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Binding of modified fragments of the *Shigella dysenteriae* type 1 O-specific polysaccharide to monoclonal IgM 3707 E9 and docking of the immunodeterminant to its modeled Fv

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Abstract

The O-specific polysaccharide (O-SP) of *Shigella dysenteriae* type 1 has been shown by others to have the structure →3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→2)-α-D-Gal*p*-(1→3)-α-D-Glc*p*NAc-(1→. We have shown in the past that IgM 3707 E9, an anti *S. dysenteriae* type 1 O-SP monoclonal antibody, binds specifically to the -α-L-Rha*p*-(1→2)-α-D-Gal*p*- determinant of the polysaccharide. In this report we show that determinant to have hydrogen bonds, necessary for binding to the antibody, involving positions 3, 4 and 6 of the galactopyranosyl residue. The hydroxyl groups of the rhamnopyranosyl moiety of the immunodeterminant appear not to partake in hydrogen-bond interactions with the antibody. A model is presented of the Fv of IgM 3707 E9 based on our previously established cDNA-sequence and two known, highly homologous immunoglobulin crystal structures. The methyl glycoside of the immunodeterminant α-L-rhamnopyranosyl-(1→2)-α-D-galactopyranose is docked to the combining area of the Fv.

Keywords: Deoxyfluoro sugars; Protein structure; Ligand/antibody binding

1. Introduction

Monoclonal IgM 3707 E9 was obtained by injection of heat-killed *S. dysenteriae* type 1 into Balb/c mice. Its interaction with a number of synthetic oligosaccharides mimicking epitopes of the bacterium's O-specific polysaccharide (O-SP), which has the structure →3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→2)-α-D-Gal*p*-(1→3)-α-D-Glc*p*NAc-(1→ [1], has been reported by our laboratory [2]. It was shown that the immunodeterminant of the O-SP consisted of the -α-L-Rha*p*-(1→2)-α-D-Gal*p*- moiety, and that the antibody was capable of binding to interior segments of the O-SP. We have since reported the sequence of cDNA copies of this antibody's heavy (H) and light (L) variable (V)

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Abbreviations: Rha*p* = rhamnopyeanosyl, Gal*p* = galactopyranosyl, Glc*p*Nac = 2-acetamido-2-deoxy-glucopyranosyl, α-L-Pha*p*-(1→2)-α-D-Gal*p*-OMe = methyl α-L-rhamnopyranosyl-(1→2)-α-galactopyranoside, etc., O-SP = O = specific polysaccharide.

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region mRNA [3], as well as that of three other murine antibodies capable of binding to the O-SP of *S. dysenteriae* type 1 [4]. For IgM 3707 E9, the V_H (μ) gene closely resembles V_H441, first identified as V_H39.1 amongst the anti-galactan family, while the V_L (λ) gene closely resembles the V_L of MOPC104E gene. We are involved in defining the molecular interactions of IgM 3707 E9 with the O-SP and here report specific hydrogen-bond interactions involved in the binding of the antibody and the disaccharide determinant. This was achieved by measuring the affinity for the antibody of saccharides (analogous to the determinant) whose structure had been specifically altered at selected positions in either of two ways, namely OH→H or OH→F [5–8]. It is recalled that substitution of a hydroxyl (OH) group by hydrogen (H) results in negation of both hydrogen-bond donation and reception at that position. Substitution of an OH group by a fluorine (F), which has a van der Waals radius slightly smaller than that of an OH group [9], although negating donation, could still allow hydrogen-bond reception in the resulting derivative [8–10]. Since crystal structures of two antibodies, one involving an H-chain, and one involving an L-chain, highly homologous to the corresponding chains of IgM 3707 E9 are known, a model of the latter could be readily constructed. Our binding studies assisted in a speculative docking of the antigenic structure with this antibody's combining site, the results of which we present herein. As before, all saccharides used in this study are in the form of methyl glycosides of the same anomeric configuration they have when occurring in the O-SP antigen.

2. Experimental

The preparation of the mono- and disaccharides **2** [11], **3** [12], **4**, **6**, **8**, **10**, **12**, **14** [13], **13** [14], **5**, **7**, **9**, **11**, **15** [15], and **16** and **17** [16] has been reported. Purification of the antibody IgM 3707 E9 was carried out as before [2]. Nuclear magnetic resonance studies on the modified disaccharides **6**, **7**, **10**, **11**, **14**, **15**, **16** and **17** indicated [17] that the synthetic modifications introduced did not alter the overall conformation of these ligands in solution when compared with that of **3**.

Affinity constants of the purified IgM with the various ligands were measured using ligand-induced tryptophanyl fluorescence changes of the protein as before [18,19]. This method has been verified by measurements on known antigen–antibody systems [20]. It is based on the perturbation by ligand of the antibody's tryptophanyl residues that are located in or near the general combining area of the antibody [8,21]. From the sequence of 3707 E9 (see Fig. 1), we know such residues to occur at positions H:33, H:98 and L:91 [3]. Briefly, measurements were performed at 25 °C in 0.01 M phosphate-buffered saline pH 7.4 (PBS) using an IgM concentration of $2.9–3.6\times10^{-8}$ M. Tryptophanyl excitation and emission wavelengths were 295 and 340 m, respectively. The instrument used was a Perkin–Elmer LS-50 spectrofluorimeter. Incremental changes in protein fluorescence due to ligand binding following additions, are called ΔF. They are divided by the maximally attained fluorescence change at infinite ligand concentration, ΔF_{max} (when all antibody sites carry ligand) to give the fraction of antibody sites that carry ligand at any given intermediate concentration of ligand: $\nu = \Delta F/\Delta F_{max}$. Ligands were added to three reference cells as well, one containing just PBS, the other two each having a known monoclonal immunoglobulin of unrelated specificity (in the case of **16** and **17**, only one immunoglobulin of unrelated specificity was used to monitor specificity). For the equilibrium between antibody sites (Abs) and ligand (lig) such as

$$\text{Abs} + \text{lig} \rightleftarrows \text{Abs} - \text{lig}$$

the equilibrium constant K_a is

$$K_a = C_{\text{Abs–lig}}/C_{\text{Abs}} \times C_{\text{lig}}$$

where C stands for concentration.

Thus, it follows that

$$K_a = \nu/(1 - \nu) \times C_{\text{lig}}$$

or

$$\nu/C_{\text{lig}} = K_a(1 - \nu)$$

Thus, a Scatchard plot of ν/C_{lig} versus ν yields the K_a as the intercept on the ordinate. The free energy of binding ($-\Delta G°$) is then obtained from $-\Delta G° = RT \ln K_a$.

The amino acid sequences of the V_H and V_L of 3707 E9 [3,4] were aligned with the most homologous sequences of known crystal structures listed in

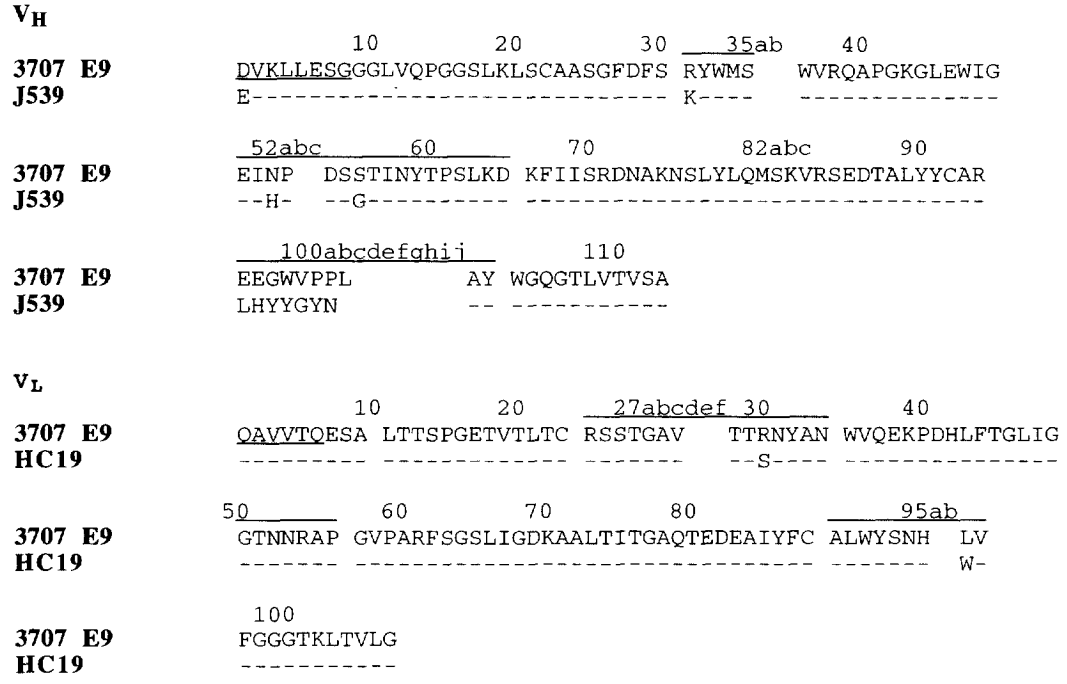

Fig. 1. cDNA-derived amino acid sequence of IgM 3707 E9, and comparison of its heavy- and light chain sequence to the corresponding known sequences of immunoglobulins J539 and HC19. The *under*scored lines indicate the sequences resulting from the expression of the primers used in the cloning. The *over*scored lines indicate the hypervariable regions.

the Brookhaven Protein Data Bank [22]. The C_α atoms of the V_H of J539 (PDB code 2FBJ) and of the V_L of HC19 (PDB code 1GIG) were aligned according to the conserved framework residues listed by Novotny and Sharp [23]. Amino acids which differed from those of 3707 E9 (except for those found in the portion of the first framework regions which are derived from the oligonucleotide primers used in cloning, Fig. 1) were substituted and the side chains were positioned either in similar alignment with the replaced side chain or by using the rotamer library for side chains according to the Ponder and Richards feature in the program Quanta 97 (Molecular Simulations, Inc.) [24]. Since all of the hypervariable loops on the light chain and the first and second on the heavy chain were homologous to those of the crystal structure templates, and were consistent with the canonical loop classes as established before [25], no modeling of these loops was deemed necessary. The third hypervariable loop of the heavy chain of 26–10 (PDB code 1IGI) [26] was spliced into the heavy chain of 3707 E9, and amino acid substitutions were made using the Quanta 97 program. The loop had to be manually adjusted to accommodate the rigid ϕ angles of its two sequential prolines (V_H 100, 100a; see Fig. 1). The overall structure of the resulting variable region was energy-minimized using a regularization script from the program, ICM (Molsoft, L.L.C.) [27] The hypervariable loop was then subjected to a Monte Carlo type conformational search using ICM.

Docking of the disaccharide **3** was also performed by a Monte Carlo type docking script using ICM as well. This in vacuo method allows for both the disaccharide and the antibody binding site to be flexible during docking. The partial charges on the disaccharide were assigned according to the Gasteiger method [28], and the structure for the disaccharide was closely based on previous NMR data [17]. During the docking, tethers were initially placed on three tryptophanyl residues in the binding area (L:91, H:33 and H:98) and the disaccharide **3**.

3. Results and discussion

First, we will discuss the measurements of affinity between ligands and antibody, and next the considerations for modeling the binding of disaccharide **3** into the antibody combining area.

The results of the affinity measurements of the IgM 3707 E9 with the haptens tested are listed in Table 1. It can be seen that the antibody shows a substantial variation in the increase of

Table 1
Binding of ligands to monoclonal, anti-*Shigella dysenteriae* type 1 IgM 3707 E9

Ligand		$\Delta G°(kcal)$	$K_a(M^{-1})$	$\%\Delta F_{max}$
1	α-D-Gal*p*-OMe	3.1	1.8×10^2	38[c]
2	α-L-Rha*p*-OMe			U[f]
3	α-L-Rha*p*-(1→2)-α-D-Gal*p*-OMe	5.1	5.8×10^3	77[a]
4	3-deoxy-α-D-Gal*p*-OMe			U[f]
5	3-deoxy-3-fluoro-α-D-Gal*p*-OMe			U[f]
6	α-L-Rha*p*-(1→2)-3-deoxy-α-D-Gal*p*-OMe	3.1	1.8×10^2	34[c]
7	α-L-Rha*p*-(1→2)-3-deoxy-3-fluoro-α-D-Gal*p*-OMe	3.0	1.7×10^2	35[e]
8	4-deoxy-α-D-Gal*p*-OMe			U[f]
9	4-deoxy-4-fluoro-α-D-Gal*p*-OMe			U[f]
10	α-L-Rha*p*-(1→2)-4-deoxy-α-D-Gal*p*-OMe			U[f]
11	α-L-Rha*p*-(1→2)-4-deoxy-4-fluoro-α-D-Gal*p*-OMe			U[f]
12	6-deoxy-α-D-Gal*p*-OMe			U[f]
13	6-deoxy-6-fluoro-α-D-Gal*p*-OMe	3.4	2.9×10^2	25[b]
14	α-L-Rha*p*-(1→2)-6-deoxy-α-D-Gal*p*-OMe			U[f]
15	α-L-Rha*p*-(1→2)-6-deoxy-6-fluoro-α-D-Gal*p*-OMe	5.1	5.6×10^3	54[b]
16	2-deoxy-α-L-Rha*p*-(1→2)-α-D-Gal*p*-OMe	5.1	5.6×10^3	74[b]
17	4-deoxy-α-L-Rha*p*-(1→2)-α-D-Gal*p*-OMe	5.5	1.0×10^4	76[d]

[a] From V. Pavliak et al., *J. Biol. Chem.* 268 (1993) 25797; disaccharide **3** was used as a reference compound to verify the antibody preparation.
[b] Value for the correlation factor (R^2) of the Scatchard plot is 0.998–0.984.
[c] Value for the correlation factor (R^2) of the Scatchard plot is 0.975–0.967.
[d] Value for the correlation factor (R^2) of the Scatchard plot is 0.934.
[e] Value for the correlation factor (R^2) of the Scatchard plot is 0.714.
[f] Unquantifiably small.

its tryptophanyl fluorescence when binding to the various ligands. The absence of a ligand-induced antibody fluorescence change for a particular ligand is taken as evidence that the antibody either does not bind that ligand, or that the subsite for that ligand is too remote to perturb the tryptophanyl residues. The latter, we know from previous work, appears unlikely for IgM 3707 E9 [2]. It can also be appreciated that methyl α-L-rhamnopyranoside (**2**) has a K_a—computed from the values for methyl α-D-galactopyranoside (**1**) and methyl α-L-rhamnopyranosyl-(1→2)-α-D-galactopyranoside (**3**)—of ca. 30. A value so low could fail to give rise to a significant ligand-induced antibody fluorescence change unless a large amount of ligand is added to the antibody solution tested[2]. Either of those two reasons could explain why **2** fails to show a measurable ΔF.

The failure of compounds **8**–**11** to bind to the IgM indicates that affinity requires the OH-4 on the galactose molecule to donate a critical hydrogen bond. Thus, the presence of a 4-deoxy- or 4-deoxy-4-fluoro group in the galactosyl moiety in either the monosaccharide (**8** or **9**) or the disaccharide (**10** or **11**) leads to cessation of binding. Not all hydrogen bonds partaking in binding need be critical. For instance, the lack of quantifiable binding of methyl 3-deoxy-α-D-galactopyranoside (**4**) and methyl 3-deoxy-3-fluoro-α-D-galactopyranside (**5**) indicates that the 3-position is involved in hydrogen-bond donation. However, the persistent binding of the disaccharides **6** and **7** (reduced by a factor of ~30 when compared to that of **3**) indicates that the chemical change at that position still allows other molecular areas of the galactosyl moiety in **6** and **7**, as well as the rhamnosyl moiety, to have binding interaction with the antibody combining site. In the case of the 6-position of the galactosyl moiety, it can be seen from ligands **12** and **13** that the 6-OH must receive a hydrogen bond to result in binding, since **12** ceases to bind, while **13** binds with approximately the same affinity as methyl α-D-galactopyranoside (**1**). We interpret this to mean that the fluorine at position 6 in **13** can accept and restore that hydrogen bond. Note that this hydrogen bond is important, since **14** also fails to bind. The disaccharide **15** binds with approximately the same affinity possessed by the disaccharide **3**, thus confirming the nearly identical affinities of **1** and **13**.

[2] Using the usual Ab solution (site conc. $\sim3.57\times10^{-7}$ M), it can be computed that the solution of methyl α-L-rhamnoside *in the cuvette* that would half-saturate the Ab sites present would be ~6 mg/mL (0.0333 M), i.e., a concentration some 10^5 times larger than the Ab site concentration.

The disaccharides **16** and **17**, having either of the two possible OH groups in the rhamnosyl moiety replaced by H respectively, showed binding, that was essentially equal to that of **3**, or better. This indicates that in the O-SP antigen the hydroxyl groups on the rhamnopyranosyl residue that is adjacent to the galactosyl residue, and that is part of the determinant, appear to have no hydrogen bonding interactions with the antibody.

To our knowledge the observation that chemically modified but conformationally identical ligands induce significantly differing maximal protein fluorescence change upon binding to this antibody is the first such observation. In previous work [8,21,29–31] we did not observe this, and we have no explanation for it.

Our proposals as to how the determinant **3** binds to anti-*Shigella dysenteriae* type 1 IgM 3707 E9 are discussed following the discussion on protein modeling. It is interesting to recall that ca. 60% of the binding energy of the determinant of *S. dysenteriae* type 1 is mediated by the galactosyl residue [2]. The affinity measurements reported here suggest that the galactosyl residue partakes in three hydrogen bonds in binding the antibody combining site. From our observations that **16** and **17** have nearly the same affinities for the IgM as does **3**, it appears that the ~2 kcal of binding energy contributed to protein binding by the rhamnosyl residue in the disaccharide determinant is due to hydrophobic interactions. Due to limitations in the availability of these ligands, no affinity measurements at differing temperatures were done.

Next we wish to address the rendition of the three dimensional model of the Fv of IgM 3707 E9 as derived from the amino acid sequence reported earlier by us [3] as well as its docking to the disaccharide **3** using that saccharide's conformation as deduced by NMR spectroscopy [17].

Criteria for good structural templates for modeling the V_H and V_L of 3707 E9 were chosen based on best primary sequence homology and best resolution of crystal structures. The V_L of HC19 shares a 98% sequence identity with the V_L of 3707 E9, and its structure has been resolved to a resolution of 2.3 Å. Similarly, the V_H of J539 shares a 97% sequence identity (excluding the first eight residues of the first framework region altered by cloning and the CDR3-H region) and that structure has been resolved to a 1.95 Å resolution. Such high homology and structure resolution made these templates excellent choices for modeling. The framework residues used for superimposing the V_H and V_L regions were in the ranges of L:35–38, L:86–88, H:36–39, and H:90–92. Superposition of these C_α atoms yielded a rms deviation of 0.48 Å.

The high sequence identity for all of the hypervariable loops obviated the need for any loop-splicing, except in the CDR3-H region. For this loop, several V_H structures were aligned with the framework regions of J539 V_H. The 26–10 CDR3-H loop was chosen since it resulted in the least amount of steric hindrance. This loop was spliced into the V_H of J539, and amino acids substituted to match the CDR3-H of 3707 E9 using Quanta 97. Since the ϕ angle of a proline is fixed at $-67°$, the backbone conformation of the 26–10 CDR3-H loop could not be maintained for these prolines at positions H:100 and 100a. In order to accommodate these fixed ϕ angles, a break in the CDR3-H loop between H:99 and H:100 had to be made so that the backbone torsion angles could be manually adjusted to allow the loop to meet. Once the loop was reconnected, the CDR3-H loop (H:95–102) was subjected to a loop search with ICM. The lowest energy loop-conformation was chosen, and the entire Fv was energy minimized to allow the other loops to adjust to this new conformation.

Since both templates used here (as well as the CDR3-H loop) were derived from crystal structures containing no bound ligand, this binding site has no bias for any particular ligand binding requirement. The binding site (Fig. 2) of the 3707 E9 Fv forms a 12 Å by 8 Å opening with a cavity that is 8 Å deep. One side of the binding site contains two tryptophans (H:33 and L:91) and there is a third tryptophan at the tip of the CDR3-H loop (H:98) which is more solvent exposed. Our earlier report on the NMR-derived conformations of **3** and various deoxy and deoxyfluoro derivatives of **3**, showed them all to have similar conformations. Various force-field computations on the conformation of α-L-Rha*p*-(1→2)-4-deoxy-α-D-Gal*p*-OMe (selected because for that compound cross peaks that result from spin diffusion can be distinguished from those that arise directly from dipole–dipole interactions) showed the intersaccharidic angles ϕ and ψ to range from $\phi = -65.1°$ to $-70.1°$, and $\psi = 98.6°$ to $109.4°$. After the docking of **3** to the antibody combining area, the disaccharide's final, resulting ϕ,ψ angles were $-71°$ and $118.6°$ (Fig. 2(a)) and $-65°$ and $135.1°$ (Fig. 2(b)). It must be remembered that antibodies have been shown to induce flexible, differing

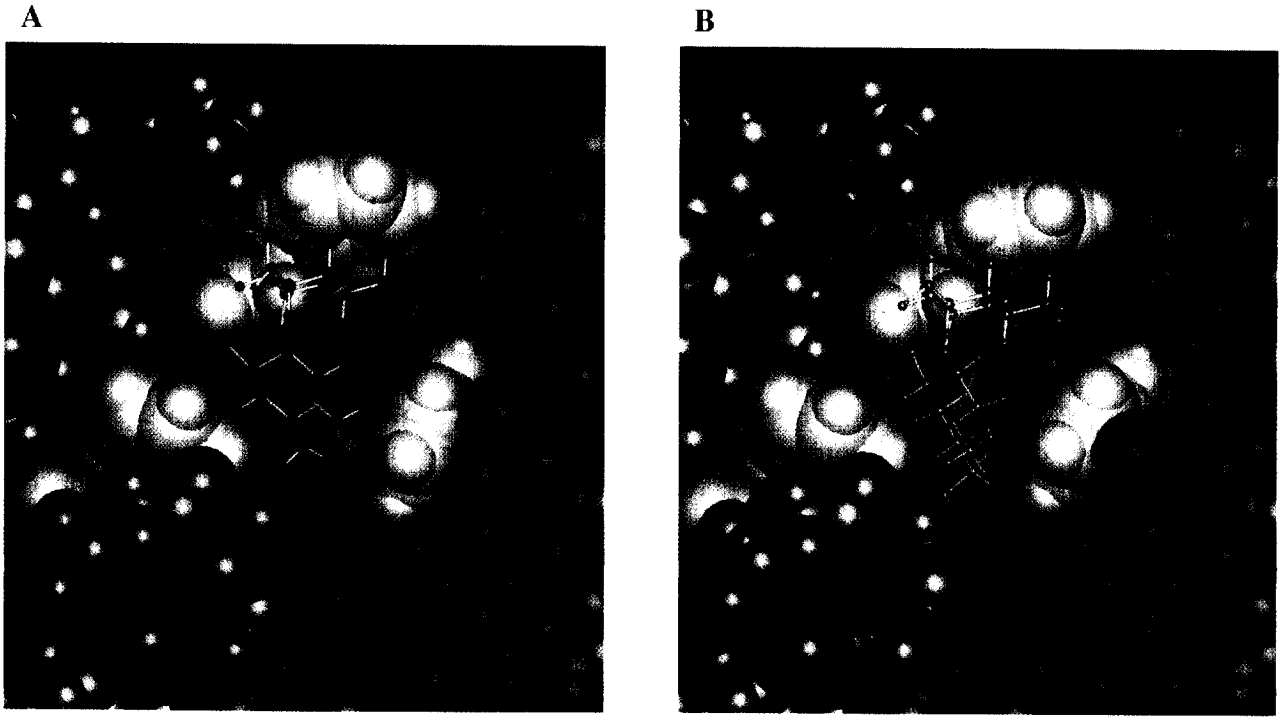

Fig. 2. The methyl glycoside of the immunodeterminant α-D-rhamnopyranosyl-(1→2)-α-D-galactopyranose (**3**) docked into the binding site of the Fv of 3707 E9. The V_H is red and the V_L is blue, and the tryptophanyl residues are indicated in yellow. A. ligand position 1 (magenta), and B. ligand position 2 (green).

conformations in the determinant to which they bind. The free energy used for that is essentially paid for by the overall free energy of binding being somewhat lessened. Two examples that come to mind are that influenza neuraminidase changes structure when it binds to an antibody, and the resulting X-ray crystallographic data on the complex are not compatible with an inflexible lock-and-key model [32]. In addition, antibody to flexible peptide fragments of myohemoerythrin bound strongly with the native protein, while antibody against less mobile sequences did not [33]. Docking of the disaccharide **3** resulted in it occupying the deepest portion of the cavity with the 3-position of the more exposed rhamnosyl portion facing outwards of the binding site directly over the galactose (Fig. 2). The latter has its C-1–OCH_3 facing the solvent. The nonpolar face of the galactosyl pyranose ring makes very close contact with the L:91 tryptophanyl ring, and the C-6 group of the galactosyl residue contacts the H:33 tryptophanyl ring to a lesser extent. The tryptophanyl ring of H:98 stacks along the nonpolar face of the rhamnosyl moiety. This is entirely consistent with the binding data which suggests that the galactosyl moiety perturbs tryptophan as evidenced by the ~40% difference in fluorescence intensity upon binding of ligand, while the rhamnosyl group perturbs a separate tryptophanyl group as shown by an additional ~40% change in fluorescence intensity upon binding the ligand **3**.

A potential hydrogen bond appears to occur between the 3-OH of the galactose and the carbonyl oxygen of H:98. For the hydrogen bond accepted by the 6-OH of galactose, two possibilities exist. The first possibility is that the proton on the 4-OH of galactose donates an intrasaccharidic hydrogen bond to the oxygen of the 6-OH group. (Ligand position 1, Fig. 2(a)). Alternatively, the oxygen at the 6-OH position of galactose could accept a hydrogen bond from the proton on the NE1 nitrogen of L:91 tryptophan (Ligand position 2, Fig. 2(b)). This would cause a slight shift of the 6-OH towards L:91 and away from H:95, providing enough room for a water molecule. That water molecule could then accept a hydrogen bond from the proton on the 4-OH of galactose in much the same manner as the oxygen of the 6-OH position of galactose described previously, thus bridging hydrogen bonds between 4-OH and the carboxyl side-chain of H:95. Since the modeling in this study was performed in vacuo, the participation of such

explicit water molecules in the ligand binding were not taken into account. No potential hydrogen bonds were observed between the Fv and the rhamnosyl moiety.

The binding of carbohydrate ligands to proteins is mediated mostly by enthalpy, and the entropic contribution is of a dual nature: restriction of motion for the ligand gives an unfavorable contribution, while the displacement of water from the surface of the protein and the surrounding of the ligand gives a favourable contribution [34].

Binding studies on the O-SP determinant of *Salmonella* serogroup B has been reported [35,36]. Those workers found the determinant to be the trisaccharide abequosyl-[galactosyl]mannosyl fragment of the O-polysaccharide whose structure was made up of abequosyl-[galactosyl]mannosyl-rhamnosyl multiple repeating units. It is interesting that there the trisaccharide and longer fragments of from two to five tetrasaccharide repeating units had nearly identical free energies of binding, as would be expected. However, the higher oligosaccharides had a more unfavorable entropic component of the free energy of binding (compensated for by a higher enthalpic contribution), again as would be expected due to the restriction in motion for the larger oligosaccharide [35].

Crystallographic studies on that same antibody/antigen system [37] showed that three tryptophanyl- and three histidyl residues of the antibody were directly or indirectly involved in H-bonding to the carbohydrate determinant of the O-polysaccharide. It is remarkable that in the interaction of the O-SP of *Shigella dysenteriae* type 1 with antibody, we also have (spectral) evidence of the interaction of multiple residues of tryptophan with the O-SP saccharide determinant, and the modeled structure of the protein shows these tryptophanyl residues to be present in the cavity at the interface of the H- and L-chains, where an antibody combining area is generally known to be situated.

Modeling of the O-specific polysaccharide of *S. dysenteriae* type 1 suggests a sharply angled, helical structure in which the immunodominant galactosyl residue occurs in the polysaccharide sequence at the solvent exposed vertex of each hairpin loop (not published). The docked position of the disaccharide **3** here shown is consistent with this hypothesis and allows extension for the O-specific polysaccharide to occur in both the downstream (from the C-1 of galactose) and the upstream (the C-3 of rhamnose) directions of the -α-L-Rha*p*-(1→2)-α-D-Gal*p*- determinant without any unfavorable steric clashes with residues of the Fv.

Lastly, it is pointed out that, although the docking of the saccharide to the antibody model is speculative, the model of IgM 3707 E9 itself is much less so. This because the amnio acid sequences of its H- and L-chains show such unusually high homologies with the H- and L-chains respectively of two known antibodies whose high-resolution crystal structures have been reported ([22] PDB code 2FBJ, and PDB code 1GIG).

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