Observation of the Anti Conformation of a Glycosidic Linkage in an Antibody-Bound Oligosaccharide

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Crystal structures of oligosaccharide−antibody complexes frequently reveal details of intermolecular interactions that are confined to the small surface area of the antigen (∼300 Å^2) buried in the antibody site.[1] The flanking saccharide residues are presumably too disordered to provide well-defined electron density. Herein we show that transferred NOE experiments provide an invaluable insight into oligosaccharide conformation for flanking residues that are crystallographically "invisible" (Cygler, et al., unpublished data).[1] It is further shown that the glycosidic linkage between α-D-Man and the first flanking hexose (α-L-Rha) of pentasaccharide **1** adopts the anti conformation (ψ_H = *ca.* 180°) (Figure 1, Supporting Information) to avoid unfavorable contacts with the protein surface. The binding of this higher energy conformation results in a significantly lower affinity without changing the interactions with the primary recognition unit. To date, only the dominant syn conformer (ψ_H = *ca.* 0°) has been observed for oligosaccharides bound to lectins or antibodies.[3]

The monoclonal antibody, Se155-4, binds the repeating unit of the *Salmonella paratyphi* B O antigen

[3)-α-D-Gal*p*(1→2)[(α-D-Abe*p*(1→3)]
α-D-Man*p*(1→4)α-L-Rha*p*(1→] (2)

Several crystal structures of the antibody Fab[1,4] or the single-chain antibody (scFv)[5] have been solved, and complementary thermodynamic[6] and NMR studies[4] have provided a detailed description of the molecular recognition between the oligosaccharide and antibody binding site. Crystallography and calorimetry data showed that the primary recognition unit was restricted to the core trisaccharide **3** and that the 3,6-dideoxyhexose residue, abequose, is buried in a binding site lined by aromatic amino acids.[1,4,6] To study interactions between the protein and flanking saccharide residues, the conformation of the synthetic pentasaccharide[7] epitope **1** was investigated in the bound state.

Dramatically different affinities observed for Se155-4 binding to the two isomeric heptasaccharides (**4**) and (**5**)[6] provided the initial impetus for this work. The K_A for binding of **4** was 50-fold lower than that of **5**.[6] Heptasaccharides **4** and **5** differ only in the relative position of the abequose residue and are obliged to bind in a monogamous fashion with respect to the position of the flanking residues (Figure 1). Modeling of the minimum energy conformation of **4** in the binding site of a single chain antibody (scFv) revealed a steric clash between the protein and the central galactose residue, whereas heptasaccharide **5** could easily

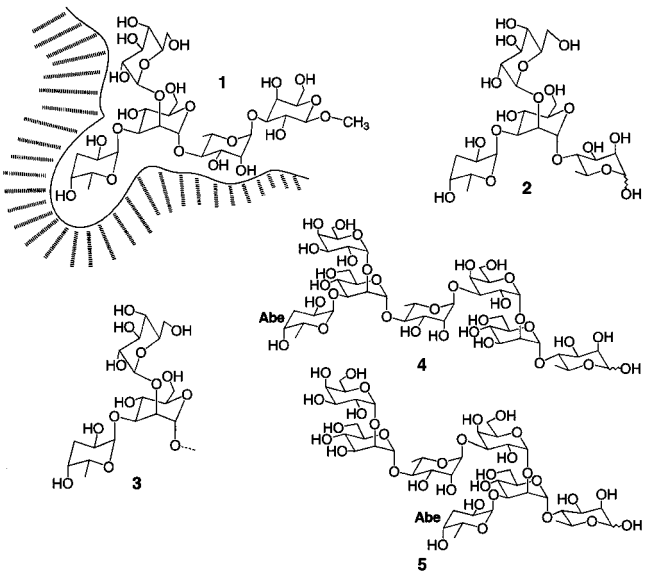

Figure 1. Oligosaccharide structures.

fit into the binding site with the terminal α-D-Man*p*(1→4)α-L-Rha*p* glycosidic linkage in the minimum energy syn conformation.[8] It was postulated that the reorientation of the rhamnose and galactose residues of **4** on binding was likely the major cause of the reduction in the K_A value.[6,8] However, molecular modeling alone could not distinguish between two possibilities for the bound conformation of the flanking residues, (i) a large conformational change confined to the Man→Rha linkage or (ii) small shifts from the global minimum in all of the glycosidic linkages combining to position the terminal galactose residue away from the protein surface. An unpublished crystal structure of the heptasaccharide **4** complexed with Fab suggested a pseudo-anticonformation for the Man→Rha conformation in the bound state; however, no electron density was observed for the subsequent three hexose residues (Gal→Man→Rha) at the reducing terminus of **4** (Cygler, et al., unpublished data), nor is it known whether crystal packing forces could have influenced the conformations of the flanking residues.

On the basis of the dissociation rate for trisaccharide **3**, the complex of **1** and Se155-4 Fab should be appropriate for the measurement of bound ligand NOEs in the fast exchange regime,[4] which permits the observation of transferred NOEs (TRNOEs). Under these conditions, the TRNOEs may be used to determine the protein-bound conformation of an oligosaccharide but only if the effects of kinetic exchange and spin diffusion are considered.[9] To treat these effects adequately, a full relaxation matrix coupled with a kinetic matrix was required[10,11] so that spin-diffusion pathways could be modelled. TRNOESY spectra may, therefore, be acquired at longer mixing times, with the advantage that correlations have improved signal-to-noise ratios. Cross-peaks due to spin diffusion also in turn carry additional structural information, since the build-up of this magnetization can be predicted from full relaxation matrix calculations.

The solution structure of pentasaccharide **1** in the absence of protein has been studied by a series of offset-compensated ROESY spectra (Bundle, Otter, et al, manuscript in preparation). The main

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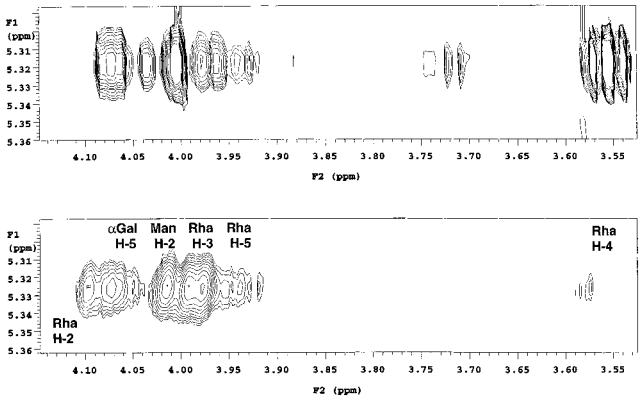

Figure 2. Comparison of ROESY and NOESY spectra showing NOE correlations with the Man H-1 resonance for the free and bound states of pentasaccharide **1**. (A) Section from a 600-MHz 2D offset-compensated ROESY spectrum (300-ms mixing time) of **1** in D_2O solution at 300 K. (B) The same region of a $T_1\rho$-filtered transferred NOESY spectrum[13] (300-ms mixing time) recorded at a temperature of 310 K for a solution of scFv (8 mM) dissolved in 14 mM PBS, pD 7.0 containing **1** (80 mM) (see Supporting Information for experimental conditions). The NOE between Man H1/Rha H2 results from spin-diffusion.

reporter region where the majority of ROE connectivities were found shows ROEs between the α-Gal, Abe, and Man residues similar to those reported previously.[4] However, of interest to the current study are the ROEs describing the α-D-Man*p*(1→4)α-L-Rha*p* linkage. ROE enhancements can be measured between Man H-1 and Rha H-3/4/5 (Figure 2A). Enhancements to Rha H-3 and H-5 are indicative of ∼6% anti conformation (ϕ_H, ψ_H ca. −30°, 180° by grid searching), whereas the very large enhancement at Rha H-4 is typical of the dominant syn conformation(s) for glycosidic linkages in free solution.

The conformation of pentasaccharide **1** in the bound state was deduced from TRNOE data measured for solutions of **1** and scFv in buffered D_2O solution (Figure 2B). Cross-peaks which arise as a result of spin diffusion were identified by the acquisition of transferred ROESY (TRROESY) spectra.[12] The NOE enhancements (Table 1, Supporting Information) suggest that the branched trisaccharide Gal[Abe]Man moiety of **1** was bound in a manner the same as that reported for the complex of **3** with Se155-4 Fab.[4]

The striking observation in the $T_1\rho$-TRNOESY[13] spectra is the almost complete disappearance of the TRNOE between Man H-1 and Rha H-4 (Figure 2B), whereas in the free solution state, a very large ROESY and NOESY (data not shown) enhancement was observed between these two protons (Figure 2A). It is also clear that there is an increase in the relative TRNOE between Man H-1 and Rha H-3, and the correlation to Rha H-5 is retained (Figure 2B). It is concluded that the sugar undergoes a significant conformational change upon binding to the protein. This is in sharp contrast to the preliminary results obtained for the tetrasaccharide moiety (**2**) (i.e., pentasaccharide **1** less the terminal galactose residue) in complex with Se155-4 scFv. In this case, the strong NOE correlation between Man H-1 and Rha H-4 is retained, and the enhancements to Rha H-3 and H-5 are lost on binding, suggesting that the dominant syn solution conformation is bound by antibody. These data are consistent with unpublished crystal structure data for a Fab−heptasaccharide **5** complex (Cygler, et al., unpublished data).

To determine the bound conformation of **1**, theoretical NOEs were back-calculated for models of the pentasaccharide complex using the full relaxation matrix program MDNOE2.[14] The NOE calculations used nonexchangeable protons of the ligand and protein binding site (Table 1 legend, Supporting Information). Models were created by generating 10 pseudorandom conformations of the pentasaccharide−scFv complex and then subjecting them to restrained simulated annealing (Table 1, Supporting Information) in the scFv binding site using a modified version (see Supporting Information) of the method described by Rutherford and Homans.[15] This created a single family of conformations [ϕ_H (−30 to −50°), ψ_H (135 to 160°)] (Figure 2, Supporting Information) that satisfied all the intramolecular TRNOE restraints for bound pentasaccharide **1**. Back-calculations of the NOEs were performed on the lowest energy conformation (Table 1, Supporting Information), and NOE ratios across the Man→Rha linkage were in general agreement with observed values including those arising from spin-diffusion. These conformations do not correspond precisely with the anti conformation predicted for the free sugar (ϕ_H/ψ_H −30°, 180°). Favorable interactions between the Rha *C*-6 methyl group and tryptophan and tyrosine residues (Figure 2 of Supporting Information) may account for this perturbation from the solution anti conformation.

It is clear from NMR and modeling data that the Man1→4Rha linkage of **1** does not bind in a slightly perturbed version of the dominant solution conformation (global minimum conformer) but undergoes a significant conformational change on binding to scFv.

A growing body of experimental evidence, primarily from NMR studies of carbohydrates in DMSO[16,17] and recently in aqueous solution,[18−22] have shown that the anti conformation is readily populated. To date, however, transferred NOE studies of oligosaccharide−antibody/lectin complexes have reported that the protein either binds the global energy minimum conformation or selects one of the major solution conformers present in conformational equilibrium.[3] In this study, the protein binds the sugar with a linkage adopting a folded conformation which is similar to the low energy anti conformation detected for the free oligosaccharide in solution. The cost of binding a high energy conformation is reflected in a reduced free energy of binding ($\Delta\Delta G = +1.4$ kcal mol^{-1}) for the binding of **1** compared to that of α-D-Gal*p*(1→2)[(α-D-Abe*p*(1→3)]α-D-Man*p*(1→4)α-D-Glc*p*-(1→4)α-D-Glc*p*, a pentasaccharide that has a minimum energy conformation where the flanking residues avoid contacts with the protein (Bundle et al., unpublished results). Although higher energy conformations have been detected for *C*-linked oligosaccharides in exchange with an enzyme,[18] this appears to be the first report of an antibody or lectin reversibly binding a high-energy conformer that is only a minor component of the global population of free oligosaccharide conformers. We note that this conformational feature is found only in that portion of an extended pentasaccharide epitope (**1**) that lies outside the formal antibody binding site and illustrates a mechanism for modulation of affinity without changing the primary recognition unit.

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Supporting Information Available: Experimental details and Table 1 and Figures 1 and 2 (7 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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