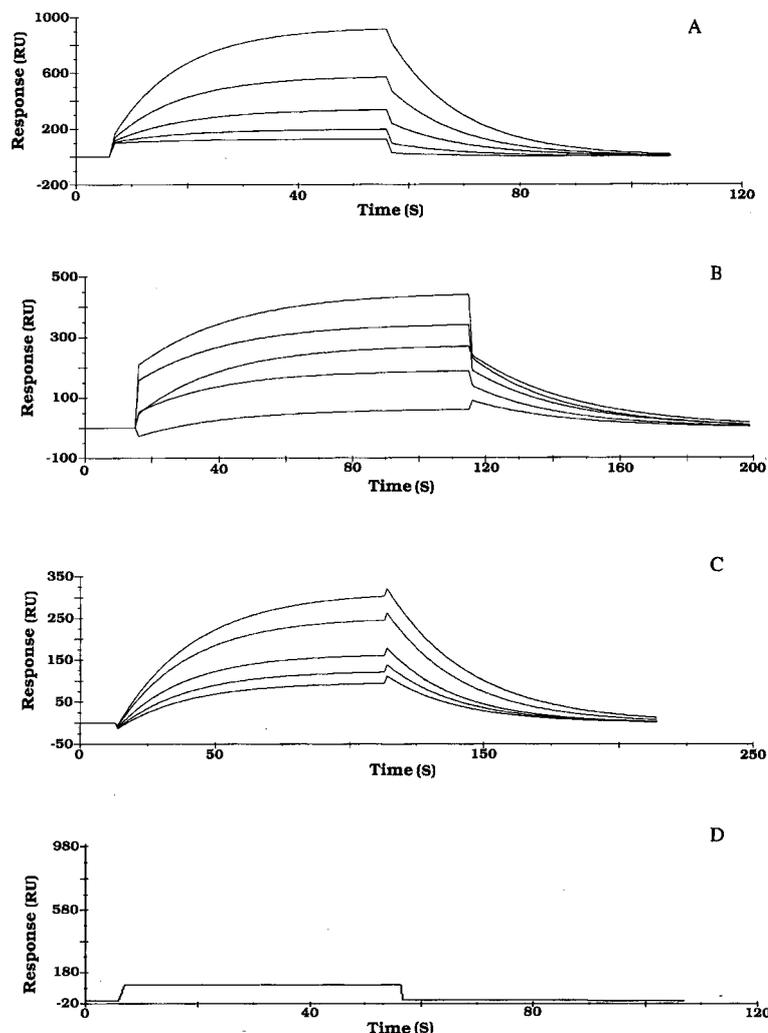
rate constant, and  $k_{\text{off}}$  is the dissociation rate constant.  $K_a$  ( $k_1/k_{-1}$ ) is the association constant, and  $K_d$  ( $k_{-1}/k_1$ ) is the dissociation constant.

**Modeling**—The coordinates of subunit A in the peanut lectin-T-antigen complex (PDB code 2TEP) were used for the modeling studies. Asn-41 was mutated to Gln, Asp, and Ala using the model-building software FRODO. The mutated coordinate set was loaded in INSIGHT II. The MODIFY option in the BUILDER module was used to modify the dihedrals  $\chi^1$ ,  $\chi^2$ , and  $\chi^{31}$  of Gln ( $\chi^1$  and  $\chi^{21}$  in the case of Asp-41) at  $10^\circ$  intervals. At each combination of  $\chi^1$ ,  $\chi^2$ , and  $\chi^{31}$  ( $\chi^1$  and  $\chi^{21}$  in the case of Asp-41), side chain contacts with sugar atoms and water oxygens (W1, W2, W3, and W4) were evaluated.

## RESULTS AND DISCUSSION

The hexopyranosyl residues of both lactose and T-antigen share an identical pattern of hydrogen bonds, nonpolar interactions, etc. with PNA. The higher affinity of PNA for T-antigen was ascribed to two additional water bridges it has with the protein over those observed with lactose (15, 17). These water-mediated contacts are established via Asn-41, Ile-101, and Leu-212 and the carbonyl oxygen of the acetamido group of the reducing end GalNAc residue of T-antigen as illustrated in Fig. 1. To address the role of one such water-mediated hydrogen bond *viz.* between Asn-41 and carbonyl oxygen of the acetamido group of the reducing end sugar (GalNAc) in T-antigen in the generation of the carbohydrate specificity of PNA, Asn-41 was mutated to a shorter uncharged nonpolar alanine residue and to two conserved amino acids, namely aspartic acid and glutamine.

**Construction of the Site-specific Mutants and Their General Properties**—The generation of N41A, N41D, and N41Q mutants were linked with the creation of a *NheI*, *AccI*, and *RsaI*



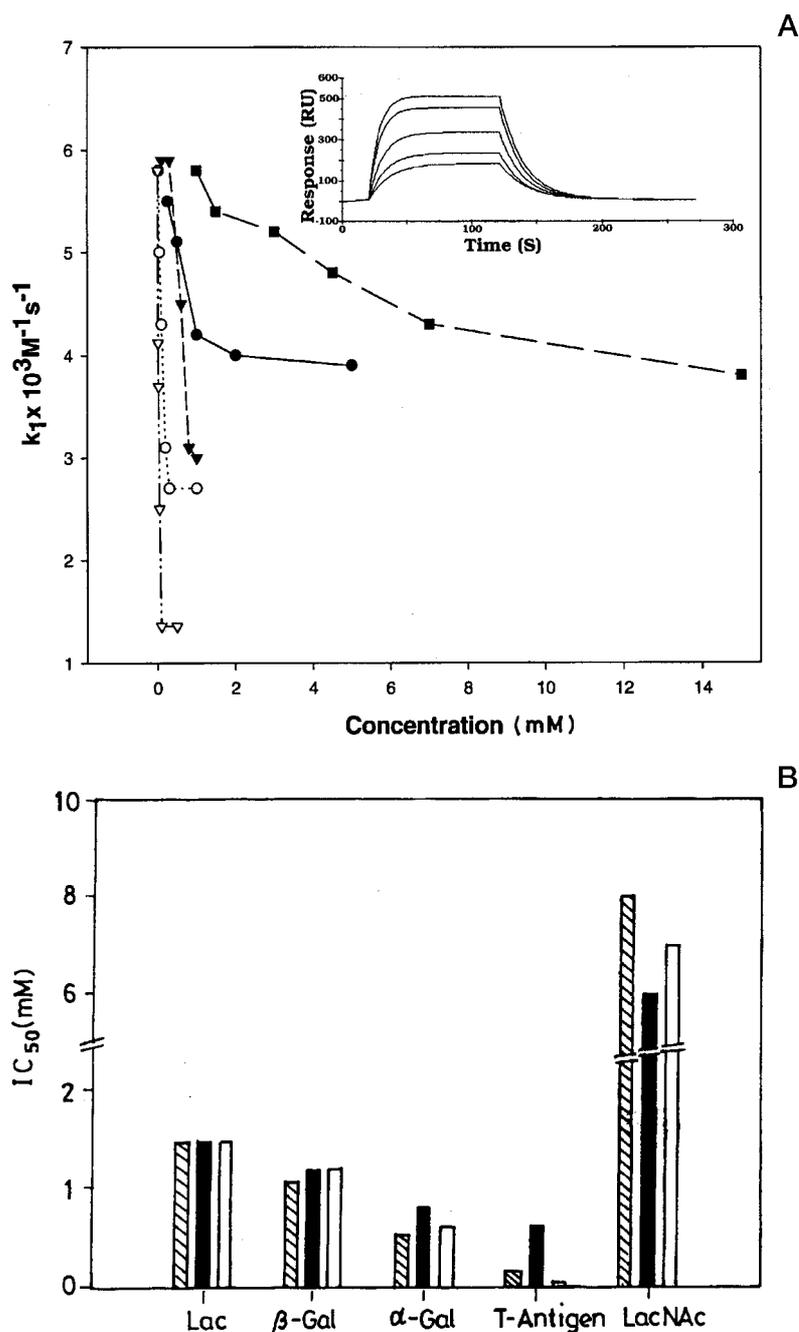


FIG. 3. A, inhibition of the binding of rPNA to immobilized T-antigen by various sugars. A plot of the decrease in the on rate in the presence of increasing concentration of various sugars is shown (●, lactose; ○, methyl- $\alpha$ -galactose; ▼, methyl- $\beta$ -galactose; ▲, T-antigen; ■, N-acetyllactosamine). *Inset*, sensorgram depicting the decrease in rate of binding of rPNA preincubated with varying concentrations of lactose. The concentrations of lactose used were 0, 0.25, 0.5, 1, and 2 mM from *top to bottom*, respectively. *RU*, response units. B,  $IC_{50}$  values for the interaction of various sugars with rPNA (crossed bars), N41D (black bars), and N41Q (empty bars).  $IC_{50}$  represents the concentration of sugar (mM) required to bring about a 50% loss in the association rate constant.  $\beta$ -Gal, methyl- $\beta$ -galactose;  $\alpha$ -Gal, methyl- $\alpha$ -galactose; LacNAc, N-acetyllactosamine.

site in the WT rPNA sequence (Table I). The mutants were confirmed by restriction analysis and sequencing. rPNA and all the mutants when expressed and purified showed a single band on an SDS-polyacrylamide gel ( $M_r = 27,000$ ). rPNA and all the mutants also eluted at the position corresponding to that of the WT PNA tetramer. All the PNA species could hemagglutinate desialylated red blood cells to comparable levels. Also all of them were inhibitable with galactose and its derivatives indicating that none of these mutations destabilize the carbohydrate binding pocket (Table II).

**Carbohydrate Specificity of the Mutants and WT PNA**—Hemagglutination inhibition studies show the binding of the various monosaccharides, lactose, and N-acetyllactosamine to the three mutants to be unaltered when compared with WT PNA. Nevertheless, when assayed for T-antigen binding, N41A shows markedly reduced inhibition when compared with the marginal decrease seen for N41D, whereas N41Q shows a 4-fold increase in the binding affinity (Tables II and III).

The binding characteristics of the mutants and WT protein were complemented with surface plasmon resonance studies, which helped to determine quantitatively the sugar binding affinities of mutants with respect to rPNA (23, 24). For this assay, biotinylated T-antigen was immobilized on a streptavidin chip followed by the injection of the protein. Sensorgrams of the concentration-dependent binding of rPNA and its mutants to immobilized T-antigen provide an estimate of the kinetic parameters involved in the interaction (Fig. 2, A–D; Table III). The on rate ( $k_1$ ) obtained for the binding of rPNA was in the range of values observed for carbohydrate-lectin interactions in general. The N41A mutant when passed over immobilized T-antigen showed no detectable change in response units. This is not surprising in view of the inability of this mutation to accommodate T-antigen in its binding site. N41D showed a marginal decrease in its association rate constant as well as its overall binding constant ( $3.5 \times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$  and  $7.0 \times 10^4 \text{ M}^{-1}$ , respectively). On the other hand, although N41Q exhibited a

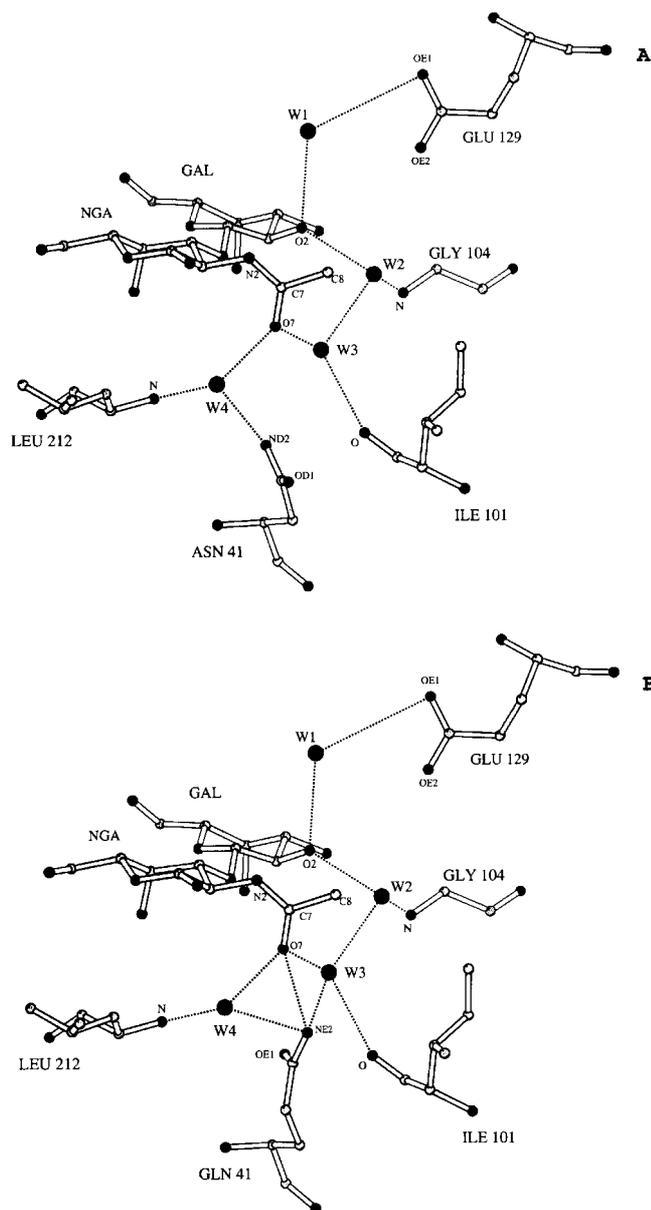


FIG. 4. A, water bridges between the wild-type lectin and T-antigen. B, interactions between the N41Q mutant and T-antigen. The figures were prepared using BOBSCRIPT (27).

marginal increase in its on rate when compared with rPNA, a lowered off rate ( $k_{-1}$ ) contributes to a 1.6-fold elevation in its overall binding constant (Table III,  $K_a = 3.15 \times 10^5 \text{ M}^{-1}$ ). Subsequently the sugars, whose binding affinities to the PNA species had to be estimated, were preincubated with the desired protein and passed over the immobilized T-antigen. The sugar binding affinity determined the extent and rate of binding of rPNA and its mutant counterparts to the immobilized T-antigen (Fig. 3). The concentration of the sugars required for 50% inhibition in the on rates ( $k_1$ ) for the binding of various sugars to rPNA and its mutant counterparts defines their potencies of binding to the lectin and its mutant counterparts.

The  $\text{IC}_{50}$  values derived from surface plasmon resonance experiments essentially corroborate the information obtained from hemagglutination inhibition studies. These studies show that while all the mutants retain their relative ligand binding affinities for the monosaccharides tested (methyl- $\alpha$ -galactose and methyl- $\beta$ -galactose) as well as the disaccharides such as lactose and *N*-acetylglucosamine, binding to T-antigen was affected in a considerable manner. Thus the N41D mutant had 4 times dimin-

ished affinity for T-antigen, whereas N41Q was 6 times more potent a receptor for the same disaccharide. A marked diminution in the affinity of N41A for T-antigen is also borne out by the fact that the appropriate sensorgrams for its binding to the immobilized T-antigen could not be obtained (Fig. 2D).

The structure and simple modeling studies provide a rationale for the activities of the mutants. The T-antigen molecule and the water bridges it makes with the wild-type lectin subunit A of the crystal structure of the complex are illustrated in Fig. 4A. Four water molecules, W1, W2, W3, and W4, are involved in the bridges. Those involving W1 and W2 are common to the lactose and the T-antigen complexes. W3 and W4 are also present in both the complexes, but only T-antigen, on account of the presence of the acetamido group, is capable of using them for water bridges. The water bridge involving Asn-41 is altogether abolished when it is mutated to alanine, leading to the decreased affinity of the sugar to the lectin. Furthermore, the removal of the side chain amide group opens up the lectin-sugar interface containing the water bridges to the solvent, which again could adversely affect binding affinity.

The carboxylate and the amide groups are similar in geometry, and the replacement of the latter by the former as in N41D cannot lead to drastic effects as in N41A. However, in the water bridge, for the same N . . . O and O . . . O distance, the former hydrogen bond would be weaker than the latter. This distance cannot be substantially reduced by a change in side chain conformation as the side chain of the residue is nearly as extended as it could be. Thus the mutation of Asn-41 to Asp could somewhat weaken the water bridge involving W4. There are of course instances where a change from asparagine to aspartic acid has led to substantially larger effects (10, 25, 26).

The mutation of Asn-41 to Gln is extremely interesting as it leads to increased affinity of the lectin to T-antigen. In a simple modeling study, the side chain of Asn-41 was replaced by that of Gln, and the three conformational angles  $\chi^1$ ,  $\chi^2$ , and  $\chi^{31}$  were systematically varied at regular intervals. This led to a very satisfactory situation, illustrated in Fig. 4B, at  $\chi^1 = -55$ ,  $\chi^2 = -65$ , and  $\chi^{31} = 35$ , which correspond to a highly plausible conformation. At these angles, the amide nitrogen forms a direct hydrogen bond with W4. Furthermore, the glutamyl side chain now forms hydrogen bonds with W3 as well. The enhancement of hydrogen-bonded interactions naturally leads to the enhanced affinity of the mutant for the sugar.

All the changes in the interactions resulting from mutations involve the acetamido group, which is present only in T-antigen among the sugars studied. Therefore, the affinity of the lectin to the other sugars remains unaffected.

In summary, these data highlight the importance of the water-mediated hydrogen bonds between the sugar atoms and functional groups in PNA in determining the exquisite specificity of the lectin for the tumor-associated T-antigenic structure. Furthermore these studies also demonstrate how three-dimensional structure can guide mutation analysis and rationalize the results of the analysis.

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