The Conformation of the T-antigen Disaccharide Bound to Maclura pomifera Agglutinin in Aqueous Solution*

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The complex of Maclura pomifera agglutinin with the T-antigen disaccharide (β-d-Gal-(1→3)-α-d-GalNAc-(1→O)-Me) was investigated by NMR spectroscopy in aqueous solution. Intramolecular transferred nuclear Overhauser enhancement (NOE) effects between the monosaccharide units in the crystal. Our results demonstrate that the crystallization of a protein-carbohydrate complex can interfere with the delicate process of carbohydrate tumor markers (4) called Thomson-Friedenreich phenomenon, within STD experiments it offers a convenient tool to map protein-ligand contacts in solution. Although saturation transfer is a well known NMR phenomenon, within STD experiments it offers a convenient tool to map protein-ligand contacts in solution.

EXPERIMENTAL PROCEDURES
Sample Preparation—M. pomifera agglutinin was prepared as described previously (14). For the NMR experiments, the protein was dissolved in phosphate-buffered saline/D2O (10 mM phosphate, 50 mM NaCl, pH 7.4) and repeatedly concentrated and diluted in Centricon transfer difference; trNOE, transferred NOE; trROE, transferred rotating frame Overhauser enhancement.

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concentrators (Amicon) with phosphate-buffered saline/D$_2$O buffer. The NMR sample finally contained 2.14 mg of protein (126 nmol binding sites) and 0.6 mg (1.52 mmol) of disaccharide yielding a protein-to-ligand ratio of 1:12.

**NMR Spectroscopy**—All spectra were acquired on a DRX500 spectrometer (Bruker) equipped with a triple probe and a Z gradient. Chemical shifts are reported relative to internal 3-(trimethylsilyl)propionic acid. trNOE and Quiet-trNOE (15, 16) experiments (50–350 ms mixing time, 2048 data points × 512 increments × 16 transitions) were performed with the standard ROESY pulse sequence. Residual water was suppressed during the recycle delay and the mixing transitions) were performed with the standard ROESY pulse sequence.

Integration of the spectra was performed with Xwinnmr (Bruker). The raw data were normalized against the decay of the signal H-4 that was fitted to an exponentially decaying function and extrapolated back to an intensity of 100% at zero mixing time. The curves for the trNOEs H-1’—H-3 and H-1’—H-4 were then fitted to an exponential function of the form $f(t) = A \times (1 - \exp(-b \times t))$ with A and -b being adjustable parameters. The initial slope of this curve determined from the first derivative of the function ($f'(0) = A \times b$) was used to calculate experimental distances with the isolated spin-pair approximation using the trNOE H-1—H-2 and a corresponding distance of 2.43 Å as reference.

**Molecular Modeling/MMC Simulations**—To compare the NMR-derived structure of the ligand with the crystal structure, protons had to be added to the protein data base structure (Protein Data Base code 1jot). This and further modeling was done with Sybyl 6.2 (Tripos). The MMC Simulation (20) was performed with ggrep 2.7 (21) on a Silicon Graphics O2 workstation. To reach all accessible conformations of the disaccharide, the MMC simulation was performed with 2 × 10$^6$ Monte Carlo steps and a temperature factor of 2000 K. A Fortran program searched all accepted conformations to find the ones that fulfilled the experimentally derived distances for the trNOEs H-1—H-3 and H-1—H-4. Glycosidic torsional angles are defined using the protons as $\phi$ (H-1’—C-1—O-1-C-x), $\psi$ (C-1—O-1—C-x—H-x) and $\omega$ (O-6—C-6—C-5—O-5), with x being the aglyconic site.

**RESULTS**

In the T-antigen structure, disaccharide 2 is $\alpha$-glycosidically linked to the peptide backbone. From an NMR spectroscopic point of view, the reducing disaccharide 2 used for the crystal structure (1) shows two sets of resonances, one for the $\alpha$- and one for the $\beta$-configuration of the ligand. For this NMR investigation we have therefore used the T-antigen disaccharide as the $\alpha$-methylglycoside 3.$^2$ The methyl group in 3 substitutes for the linkage to the amino acid serine or threonine and thus serves as a probe to determine protein-ligand interactions at the reducing end of the disaccharide. A titration of the lectin with ligand was performed to find the protein-to-ligand ratio giving intense trNOEs and a good signal-to-noise ratio. This yielded a protein-to-ligand ratio of 1:12, which was also used for all NMR experiments.

$^2$ R. Bukowski and T. Weimar, manuscript in preparation.

![Chart with chemical structures and spectra](chart.png)
**STD Experiments**—To verify the binding mode of the T-antigen disaccharide bound by MPA in aqueous solution, one-dimensional STD experiments are the method of choice since this type of NMR experiments creates a map of proton resonances of the ligand which are accessible for the protein. The STD experiment makes use of the effective magnetization transfer (spin diffusion) of the slow tumbling protein and complex to point out ligand protons in close proximity to protein protons during the time the ligand spends in the complex. During the experiment, protein resonances are saturated at a position in the spectrum where no ligand resonances appear and part of this magnetization is transferred to the ligand in the binding site. After subtraction of a reference spectrum, with the frequency of the saturation pulse set far away from all resonances, the STD spectrum remaining shows only ligand resonances in contact to the protein (Fig. 1B). Since only the resonances from the GalNAc moiety and H-1 from Gal are significantly above the noise level in the STD spectrum, this experiment supports the binding mode of the crystal structure. A closer inspection of the crystal structure (1) reveals that the proton H-5* of the ligand in the crystal has a distance of only 2.58 Å to the ε-proton of Tyr-78. This should allow an effective magnetization transfer to proton H-5* but the actual enhancement is, at best, very weak. This fact points to a deviation of the ligand conformation in solution from the crystal structure (compare below).

**Intermolecular trNOEs**—Like many lectins, MPA has a specific but low affinity for ligands, here α-galactose-containing carbohydrates (22). For the ligand molecule, this leads to a fast exchange between the protein-bound state and the free state permitting the observation of trNOEs. Often only trNOEs involving the proton resonances of the ligand molecule are observable; however, for the complex investigated here we also observed intermolecular trNOE effects between protein and ligand resonances (Fig. 1D). This is attributed to the fact that MPA has a moderate size (68 kDa as a tetramer), and therefore, some individual resonances of protein protons are relatively narrow and could thus be observed in a two-dimensional spectrum. As can be seen from Fig. 1D, intermolecular trNOEs are found between aromatic amino acid protons, and all ligand protons that show an enhancement in the STD spectrum. Thus, major mediators for the magnetization transfer from the protein to the ligand are the aromatic residues in the binding site of the lectin. These amino acid residues (Phe-47, Tyr-78, Tyr-122, and Trp-123) form a hydrophobic pocket around the GalNAc unit and provide favorable van-der-Waals interactions to the ligand in the binding site (1). On the basis of the crystal structure of the complex, some of the aromatic residues could be assigned (see Table I). Based on the contact to H-2 an unequivocal assignment for the δ and ε proton resonances of Phe-47 was possible. Unfortunately, the other cross-peaks could only be assigned to the amino acid residues. Particularly for Tyr-78, which provides a stacking interaction to the GalNAc moiety, the δ and ε resonances seem to be degenerate since H-3, H-4, H-5, H-6, H-1’, and the O-methyl group give a cross-peak to resonances at 6.93 ppm. Analogous to the STD experiments no intermolecular trNOEs involving proton H-5* of the disaccharide are observed. The reason for the discrepancies involving H-5* will become clear when the conformation of the ligand in the bound state is derived in the following.

**The Bound Conformation of the Ligand**—Intramolecular trNOEs of ligand resonances, especially across the glycosidic linkage, provide information about the conformation of the bound disaccharide in aqueous solution. Two intense interglycosidic trNOEs, namely H-1’—H-3 and H-1’—H-4, and a very weak effect H-1’—H-5 can be observed (Figs. 1C and 2). Given the r^6 dependence of the NOE effect from the distance between the protons, the integers of H-1’—H-3 and H-1’—H-4 translated into almost the same experimental internuclear distance. By using the two-spin approximation, we calculated 2.65 and 2.75 Å for the distance between H-1’ and H-3 or H-4, respectively. In the crystal structure of the disaccharide complexed by MPA (ψ0 = 39°—8°) these interproton distances are 2.05 and 3.7 Å (1). Thus an interglycosidic trNOE H-1’—H-4 would not be observable in this conformation of the disaccharide. Such a discrepancy can either be caused by an indirect magnetization transfer (spin diffusion) or a variation of the conformation at the interglycosidic linkage. Although it is very unlikely that a strong trNOE such as H-1’—H-4 arises entirely through spin diffusion, we performed trROE (19) and Quiet-trNOE (15, 16) experiments (data not shown) which gave no indication for a indirect magnetization transfer being responsible for this trNOE. For the small interglycosidic trNOE H-1’—H-5, as well as for all other very weak effects, the situation is different. These effects had an integral much smaller than 1% at 350-ms mixing time, and the control experiments identified them to be spin diffusion artifacts. Therefore, the

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**Table I**

Proposed assignment of some aromatic amino acid resonances of MPA based on the crystal coordinates of the lectin with 2 (1) and intermolecular trNOE effects to disaccharide 3 (compare Fig. 1D)

| Ligand proton | ppm | Aromatic residue from MPA |
|---------------|-----|---------------------------|
| O-Me          | 6.65| Tyr-122                   |
|               | 6.87| Tyr-122                   |
|               | 6.93| Tyr-78                    |
| H-1           | 7.41| He Phe-47                 |
|               | 7.27| H6 Phe-47                 |
|               | 6.87| Tyr-122                   |
| H-2           | 7.41| He Phe-47                 |
| H-3           | 6.93| Tyr-78                    |
| H-4           | 6.93| Tyr-78                    |
| H-5           | 6.93| Tyr-78                    |
| H-6 + H-6**   | 6.65| Tyr-122                   |
|               | 6.87| Tyr-122                   |
|               | 6.93| Tyr-78                    |
|               | 7.48| Trp-123                   |
|               | 7.54| Trp-123                   |
| H-1’          | 6.93| Tyr-78                    |

*a* The resonances of the protons H-6 and H-6* cannot be separated in one-dimensional experiments.

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**FIG. 2.** TrNOE build-up curves for the trNOEs H-1—H-2, H-1’—H-3 and H-1’—H-4 from which interproton distances have been extracted. The curves have been fitted to an exponential function (see “Experimental Procedures” for details).
trNOE H-1′—H-5 does not have any value for the structure elucidation of the bound disaccharide.

From the trNOE data it is therefore obvious that the experimental distances for the trNOEs H-1′—H-4 and H-1′—H-3 in fact point to conformational differences of the bound disaccharide in aqueous solution as compared with the crystal structure. By using the experimentally derived distances between the protons H-1′ and H-3, respectively H-4, we aimed to define the bound conformation of the disaccharide in aqueous solution with an MMC simulation (20). Such a simulation for the disaccharide was performed with a temperature factor of 2000 K. Although this simulation gave a relative distribution of conformational families that differed slightly from the distribution at ambient temperature, the elevated temperature factor enables the simulation to reach all accessible conformations of the disaccharide. Based on this MMC simulation, all conformations resembling the experimentally derived data were searched and are displayed in a population plot in Fig. 3. For this the distances between H-1′—H-3 and H-1′—H-4 were defined to be in a range of 2.4–3.0 and 2.5–3.1 Å, respectively. These ranges took into consideration the inaccuracy of the experimental data and of the two-spin approximation. In Fig. 3 the conformations that satisfied these distance ranges are overlaid with the population plot of 3. Depending on the range of distances used, slightly narrower or wider regions of the population plot were found to be in accord with the experimental data, but the general shape of the highlighted region did not change.

Fig. 3 clearly shows that the bound conformation of the disaccharide in aqueous solution was not identical to the bound conformation in the crystal structure. Additionally, two conformational families were in accord with the experimental distances, and these conformational families did not belong to regions that are highly populated by the unbound disaccharide in solution. The two conformations that satisfied the experimental restraints best (ψ/φ = 45°–65° and –65°–18°) are displayed in Fig. 4. It is interesting that the protons H-1′, H-3, and H-4 are in the same relative position in these two conformations and that both conformations seem to be populated by the uncomplexed disaccharide with approximately the same probability (compare Fig. 3). Modeling of the complex based on the crystal structure did not reveal forbidden contacts with the protein that would exclude one of these conformations. More-
over, these conformations place proton H-5’ in distances between 3.0 and 4.7 Å to the α- and ε-protons of Tyr-78 which readily explained why a very weak STD effect but no intermolecular trNOE was observed. Unfortunately, the observed experimental effects did not allow us to decide whether one of the two conformations or a range of conformations is selected by MPA in solution.

A further point to address is the conformation of the α-linked O-methyl group in the complex. In the crystal structure with MPA the reducing oxygen of 2 (at 2.2-Å resolution no hydrogen atoms are resolved) in the α-position of GalNAc points directly toward the aromatic ring system of Tyr-122. In this arrangement the structure does not allow the accommodation of a methyl group or a linkage to the amino acids serine or threonine. Intermolecular trNOEs and STD effects (Fig. 1) indicated a short distance between the O-methyl protons of 3 and Tyr-122 and Tyr-78 of MPA in solution. Thus, in the bound form of 3 the methyl group has the same protein environment as the hydroxyl group of 2 in the crystal structure. There was also a strong intramolecular trNOEs from the O-methyl group to H-1 and small effects to all other protons of the GalNAc moiety; the latter could be shown to be spin diffusion artifacts by means of trROE and Quiet-trNOE experiments. From this we concluded that the α-angle of the GalNAc unit in the complex has a small negative value (between approximately −20° and approximately −60°) because such an orientation satisfied the trNOE to H-1 and is in accord with the exo-anomeric effect. Support for this assumption came from the closely related crystal structure of jacalin with methyl-α-d-galactopyranoside (9), where four carbohydrate ligands in four binding sites of the tetrameric lectin also display φ angles in the range between −24 and −73°. We have, therefore, used a φ angle of −25° for the models presented in Figs. 4 and 5. Independent of the exact torsional angle φ, the O-methyl group always clashed into the aromatic ring of Tyr-122 when the crystal coordinates of MPA were used (Fig. 5). A torsional angle Ca-Cβ-Cy-Cδ of Tyr-122 of −48° is responsible for this steric hindrance. In the crystal structure, this angle yields favorable interactions between the aromatic ring and the reducing α-hydroxyl group of 2 but places the aromatic ring too close to the carbohydrate to accommodate the O-methyl group of 3. Jacalin has the same amino acid arrangement around the binding site as MPA but in the complex with methyl α-d-galactopyranoside the torsional angle Ca-Cβ-Cy-Cδ of the four Tyr-122 residues have values between 10° and 30°. Such a torsional angle also opens up a cleft on the protein surface between Tyr-78 and Tyr-122 so that the O-methyl group in the α-position of Gal or GalNAc can be accommodated in the binding site and, additionally, provides hydrophobic and van-der-Waals interactions to the methyl group. We therefore propose the torsional angle Ca-Cβ-Cy-Cδ for Tyr-122 in the complex of MPA with 3 to be between 10° and 30°, as used in the presentation of Fig. 5.

**DISCUSSION**

Is the crystal structure of the T-antigen-MPA complex also representative for the recognition of the ligand by the lectin in aqueous solution? We have demonstrated here that the answer has to be divided into two parts. The general recognition of the ligand, i.e. the manner the α-GalNAc unit of the disaccharide is bound by the lectin and the arrangement of the aromatic amino acids around the ligand in the binding site, is verified by STD and trNOE experiments. These experiments prove that the aromatic amino acids are in close vicinity to the ligand either by direct saturation transfer from protein to ligand or by intermolecular trNOE effects between ligand and protein. From our experiments there is no doubt that the GalNAc moiety in aqueous solution is in fact in the same position as in the crystal. On the other hand the NMR experiments uncover structural differences between crystal and solution. The interpretation of the trNOE data demonstrates that the water-mediated hydrogen bonds observed in the crystal structure between hydroxyl protons of the Gal unit and the protein are crystal-specific and artificially stabilize the β(1→3) linkage to GalNAc in a low energy conformation. In solution, when these H bonds are absent, the lectin recognizes one or more conformational families of the ligand that are not highly populated by the free ligand. Although our NMR data give no indication whether a single conformation or a mixture of the two conformational families are bound by the lectin, the advantage for the selection of a range of conformations would be a smaller loss of entropy and, hence, a larger free enthalpy gain during complex formation. Preliminary thermodynamic investigations of different complexes of MPA are in accord with a flexible model of ligand recognition.

Our investigation also provides strong evidence for flexibility of the protein during the recognition of the ligand. The aromatic ring of Tyr-122 has to move from its position in the crystal structure complex with 2 in order to accommodate the O-methyl group of 3. The cleft which opens up when the torsional angle Ca-Cβ-Cy-Cδ is around 20° should also provide enough space for the glycosidic linkage to the peptide backbone of T-antigen structures like 1.

Carbohydrate recognition, like many other biological processes, takes place in solution or the interface between solution and a surface, i.e. membranes. This report demonstrates that NMR spectroscopy is an important tool to validate and improve crystal structures of biomolecular complexes by taking the solution environment into account. Since it is important for the general understanding of carbohydrate recognition whether and how the mobility of amino acid side chains such as Tyr-122 on the protein surface influences the ligand recognition of MPA, we have started long time molecular dynamics simulations of MPA-T-antigen complexes in water. These simulations should also help to decide whether a single or a range of conformations of the ligand is selected by MPA.

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