

Structure of a single-chain antibody variable domain (Fv) fragment complexed with a carbohydrate antigen at 1.7-Å resolution

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ABSTRACT We describe here the 1.7-Å resolution structure of a single-chain antibody variable domain (scFv) molecule, based on the carbohydrate-binding antibody Se155-4, complexed with the trisaccharide ligand α -D-Gal(1→2)[α -D-Abe(1→3)] α -D-Manp1→OMe, where Abe is abequose. The scFv expressed in *Escherichia coli* has the variable region light chain to heavy chain polarity with the domains connected by a 19-residue linker. Although the linker is partially disordered in the crystal, the packing of the molecules suggests a monomeric state of the scFv. The carbohydrate adopts a different conformation about the Man-Gal linkage than was observed previously in the Fab-trisaccharide complex. Instead of a direct hydrogen bond between O2^{Abe} and O2^{Gal}, these two atoms are bridged by a water molecule in the present complex.

The demonstration that the separate light chain variable region (VL) and heavy chain variable region (VH) domains, which form the antibody variable domain (Fv) and contain the antigen binding site, could be fused into a single tandem polypeptide was a landmark in antibody engineering (1, 2). Crystallization of a single-chain Fv (scFv) was described recently (3) followed by a preliminary report of the structure,[¶] yet a detailed structure of such a molecule has not been reported. Only the structures of recombinant Fv molecules consisting of separate VL and VH domains were described (refs. 4–6, also ||). We report here the high-resolution crystal structure for a scFv molecule, complexed with its carbohydrate antigen, complementing the data for the parent Fab of the murine antibody Se155-4 in both its complexed and native Fab forms (7, 8).

Although Fv molecules consisting of separate VL and VH domains associate in a way very similar to that in their parent Fab fragments and display full antigen recognition (4), stability is greatly enhanced when they are expressed as a single-chain protein, in which the two domains are connected by a linker segment of \approx 15 amino acid residues (9). When combined with toxins or radioisotopes, the scFv proteins may be superior to other approaches for cancer therapy because their small size allows greater tumor penetration and more rapid clearance rates (10). Two practical considerations in scFv design are (i) maintaining solubility and (ii) optimizing linker sequences to minimize the possibilities of protease attack and/or minimize scFv oligomerization.

We have produced (11) in *Escherichia coli* several versions of a Se155-4 scFv specific for an oligosaccharide epitope that is characteristic of *Salmonella* serogroup B LPS. Molecules with a VL → VH order and a linker sequence derived from the elbow region of the light chain exhibited good antigen binding characteristics. The phage display system identified a fully active scFv framework mutant 3B1 (Ile-77H → Thr or Ile77HThr) with improved solubility (12) and its crystal

structure in complex with the trisaccharide ligand, α -D-Gal(1→2)[α -D-Abe(1→3)] α -D-Manp1→OMe (where Abe is abequose), is reported here.**

MATERIALS AND METHODS

Gene Constructions. The scFv gene, preceded by the *ompA* secretory sequence, encoded Se155-4 scFv with a VL–VH domain polarity and Val-106L and Glu-1H linked by a 19-residue sequence derived from the light-chain elbow region (11). After random mutation of the VH region, a phage display library was constructed from which a mutant (Ile77HThr) with superior secretory properties in *E. coli* and improved solubility was selected (12).

Expression and Purification. Expression was carried out in *E. coli* TG1 under control of the *lac* promoter. One-liter cultures were grown at 30°C in M-9 medium supplemented with 0.4% Casamino acids and ampicillin (100 mg/ml). At 24 h, the cultures were induced with supplementary nutrients (12 g of tryptone/24 g of yeast extract/4 ml of glycerol per liter) and 1 mM isopropyl β -D-thiogalactoside. The cultures were harvested 72 h later and periplasmic extracts were prepared by an osmotic-shock procedure (11). Functional scFv was eluted from an antigen-Sepharose affinity column with 0.5 M NaCl/0.1 M sodium acetate, pH 4.5 (11).

Crystallization. α -D-Gal(1→2)[α -D-Abe(1→3)] α -D-Manp1→OMe was obtained by chemical synthesis (E. Eichler and D.R.B., unpublished data). The cocrystals were grown by the hanging-drop vapor-diffusion method at 18°C. A 3- μ l aliquot of protein solution (19 mg/ml in 10 mM Tris-HCl, pH 8.0/10 mM NaCl) was mixed with 0.5 μ l of a 10 mM trisaccharide solution and 4.5 μ l of the well solution containing 14% (wt/vol) PEG (M_r , 8000), 50 mM Tris-HCl (pH 8.5), and 0.1 M NaCl. The crystals belong to the space group $P2_12_12_1$ with unit-cell dimensions of $a = 53.1$, $b = 61.0$, and $c = 74.8$ Å.

Data Collection. Diffraction data were collected on a RAXIS-IIC image plate area detector in 73 oscillation frames ($\Delta\phi = 1.4^\circ$, 30 min per frame) from a single crystal. A total of 177,018 reflections was measured, of which 93,140 with I

Abbreviations: Fv, antibody variable domain; scFv, single-chain Fv; VL, light chain variable domain; VH, heavy chain variable domain; Abe, abequose.

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[¶]Malby, R. L., Lawrence, M. C., Tulip, W. R., Harley, V. R., Webster, R. G., Hudson, P. J. & Colman, P. M. (1993) *Collected Abstracts of XVI Congress of International Union of Crystallography*, August 21–29, 1993, Beijing, China, p. 110.

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**The atomic coordinates have been deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973 (reference 1 MFA).

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Table 1. Affinity constants and thermodynamics for methyl glycoside-antibody fragment binding

Antibody molecule	K_a , ($\times 10^5$) M^{-1}	ΔG^*	ΔH , kJ/mol	$-T\Delta S$	Ref.
IgG	2.1 ± 0.3	-30.5 ± 0.4	-20.5 ± 1.7	-10.0 ± 0.8	14
<i>E. coli</i> wt Fab	2.1 ± 0.3	-30.3 ± 0.4	-24.8 ± 0.6	-5.5 ± 0.7	12
wt scFv	1.3 ± 0.5	-29.1 ± 0.9	-23.7 ± 1.0	-5.4 ± 1.8	12
3B1 scFv	1.1 ± 0.2	-28.8 ± 0.4	-31.1 ± 0.8	2.3 ± 0.9	12
Fv (proteolysis of scFv)	0.6 ± 0.5	-27 ± 2	-26 ± 6	1 ± 8	11

wt, Wild type. Data are the mean \pm SD. Uncertainties represent three SDs obtained from regression analysis.

$\geq \sigma(I)$, where I is intensity and σ is standard deviation, were accepted. Averaging of symmetry-related reflections resulted in 22,712 reflections with a residual error of $R_{\text{sym}} = 4.9\%$. The completeness of the data to 1.7-Å resolution is 84.3%.

Structure Solution and Refinement. The structure was solved by the molecular replacement method using the coordinates of the variable domain of the parent Fab fragment (7) as a starting model. After several cycles of minimization (13) and refitting, the difference electron density showed clearly the position of the trisaccharide and allowed extension of the heavy chain to residue 112H. Further refinement was carried out by a simulated annealing algorithm with intermittent refitting of the model against the $3F_o - 2F_c$ electron density map, where F_o and F_c are the observed and calculated structure factors, respectively. The linker region is mostly disordered and only its first two residues and last one residue could be localized, albeit with half occupancy. The present model also includes 164 solvent molecules. The final R factor is 0.166 for reflections with $I > 2\sigma(I)$ in the 8.0- to 1.7-Å resolution shell (0.178 for all reflections); the rms deviation of bond lengths and valence angles from the ideal values are 0.014 Å and 2.8°, respectively.

RESULTS AND DISCUSSION

Se155-4 Mutant Characterization. The single amino acid substitution in the wild-type sequence, Ile-77H \rightarrow Thr, significantly increased the yield of a fully functional scFv secreted in *E. coli*. The yield of this mutant was ≈ 100 mg/liter compared to the yield of ≈ 10 mg/liter for the wild type. In addition, the solubility of the mutant scFv was 20 mg/ml compared to 5 mg/ml for the wild type. The mutated residue resides in the framework segment and its side chain does not

contact the antigen. The antigen-binding affinities of the wild-type and mutant scFv molecules were similar. The K_a of the mutant and wild-type forms for the trisaccharide antigen, as determined by titration microcalorimetry, were $1.1 \pm 0.2 \times 10^5 M^{-1}$ and $1.3 \pm 0.2 \times 10^5 M^{-1}$, respectively (Table 1).

The Structure of Se155-4 scFv. Fv molecules are compact molecules and tend to form tightly packed crystals that diffract to high resolution (refs. 4–6 and 15 and ||). The present scFv molecule is no exception, forming crystals that diffract to a 1.7-Å resolution, and provides the opportunity to obtain very precise structural parameters for the scFv-trisaccharide complex, including the ordered solvent structure. The crystal structure was readily solved by the molecular replacement method and refined to $R = 0.166$ for 8- to 1.7-Å resolution data.

Despite careful refinement only the beginning and the end of the linker could be localized. Residue Leu-106aL was well resolved, whereas the next two amino acids, Gly-107L and Gln-108L, and the last residue of the linker, Gly-124L, were refined with only half occupancy. The remainder of the linker is disordered. Nevertheless, conclusions regarding the oligomeric state of the molecules could be reached and a shorter linker was designed.

The linker stabilizes the Se155-4 Fv molecule in that it increases the affinity for the trisaccharide antigen by 2-fold (Table 1) and overcomes an apparent problem of VL-VH dissociation at high dilution (9). However, as noted also for other scFv molecules (16), the linker is susceptible to proteolytic attack (11). The intact nature of the scFv used for the present study was confirmed by SDS/PAGE of the dissolved crystal used for data collection, which showed a single band with the expected molecular mass of ≈ 26.5 kDa (data not shown).

The association of VL and VH in scFv is very similar to that observed in the Se155-4 Fab fragment. Since the previ-

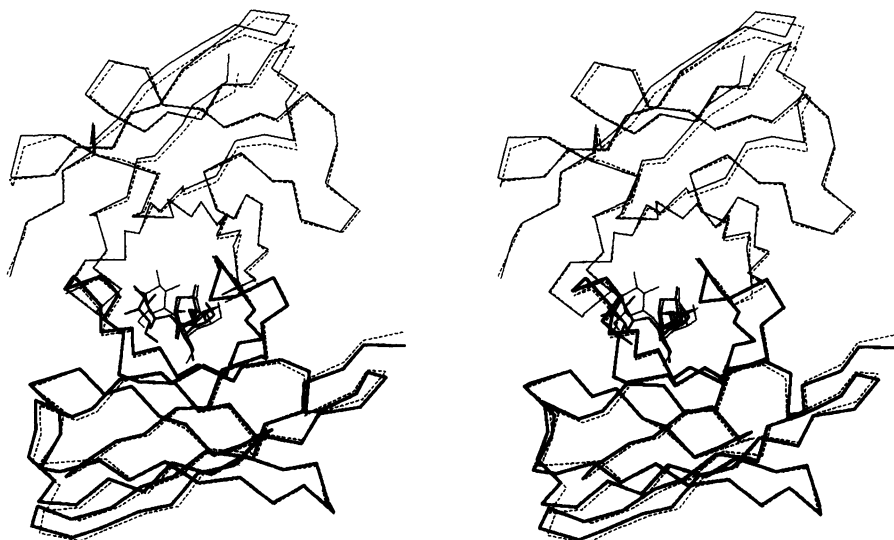


FIG. 1. Superposition of the $C\alpha$ tracing of the scFv and the Fv domain of the Fab as determined in the Fab-trisaccharide complex. The transformation matrix is based on the main-chain atoms of residues within a 5-Å shell around the trisaccharide. The scFv is shown by solid lines, the Fv from the Fab fragment is shown by dashed lines, and the VH is shown by thick lines.

ously reported Fab–oligosaccharide complex contained a dodecasaccharide (7), we have also determined (17) the structure of a Fab–trisaccharide complex at a 2.1-Å resolution for an unbiased comparison with the scFv. The superposition of the scFv and Fv fragment of the Fab gave a rms deviation of 0.43 Å for C α atoms, and the superposition of individual VL (residues 1L–106L) or VH (residues 1H–111H) domains gave a somewhat smaller rms of \approx 0.35 Å. When, however, the superposition was based only on either VL or VH, the rms deviation for the other domain was roughly twice as large. In the complementarity-determining regions, where protein–antigen interactions occur, the deviations were significantly smaller. The best superposition of 15 residues within a 5-Å shell around the antigen gave a rms of 0.19 Å for C α atoms and 0.34 Å for all atoms of these residues. These numbers indicate that the arrangement of the VL and VH domains in scFv molecule is slightly different from that in the Fab fragment (Fig. 1). Another indicator of these differences is provided by a comparison of the rotation angle and the translation vector that are required to superimpose VL on VH (pseudo twofold symmetry). For the Fab fragment these values are 177.3° and 0.14 Å and for the scFv they are 174.5° and 0.32 Å, respectively. The difference is largely in the rotation angle (\approx 3°). Of the complementarity-determining-region loops, only the end of L1 (residues 26 and 27), which does not interact with the antigen, shows differences in the C α positions exceeding 1.0 Å. This region is involved in the interactions with the symmetry-related molecules in the scFv crystal. The observed differences in the VL–VH association may stem from removing the constraints in scFv imposed by the presence of the constant domain in the Fab fragment. The linker in the present construct is longer than is required for the shortest connection between the VL and VH domains and imposes no restraints on VL–VH association. It is equally likely that the observed changes reflect differences in interactions with neighboring molecules in the two crystal environments.

The structures of two Fv molecules for which the Fab structures were also known have been reported (4, 6). Similar small differences in the VL–VH association between the Fab fragments and the Fv domains were also noted.

Linker. There are two ways to link VL and VH to construct a scFv. Bird *et al.* (1) were the first to make a VL \rightarrow VH construct, and a scFv with a VH \rightarrow VL polarity was first described by Huston *et al.* (2). While the end of the linker is uniquely defined by the first residue of the second domain (VH or VL, depending on the polarity), various authors define the beginning of the linker differently. Here we consider residue 106L [numbering of Kabat *et al.* (18)], the last involved in the formation of β -sheet, as the end of VL and the analogous 111H as the end of VH. Many scFv constructs utilize the (Gly₄-Ser)₃ sequence, designed by Huston *et al.* (2), to link the VL and VH chains in either of the two polarities. In the present molecule, the VL \rightarrow VH 19-residue linker (underlined), V^{106L}LGOPKSSPSVTLPSSNGE^{1H}, corresponds to the extension of the light chain. This linker, significantly longer than the minimum length required to connect the two domains, is disordered in the crystal and its position has not been determined. Only the two ends of the linker could be traced, and even then partial occupancy had to be assigned to them. That leaves open the question of the oligomeric state of the scFv molecules in the crystal since, in principal, the linker can connect the VL and VH from the same Fv (monomers) or from two different Fv molecules (oligomers). Examination of the packing of the Fv molecules in the crystal strongly suggests that they are in a monomeric form. Our reasoning is as follows. We find two possible routes from the end of VL to the beginning of VH in the same Fv or between neighboring symmetry-related molecules with distances between the end points of \approx 32 Å. A second group

of inter-scFv routes has the end points 55–60 Å or more apart but since these routes meander among the scFv molecules, their pathways are significantly longer. The 16 residues of the linker that have not been located in the structure can span at most \approx 59 Å in a fully extended conformation and \approx 55 Å in a β -sheet conformation, which makes these routes unlikely as possible paths for the linker. That leaves only the two shorter routes for further consideration. One path connects the VL and VH domains of the same Fv (Fig. 2a), and the other connects the chains from two molecules related by a 2₁ screw rotation along the y axis (Fig. 2b). Both paths pass through an empty space between symmetry-related molecules and could be easily occupied by a disordered linker with many different conformations. While the first route of the linker corresponds to monomers of scFv, the second route is equivalent to the existence of scFv in the form of long polymers extending along the 2₁ screw axis. Although the equilibrium between the monomers and higher oligomeric forms in solution is quite possible, one would not expect a large population of long polymers. Our analysis of the scFv population by gel filtration showed that the majority of molecules in solution are in a monomeric form with only a small fraction of dimers (data not shown). Formation of long polymers could happen during the growth of the crystal but it would require a significant population of scFv in a dissociated form, first for the nucle-

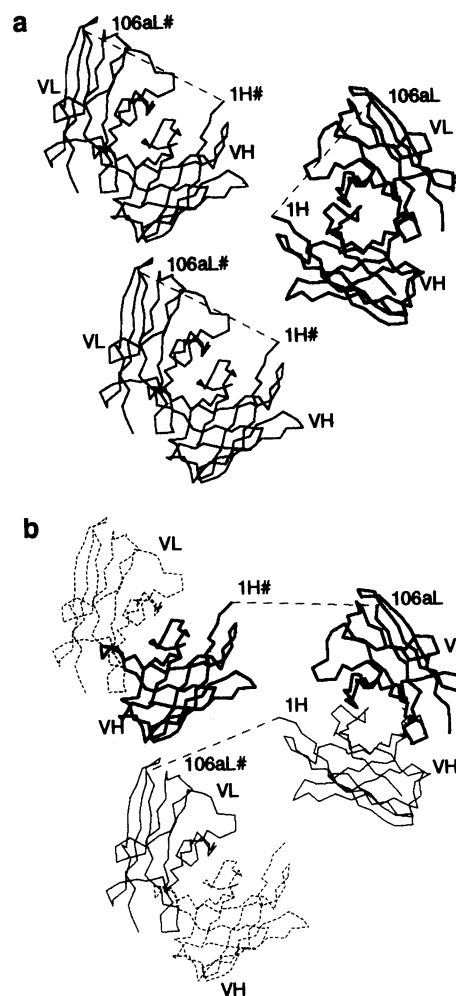


FIG. 2. Three neighboring Fv domains with two possible scFv arrangements. (a) Linker within the same Fv molecule—monomers. (b) Linker between Fv molecules related by a twofold screw axis—long polymers. One of the scFv molecules is shown by thick lines; others are shown by thin or dashed lines. Symmetry-related molecules are indicated with the symbol #.

ation step and later for the extension of the crystal by pairing of the free VL with the overhanging VH (or vice versa). In our case, the presence of the trisaccharide antigen during the crystallization provides additional stabilization of the Fv domain and shifts the equilibrium further toward the association, rather than dissociation of VL and VH. The formation of long polymers in highly concentrated protein solution would likely lead to low solubility of such material and easy formation of precipitate. None of this was observed, either during protein concentration or during crystallization.

Some local differences between the scFv and the Fab have been found in the region where the linker is most likely located. The loops 40L–43L and 55L–58L are not as well defined in the scFv as in the parent Fab, indicating larger motional flexibility of this region, possibly due to the influence of a nearby disordered linker.

The present linker, being longer than required for connection of VL to VH, does not pack tightly against the rest of the protein, which makes it susceptible to proteases. Modeling suggests that the domains could be connected by a 12- to 13-residue linker in a relatively unconstrained conformation, somewhat shorter than the most commonly used (Gly₄Ser)₃ design.

Epitope Binding. The trisaccharide epitope binds to Se155-4 scFv in the depression between the VL and VH chains. Abe, the immunodominant sugar of *Salmonella* O antigen, is totally buried in the depression, whereas Man and Gal are on the protein surface. The positions of all amino acid residues that interact with the trisaccharide epitope in the scFv are nearly the same as in the Fab fragment (Fig. 1). However, the conformation of the trisaccharide differs in the two complexes. The positions of the Abe and Man sugars are the same in both complexes with very similar torsion angles about glycosidic bonds. They make the same interactions with the protein. The difference occurs in the position of the Gal residue (Fig. 3). In the Fab–trisaccharide complex, the glycosidic torsion angles $O5^{Gal}-C1^{Gal}-O2^{Man}-C2^{Man}$ and $C1^{Gal}-O2^{Man}-C2^{Man}-C3^{Man}$ are 104° and 89° , and in the scFv complex, they are 77° and 144° , respectively. As a result, the intramolecular hydrogen bond $O2^{Abe} \dots O2^{Gal}$, observed in the Fab–trisaccharide and Fab–dodecasaccharide complexes (7, 8), does not exist in the scFv–trisaccharide complex. Instead, these two hydroxyl groups are bridged by a water molecule, which is found in a position nearly identical to that

of $O2^{Gal}$ in the Fab complex. This water molecule is a part of the network of well-ordered solvent molecules surrounding the antigen (Fig. 3). The comparison of these two conformations with the minimum energy conformation obtained from theoretical calculations shows that the calculated structure assumes a conformation intermediate between the one observed here and that in the Fab–trisaccharide complex (17). It is not quite clear why the trisaccharide assumes different conformations in complexes with Fab and scFv. There are no contacts with the symmetry-related molecules that could be credited with the influence on the conformation of the Man–Gal linkage. However, differences in the packing of molecules in the two crystals result in a rearrangement of ordered water molecules near the Gal (Fig. 3) and this change may have triggered the observed rotation of Gal along the glycosidic linkage. There are significantly more ordered solvent molecules around the trisaccharide in the scFv structure than in the Fab crystals. NMR measurements of the free trisaccharide in solution indicate flexibility around the Gal–Man glycosidic bond and suggest that in the presence of Fab the trisaccharide undergoes a conformational change toward that observed in the crystal structure of Fab–trisaccharide complex (17). The results in solution, combined with the crystal structures of Fab–trisaccharide and the present scFv–trisaccharide complexes, indicate clearly that the Man–Abe linkage is relatively rigid whereas the oligosaccharide displays some flexibility along the Gal–Man linkage. In the bound state, the hydrogen bonds to water molecules help to stabilize conformations that are sampled but not highly populated by the free oligosaccharide in solution.

Solution binding constants have been determined by titration microcalorimetry for a range of oligosaccharide structures of various length (14) and the binding of the trisaccharide methyl glycoside has been measured with IgG, *E. coli*-expressed Fab, scFv mutants, and Fv molecules (11, 12). These data show that the free energy of binding levels off for the trisaccharide epitope and stays nearly constant for longer oligosaccharides (14). Minor differences in the association constants of IgG, Fab, and scFv fall within the experimental error (Table 1). This is consistent with a uniform binding mode for each protein and suggests that the small differences in relative packing of VH and VL domains in the scFv and Fab structures may be attributed to either a lack of constraints imposed by the constant domain on the Fab or to

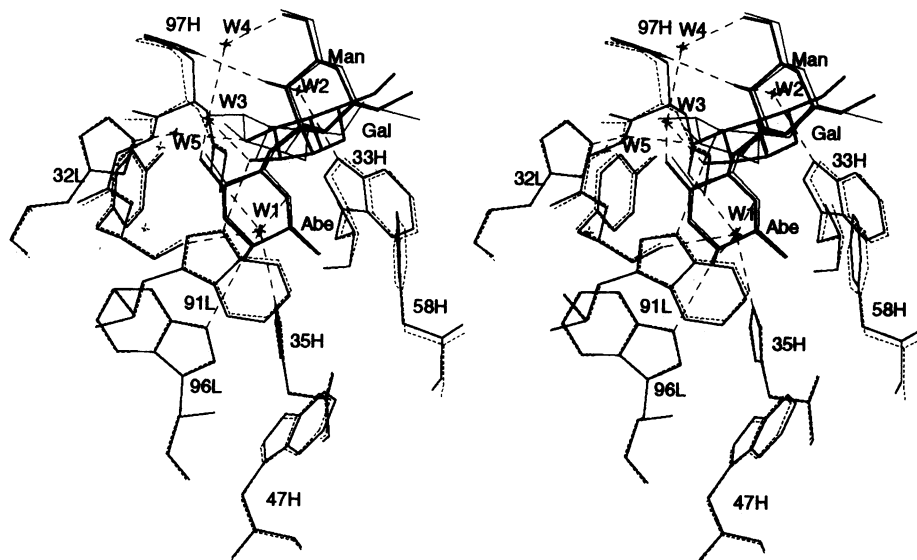


FIG. 3. Superposition of oligosaccharide antigen in scFv and Fab fragment with residues within a 5-Å distance from the oligosaccharide. The scFv is shown by solid lines, Fab is shown by short dashed lines, water molecules of the scFv complex are shown by thick crosses, waters in the Fab are shown by thin crosses, and hydrogen bonds in the scFv complex are shown by long dashed lines.

crystal lattice forces, while the large differences in the bound saccharide conformation may reflect the influence of ordered water molecules on the exposed and relatively flexible Gal-Man linkage of the trisaccharide epitope. The latter does not affect ΔG due to entropy–enthalpy compensation but may be inferred from the relative changes in the enthalpy ΔH and entropy $T\Delta S$ terms (Table 1).

With respect to mobility and antigen–antibody interactions, the present example suggests that the carbohydrate antigen undergoes an induced fit with antibody (17). A similar situation was reported for the interaction of a β -galactan ligand with J539 (19) and for peptides (20). Generally, a spectrum of relative flexibility for both antibody and antigen has been observed in published crystal structures (21, 22). In the example here, we see that the saccharide flexibility is restricted to the most exposed pyranose residue and that the extent of glycosidic torsional angle adjustment is modulated by ordered water molecules.

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