Molecular Recognition of a *Salmonella* Trisaccharide Epitope by Monoclonal Antibody Se155-4

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ABSTRACT: The binding site of monoclonal antibody Se155-4, which has been the object of successful crystallographic and antibody-engineering studies, is shown by solid-phase immunoassays to be complementary to a branched trisaccharide, α-D-Gal*p*(1→2)[α-D-Abe*p*(1→3)]-α-D-Man*p*(1, rather than to the tetrasaccharide repeating unit α-D-Gal*p*(1→2)[α-D-Abe*p*(1→3)]-α-D-Man*p*(1→4)α-L-Rha*p*(1- of the bacterial antigen. Specificity for the 3,6-dideoxy-D-*xylo*-hexose (3,6-dideoxy-D-galactose) epitope present in *Salmonella paratyphi* B O-antigens was ensured by screening hybridoma experiments with glycoconjugates derived from synthetic oligosaccharides. Detailed epitope mapping of the molecular recognition by modified and monodeoxy oligosaccharide derivatives showed that complementary surfaces and three antibody–saccharide hydrogen bonds are essential for full binding activity. Both hydroxyl groups of the 3,6-dideoxy-D-galactose residue were obligatory for binding and consistent with the directional nature of their involvement in carbohydrate–protein hydrogen bonds; related tetrasaccharides built from the isomeric 3,6-dideoxyhexoses, 3,6-dideoxy-D-glucose, paratose, and 3,6-dideoxy-D-mannose, tyvelose were not bound by the antibody. Titration microcalorimetry measurements were consistent with the hydrogen-bonding map inferred from the crystal structure and suggest that the displacement of water molecules from the binding site accounts for the favorable entropy that accompanies binding of the native trisaccharide determinant. The protein sequences determined for the antibody V_L and V_H domains reveal somatic mutation of the V_L germ line gene, implying that this antibody-binding site results from a mature antibody response.

Carbohydrate recognition by proteins is a subject of intrinsic interest (Quiocho, 1986; Lemieux, 1989) with many practical implications once the specifics of the molecular recognition process are understood. Thus, in order to realize the full potential of antibody engineering of carbohydrate-binding sites, a deeper appreciation is required of intermolecular interactions that govern the affinity and specificity of antigen binding. Although an antibody to lysozyme has been altered to provide higher binding affinity (Roberts *et al.*, 1987), the design of antibody molecules with improved binding sites is a field of study that is in its infancy (Cheetham, 1992). Carbohydrate–antibody systems are well suited to addressing certain aspects of this task and are of special interest since antibodies to tumor-associated carbohydrate antigens are used for therapeutic applications (Vadhan *et al.*, 1988; Honsik *et al.*, 1986).

Several antibody- and lectin-binding sites have been extensively mapped by natural ligands and ones modified by single-site deoxygenation (Lemieux, 1989; Glaudemanns, 1991; Bundle, 1989; Rivera-Sagredo *et al.*, 1991). These conservative replacements, such as the substitution of fluorine or hydrogen for a hydroxyl group, not only allow a detailed appreciation of the molecular and stereochemical requirements of binding but have also, in some instances, resulted in improved binding energy, with changes as high as 8 kJ mol^{-1} (Hanna & Bundle, 1993). Previously, only studies of the β-galactan antibody J539 and the *Griffonia simplicifolia* lectin GSIV (Delbaere *et al.*, 1990, 1993) have been able to draw on a solved X-ray structure to aid the interpretation of thermodynamic binding data. To understand more fully the nature of carbohydrate recognition in this type of system, it is desirable to have more examples of crystal structures, complemented by epitope mapping and thermodynamic data, to guide protein engineering of carbohydrate antibodies. Full thermodynamic characterization (ΔG, ΔH, and ΔS), which is conveniently obtained by titration microcalorimetry, provides details on the overall energetics of the binding interactions that structural studies cannot give (and vice versa) (Raffa & Porreca, 1989). In this paper, we report the selection, characterization, and epitope mapping of an antibody of known crystal structure (Cygler *et al.*, 1991) that has also been expressed in *Escherichia coli* as both Fab[1] (Anand *et al.*, 1991a) and single-chain Fv products (Anand *et al.*, 1991b) with antigen-binding activity and in mutant forms (Brummell *et al.*, 1993).

The chemical repeating unit of the *Salmonella* group B O-antigen (Figure 1) is based on a main-chain trisaccharide ManRhaGal that is common to *Salmonella* of groups A, B, and D. In these antigens, a 3,6-dideoxyhexose residue forms a branch point to the α-D-Man residue and is an immunodominant monosaccharide, *i.e.*, antibodies specific for the

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[1] Abbreviations: Abe, abequose, 3,6-dideoxy-D-*xylo*-hexose; BSA, bovine serum albumin; EIA, enzyme-linked immunoassay; LPS, lipopolysaccharide; PTH phenylthiohydantoin; V_L, variable domain of immunoglobulin light chain; V_H, variable domain of immunoglobulin heavy chain; Fab, *ca.* 50 000-kDa antigen-binding fragment composed of V_L, C_L, and V_H CH1 domains and released from IgG by papain proteolysis; Fv, *ca.* 25 000-kDa antigen-binding fragment composed of V_L and V_H domains; CDR, complementary determining regions or hypervariable loops.

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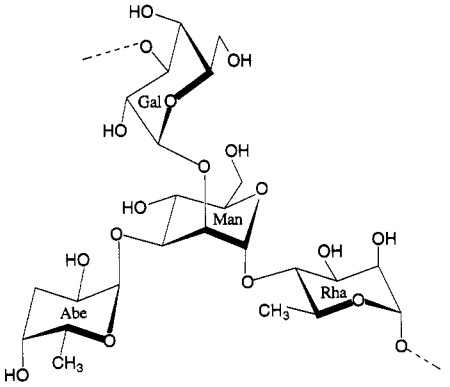


FIGURE 1: Structure of the chemical repeating unit of the *Salmonella* serogroup B *O*-polysaccharide.

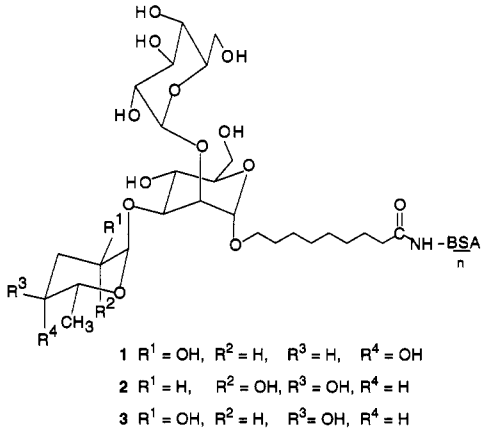
three serogroups A, B, and D use the dideoxyhexose as the principal site for binding (Lüderitz *et al.*, 1966). The stereochemistry of the 3,6-dideoxy-D-hexose (abequose in serogroup B) is the sole variable in this family of structurally related antigens, and hence, the stereochemistry of single hydroxyl groups on this moiety can determine antibody binding. Conformational analysis suggests that the accessibility of the Abe-Man element in the serogroup B determinant as well as the constrained flexibility of this glycosidic linkage accounts for this property (Bock *et al.*, 1984a,b). In the work reported here, glycoconjugates derived from synthetic ligands were used to characterize the binding profile of an antiserogroup B monoclonal antibody, Se155-4. A panel of antigen fragments and monodeoxy trisaccharide derivatives were used to identify those portions of the antigen that are hydrogen bonded to the antibody and to investigate the thermodynamic features of the antigen–antibody interactions. The determination of the amino acid sequence of the antibody is also reported.

MATERIALS AND METHODS

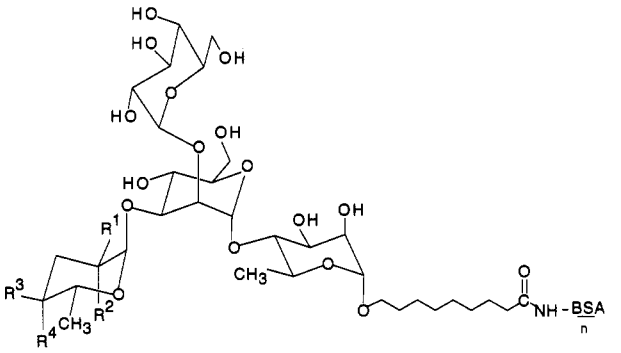
General procedures employing glycoconjugates and lipopolysaccharide antigens for hybridoma experiments and EIA followed previously published methods (Bundle *et al.*, 1982; Carlin *et al.*, 1986) unless otherwise indicated.

Bacterial Antigens. *Salmonella* strains belonging to the Kauffmann–White serogroups A, B, and D_1 were as follows: *S. paratyphi* A var. *durazzo* (O-factors 2 and 12), *S. essen* (O-factors 4 and 12), *S. paratyphi* B (O-factors 4, 5, and 12), and *S. typhi* (O-factors 9 and 12) (Kauffmann, 1975). Lipopolysaccharides were extracted from bacterial cells by the phenol–water method and purified by standard procedures (Westphal *et al.*, 1952; Johnson & Perry, 1976). Polysaccharide antigen was liberated from LPS by first incubating LPS with 0.25 M sodium hydroxide solution at 56 °C for 1 h; then, the solution was brought to pH 3.5 by addition of acetic acid followed by mild acid hydrolysis at 100 °C for 2 h. Purified polysaccharide was recovered by gel filtration (Caroff *et al.*, 1984).

Immunization and Generation of Hybridoma Antibodies. Phenol-killed cells suspended in sterile saline at $\sim 2 \times 10^8$ cells/mL were used to immunize groups of 6-10 BALB/c mice in one of two immunization protocols. Alkali-treated *S. paratyphi* B cells (10^8 cells, 2 h at 20 °C) were given by intraperitoneal injection on days 0 and 7. A third intravenous injection was given on day 28. The second protocol employed eight injections of 10^8 *S. essen* cells. The first six intraperitoneal injections were spaced 2 weeks apart followed after a 3 week rest by the seventh injection. A final injection was



1 R^1 = OH, R^2 = H, R^3 = H, R^4 = OH
2 R^1 = H, R^2 = OH, R^3 = OH, R^4 = H
3 R^1 = OH, R^2 = H, R^3 = OH, R^4 = H



4 R^1 = OH, R^2 = H, R^3 = H, R^4 = OH
5 R^1 = H, R^2 = OH, R^3 = OH, R^4 = H
6 R^1 = OH, R^2 = H, R^3 = OH, R^4 = H
7 R^1 = H, R^2 = H, R^3 = H, R^4 = OH
8 R^1 = OH, R^2 = H, R^3 = H, R^4 = H

FIGURE 2: Structures of the synthetic glycoconjugates used in antibody screening and characterization. The degree of hapten substitution on BSA, *n*, ranged from 10 to 30 (Pinto & Bundle, 1983).

given intravenously 3 weeks later. Antibody response was measured by EIA at appropriate intervals. Fusion experiments used pooled spleen cells collected 3 days after the last injection from two mice with strong EIA titers. The fusion protocol employed the Sp2/0 plasmacytoma cell line in a standard procedure used in this laboratory for other glycoconjugate-binding antibodies (Bundle *et al.*, 1982; Carlin *et al.*, 1986). One fusion experiment each was performed using spleen cells from mice immunized with *S. essen* and *S. paratyphi* B.

Screening and Selection of Hybridoma Cell Lines. Culture supernatant (100 μL) from hybridomas grown in 96-well culture plates (Nunc, GIBCO, Burlington, Ontario) were assayed by EIA with either serogroup B LPS- or glycoconjugate-coated EIA plates (Linbro, ICN Biochemicals, Mississauga, Ontario). LPS-coated plates were incubated with antibody-containing culture fluid with or without *O*-polysaccharide inhibitor. Positive clones with OD readings greater than 0.4 were further assayed against two glycoconjugates (**1** and **4**), a trisaccharide (Gal[Abe]Man), and a tetrasaccharide (Gal[Abe]ManRha) each conjugated to BSA (Figure 2) and against LPS samples from *Salmonella* of serogroups A, B, and D_1 (Figure 3). Nine clones positive for LPS binding were obtained from the *S. paratyphi* experiment, and none showed significant titers with any glycoconjugates. From the *S. essen* fusion, five clones were identified with high titers against the glycoconjugates and serogroup B LPS. Clone Se155-4, an

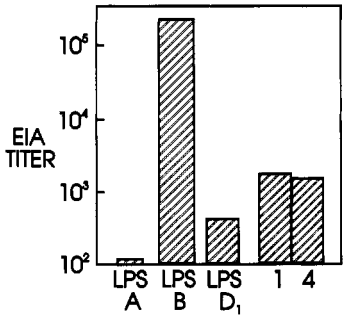


FIGURE 3: Specificity of Se155-4 for LPS of different serogroups and synthetic glycoconjugates in EIA.

IgG antibody, was selected for detailed study.

Heavy-chain isotypes were determined using an EIA mouse antibody kit (Hybri-Clonal, Kirkegaard and Perry Lab., Mandel Scientific Co. Ltd., Guelph, Ontario). Light-chain and IgG subclasses were established by immunodiffusion with antisera specific for mouse λ and κ chains (nordic Immunological Reagents, El Toro, CA) and for IgG subclasses (Zymed, Dimension Laboratories Inc., Mississauga, Ontario).

Enzyme Immunoassays. Indirect EIA employing LPS- or glycoconjugate-coated assay plates was performed as previously described using an anti-mouse Ig reagent coupled to alkaline phosphatase as the disclosing reagent (Bundle *et al.*, 1982).

Direct EIA was done with affinity-purified Se155-4 antibody Coated onto EIA plates and *O*-polysaccharide coupled to alkaline phosphatase (Vorberg & Bundle, 1994) or biotin (Meikle *et al.*, 1990). In the latter case, streptavidin-HRP conjugate (Sigma Chemical Co., St. Louis, MO) served as the disclosing reagent. Inhibition experiments with monovalent or polyvalent inhibitors were done according to previously reported protocols (Vorberg & Bundle, 1990).

Antibody Purification by Affinity Chromatography. O-Chain polysaccharide was aminated and coupled to epoxy-activated Sepharose (Altman & Bundle, 1994). Ascites fluid was centrifuged for 3–5 min in an Eppendorf microfuge. The supernatant was filtered through 0.8 and 0.22-μm filters and finally diluted with three volumes of 0.15 M NaCl in 0.05 M Tris buffer, pH 8.0. The affinity column (8 mL of gel in a 1.5- × 5-cm jacketed column cooled to 5 °C) was equilibrated with the Tris buffer, and 25 mL of diluted ascites fluid was applied to the column at a flow rate of 1 mL/min. The column was washed with 0.15 M NaCl in the Tris buffer, and then, the specific Se155-4 IgG was eluted with 0.5 M NaCl and 0.1 M sodium acetate buffer, pH 4.5. Typical recoveries were 3.5–5 mg of antibody/mL of ascites.

Preparation of Fab. Se155-4 in 0.05 M Tris buffer, 0.15 M NaCl, and 2 mM EDTA, pH 8.0, was reduced with 1.5 mM dithiothreitol for 30 min and digested with papain, freshly prepared from mercuripapain, with an enzyme–substrate ratio of 1:100 for 1 h at room temperature. The Fab and Fc products were separated by the above affinity chromatography procedure, without prior alkylation thus permitting the H–L interchain disulfide bond to reform during the affinity chromatography.

Preparation and Sequencing of Fd and L Chain. Ion-exchange chromatography in 6 M urea was used to obtain the two chains from fully reduced and alkylated (with iodoacetic acid) Fab. The protein was dissolved in 20 mM acetate buffer, pH 5.5, containing 6 M urea, applied to a column of Whatman CM-52 CM-cellulose (1 × 30 cm^2) in the same buffer, and eluted with a linear gradient to 0.25 M NaCl. To obtain peptide fragments, samples of Fd or L chain (200–500 μg) were dissolved in 50 mM NH_4HCO_3 and trypsin or chymotrypsin purified by reverse-phase HPLC (Titani *et al.*, 1982) was added in a 1:100 proportion. A sample of Fd was similarly digested with 1:50 thermolysin. After digestion at 37 °C for 3 h, a second portion of enzyme was added and the mixture was incubated for a further 3 h. L chain (200 μg) was cleaved at aspartic residues by mild acid hydrolysis (Schultz, 1967) using 0.25 M acetic acid at 110 °C for 8 h in vacuo. The products of these digestions were freeze-dried three times, and the peptides were purified by reverse-phase HPLC on Synchropak RP-P and RP-8 columns (4.1 × 250 mm^2) equilibrated in 0.1% trifluoroacetic acid and eluted with an acetonitrile gradient (1%/min) at a flowrate of 1.5 mL/min. Peptide peaks that were mixtures were refractionated using 10 mM potassium phosphate buffer, pH 7.0, and a *n*-propanol gradient. Fd (1.5 mg) was cleaved at methionine residues with CNBr in 70% formic acid at room temperature for 16 h under argon. The fragments were separated by HPLC using four TSK2000 size-exclusion columns run in series in 6 M guanidinium hydrochloride, 0.1 M Na_2SO_4, and 0.05 M phosphate buffer, pH 5.9. The products were simultaneously desalted and purified further by reverse-phase chromatography on an Altex RPSC-C3 column.

Amino acid compositions were determined on a Durrum D500 analyzer for peptide samples hydrolyzed in 6 M HCl at 110 °C for 20 h.

Automated gas-phase sequencing was performed with an Applied Biosystems Model 470A sequencer using 0.1–0.5 nmol of purified peptides. The samples in 30 μL of water were applied to glass-fiber discs containing 1.5 mg of precycled polybrene. The PTH derivatives were identified by on-line HPLC on an Applied Biosystems Model 120A chromatograph. In cases of ambiguity, the PTH derivatives were checked by chromatography on a cyanopropyl column using a Varian Vista 56 HPLC system. The N-terminal sequence of the L chain was determined on samples with and without treatment with the enzyme pyroglutamate aminopeptidase (Boehringer Mannheim Ltd., Laval, Quebec) by the method of Podell and Abraham (1978).

Antigen Fragments and Synthetic Ligands. Synthetic oligosaccharides were prepared according to published procedures (Pinto & Bundle, 1984; Bock & Meldal, 1984a,b) and coupled to proteins by standard methodology (Lemieux *et al.*, 1975; Pinto & Bundle, 1983). Oligosaccharide methyl glycosides and deoxygenated derivatives were prepared by elaboration of new synthetic schemes (Bundle and Eichler, 1994). Dodecasaccharide and octasaccharide fragments were obtained by phage degradation of the O-chain, and univalent heptasaccharide ligands were obtained by controlled acid hydrolysis of the octasaccharide (Baumann *et al.*, 1993). The structures of the polysaccharides, phage-derived oligosaccharides, and synthetic compounds were confirmed by ^1H NMR spectroscopy at 500 MHz.

Microcalorimetry. The thermodynamics of the binding of congeners **10–29** by antibody were determined by titration microcalorimetry using an OMEGA titration microcalorimeter (Wiseman *et al.*, 1989) from MicroCal Inc. (Northampton, MA). Samples of approximately 50 μM antibody in 50 mM Tris and 150 mM NaCl, pH8.0, were titrated with solutions of the congeners of an appropriate precisely known concentration in the same buffer at 25 °C. The ligand was injected in 20 portions of 12.5 μL from a 250-μL syringe with stirring at 400 rpm. The instrument was calibrated by standard heat pulses. Base-line determination and integration of the recorded thermograms were carried out using the ORIGIN

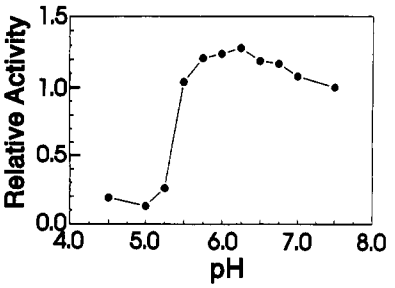


FIGURE 4: Effect of pH on the activity of Se155-4 in EIA.

software (MicroCal, Inc.), and the binding isotherms were analyzed as described previously (Sigurskjold *et al.*, 1991; Sigurskjold & Bundle, 1992).

RESULTS

Production and Characterization of Se155-4. Mouse hybridomas were generated from spleen cells of mice immunized with killed whole-cell vaccines of *S. essen* and *S. paratyphi* B. Both bacteria express the Kauffmann–White O-factor 4 that is associated with the immunodominant abequose residue (Lüderitz *et al.*, 1966), but the *S. paratyphi* B O-antigen also carries an *O*-acetylated form of this monosaccharide that results in the production of O-factor 5 antibodies (Bundle, 1990). This structural element was destroyed prior to immunization by incubation of bacterial cells with alkali to hydrolyze the acetate ester. The *S. paratyphi* fusion experiment using spleen cells from mice given three injections with these bacterial cells gave *nine* antibody-producing cell lines with high titers for the homologous LPS antigens of serogroup B *Salmonella*. Apart from three low-titer IgM antibodies, none showed significant EIA titers (OD > 0.2 at ascites dilutions of 1:100) with the glycoconjugates **1** and **4** (Figure 2). However, five hybridomas that did react with these glycoconjugates were obtained from the fusion experiment that used spleen cells from mice hyperimmunized (eight injections) with *S. essen* bacteria. Four of these belonged to the IgM class, and ascites fluid from these cell lines exhibited antiglycoconjugate titers of 10^4. A fifth antibody, Se155-4 (an IgG), also had a similar titer (10^3) against the two conjugates **1** and **4**. All five antibodies precipitated the homologous, alkali-treated LPS and glycoconjugates **1** and **4**, implying binding to a multivalent epitope. Negligible or weak titers were observed for Se155-4 with the LPS from the serogroup D_1 and serogroup A LPS and the glycoconjugates with the corresponding tri- and tetrasaccharide epitopes **2**, **3**, **5**, and **6**. The antibody did not bind to glycoconjugates **7** and **8** modified at C-2 and C-4 of the abequose residue (Figure 3). These experiments, together with immunodiffusion results (not shown), established that Se155-4 precipitated only *O*-polysacchardies containing the abequose epitope of serogroup B *Salmonella*.

Antibody Purification and Chemical Characterization. Heavy- and light-chain isotyping showed that Se155-4 belonged to the IgG_1 subclass and had a λ light chain. For purification of the antibody from ascites fluids, an affinity matrix was prepared from bacterial *O*-polysaccharide, selectively aminated to provide a site for reaction with epoxy-activated Sepharose gel (Altman & Bundle, 1994). The capacity of the affinity column prepared in this way was at least 5.8 mg of antibody/mL of gel. A pH profile obtained by EIA (Figure 4) showed that the Se155-4 antibody lost activity as the pH approached 5.0. It could therefore be readily eluted from the affinity column with sodium acetate buffer, pH 4.5. The antibody's pH behavior is consistent with the presence of histidine residues in the binding site (see below). Antibody purified in this very mild way was shown to retain full activity and was used for the amino acid sequencing, EIA competitive binding assays, and microcalorimetry. In the latter case, antibody was also recovered for successive measurements by rechromatography on the matrix, washed free of ligand, and the fully active antibody was recovered.

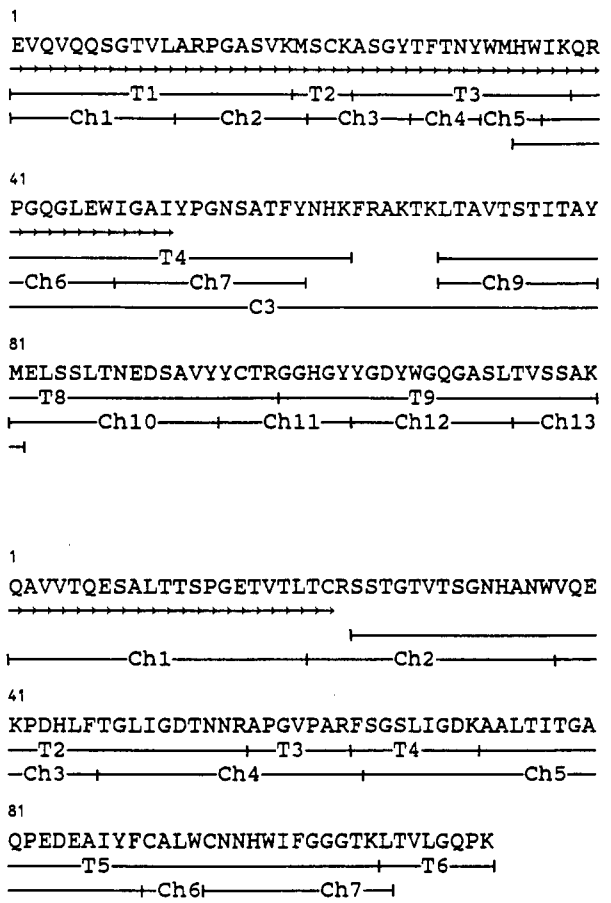


FIGURE 5: Sequencing of the V_H domain (upper diagram) and V_L domain (lower diagram) of Se155-4. The peptide fragments are T, tryptic, Ch, chymotryptic, and C, CNBr. Confirming sets of thermolytic peptides for V_H and acid-cleavage peptides for V_L were also obtained.

Conditions were established in preliminary experiments that gave a high yield of Se155-4 Fab by papain digestion of mildly reduced IgG_1. The Fab and Fc products were readily separated with the affinity column. If the protein was not alkylated after the digestion, the H–L interchain disulfide bond reformed during the affinity chromatography, giving a stable species for crystallization (Rose *et al.*, 1990).

For sequencing, the Fd and L chains were obtained from reduced and alkylated Fab by ion-exchange chromatography in urea. N-terminal sequencing of the two chains showed that the majority of the L chains had blocked N-termini. Though a sequence was obtained from the minor unblocked species, it was subsequently found that it was missing the first two residues when compared to the main blocked population of L chains. The amino acid sequences of the V_H and V_L domains and the peptides used to establish them are shown (Figure 5), and composition data for peptides are given in Tables 1 and 2.

Complete sets of tryptic, chymotryptic, and thermolytic peptides for the CH1 domain were also sequenced. Differences from the reported sequences for this domain (Kabat *et al.*,

Table 1: Amino Acid Compositions of V_H Peptides[a]

	T1- (1–19)	T2- (20–23)	T3- (24–38)	T5- (39–63)	T6- (70–98)	T7- (99–120)	C3- (35–81)
CMC		(1)			(1)		
Asp			0.9 (1)	2.0 (2)	2.1 (2)	1.1 (1)	2.2 (2)
Thr	0.9 (1)		1.8 (2)	0.9 (1)	5.6 (6)	0.9 (1)	4.4 (5)
Ser	2.8 (2)	0.9 (1)	0.9 (1)	0.8 (1)	3.6 (4)	2.7 (3)	1.4 (2)
Glu	4.0 (4)			3.2 (3)	2.2 (2)	1.0 (1)	3.4 (3)
Pro	1.1 (1)			2.1 (2)			2.0 (2)
Gly	2.1 (2)		1.2 (1)	4.3 (4)		6.5 (6)	4.3 (4)
Ala	2.2 (2)		1.0 (1)	2.1 (2)	3.2 (3)	2.1 (2)	6.4 (6)
Val	3.8 (4)				1.9 (2)	0.9 (1)	1.2 (1)
Met		0.7 (1)	0.7 (1)		0.7 (1)		(1)
Ile			1.0 (1)	1.9 (2)	1.0 (1)		3.2 (4)
Leu	1.1 (1)			1.0 (1)	3.2 (3)	1.0 (1)	2.0 (2)
Tyr			1.9 (2)	2.0 (2)	2.9 (3)	2.9 (3)	2.4 (3)
Phe			1.1 (1)	1.1 (1)			2.1 (2)
His			0.9 (1)	0.9 (1)		0.9 (1)	1.6 (2)
Lys	1.1 (1)	1.0 (1)	1.1 (1)	1.0 (1)		1.1 (1)	4.0 (4)
Arg	0.9 (1)			0.9 (1)	1.0 (1)		2.0 (2)
Trp			(2)	(1)		(1)	(2)
total	(19)	(4)	(15)	(25)	(29)	(22)	(47)

[a] Tryptic peptides, T; CNBr peptide, C. Values in parentheses are the predicted numbers of residues from the sequence.

Table 2: Amino Acid Compositions of V_L Peptides[a]

	Ch1- (1–18)	CH2- (19–35)	T2- (23–56)	T3- (57–63)	T4- (64–72)	T5- (73–105)	T6- (105–113)
CMC		(1)				(2)	
Asp		2.1 (2)	5.9 (6)		1.0 (1)	3.0 (3)	
Thr	4.7 (5)	3.7 (4)	4.9 (5)			2.8 (3)	1.0 (1)
Ser	1.9 (2)	2.7 (3)	2.8 (3)		1.8 (2)		
Glu	4.2 (4)		2.1 (2)			3.1 (3)	1.0 (1)
Pro	1.1 (1)		0.9 (1)	2.1 (2)		1.1 (1)	1.0 (1)
Gly	1.2 (1)	2.1 (2)	3.9 (4)	1.1 (1)	2.1 (2)	4.2 (4)	1.0 (1)
Ala	2.1 (2)	1.1 (1)	1.0 (1)	2.0 (2)		5.3 (5)	
Val	3.1 (3)	0.9 (1)	1.9 (2)	0.9 (1)			0.9 (1)
Met							
Ile			0.9 (1)		1.0 (1)	2.9 (3)	
Leu	2.0 (2)		2.0 (2)		1.1 (1)	2.1 (2)	1.9 (2)
Tyr						1.0 (1)	
Phe			1.0 (1)		1.0 (1)	2.0 (2)	
His		0.9 (1)	2.2 (2)			0.9 (1)	
Lys			1.2 (1)		0.9 (1)	1.2 (1)	1.1 (1)
Arg		1.0 (1)	1.1 (1)	0.9 (1)			
Trp		(1)	(1)			(2)	
total	(18)	(17)	(33)	(7)	(9)	(33)	(8)

[a] Tryptic peptides, T; chymotryptic peptides, Ch. Values in parentheses are the predicted numbers of residues from the sequence.

Table 3: Amino Acid Compositions of CH1 Peptides[a] from the 198–199 Region

	Th193–204	Th187–209	Ch186–208
CMC		(1)	(1)
Asp		1.1 (1)	
Thr	2.8 (3)	3.6 (4)	4.6 (5)
Ser	2.7 (3)	5.4 (6)	5.4 (6)
Glu	1.1 (1)	1.2 (1)	.8 (1)
Pro	2.3 (2)	2.2 (2)	2.1 (2)
Val	2.3 (2)	2.8 (3)	2.8 (3)
Leu		1.1 (1)	1.1 (1)
Trp	(1)	(1)	(1)
total	(12)	(20)	(20)

[a] Thermolytic peptides, Th; chymotryptic peptide, Ch. Values in parentheses are the predicted numbers of residues from the sequence. The overall segment sequence is [numbered according to Kabat *et al.* (1991)] ^{187}LSSSVTVPSS-TWP-SETVT-CN^{209}.

1991) were found in two places, residue 135 being Asp rather than Asn and residues 198 and 199 being Thr and Trp rather than Pro and Arg, respectively. The composition data for peptides from the latter region are shown in Table 3; the assignments are confirmed by the Pro and Thr contents and the lack of Arg. These sequence assignments bring the IgG_1 CH1 domain into greater homology with the other three IgG isotypes. A peptide set for the Cλ1 domain was also checked, and it was fully consistent with the literature sequence.

Table 4: Relative Inhibitory Activity in EIA of *Salmonella* Serogroup B O-Antigen Analogues

inhibitor	$\Delta(\Delta G)$ (kJ mol^{-1})
tetrasaccharides:	
(**9**) Gal[Abe]ManRha-OCH_3	0.0
(**7b**) Gal[2-deoxy-Abe]ManRha-OCH_3	≫9
trisaccharides:	
(**10**) Gal[Abe]Man-OCH_3	1.7
(**1b**) Gal[Abe]Man-$O(CH_2)_2CO_2H$	−6.3
(**2b**) Gal[Tyv]Man-$O(CH_2)_2CO_2H$	≫10
disaccharides:	
(**18**) AbeMan-OCH_3	4.7
(**17**) Abe(2-*O*-CH_2OCH_3-Man)-OCH_3	2.1

Ligand-Binding Characteristics. Direct competitive EIA was used to evaluate the activity of inhibitors prepared by synthetic chemistry. EIA plates coated with Se155-4 were incubated with synthetic haptens and polysaccharide conjugated to alkaline phosphatase or to biotin. This competitive EIA provided for the estimation of K_A and $\Delta(\Delta G)$ values for ligands with single-site structural modifications (Vorberg & Bundle, 1990; Meikle *et al.*, 1990). The data (Table 4) shows that abequose trisaccharide epitope **10** bound almost as well as the corresponding tetrasaccharide **9** (Figure 6). Consequently, the rhamnose residue contributes little binding energy, implying that the site is complementary to a trisaccharide. It is also apparent that the largest part of the binding energy derives from the Abe-Man disaccharide **18**, since it is only ~3 kJ mol^{-1} less active than trisaccharide **10**. Replacing the rhamnose residue by a nine-carbon aglycon produced a more potent inhibitor, suggesting that hydrophobic interactions could occur in this peripheral region of the binding site. Epimerizing the axial hydroxyl group of abequose to give the *arabino* configuration (tyvelose) abolished activity, and introduction of hydroxyl groups at the former deoxy centers C-3 and C-6 (compounds not shown) had the same effect, as did the introduction of a 2-deoxy group (compound **7b**, Table 4). This implies that abequose and the antibody site must possess highly complementary surfaces, since steric repulsion is known to be an important mechanism for achieving high biological specificity (Fersht & Dingwall, 1979).

Microcalorimetry. Microcalorimetry was carried out with 16 synthetic inhibitors (Figure 6) including trisaccharide and disaccharide analogues **10–18**, monodeoxygenated derivatives **19–22**, and simple monosaccharides **23–25** that possessed association constants in the range 10^2–10^5 M^{-1}. The results of these measurements conducted with 6 mg of whole IgG per experiment confirm that the EIA data do in fact provide a reliable ranking of inhibitory power and, at least for compound **10**, an estimate of K_A. All of the ligands display relatively strong enthalpic interactions, and for several trisaccharides, most notably the native trisaccharide **10**, a favorable and substantial entropy contribution was observed (Table 5).

The dependence of binding free energy on ligand size has been investigated earlier by titration microcalorimetry, and pertinent data for octasaccharide **26** and dodecasaccharide **27** (two and three repeating units) are summarized together with new data for the univalent heptasaccharides **28** and **29** in Table 6. For trisaccharide **10** and oligomers **26** and **27**, the molar free energy is virtually constant, while the molar enthalpy and entropy show strong but mutally compensating dependencies on the chain length, suggesting that this relatively small trisaccharide structure virtually fills the binding site.

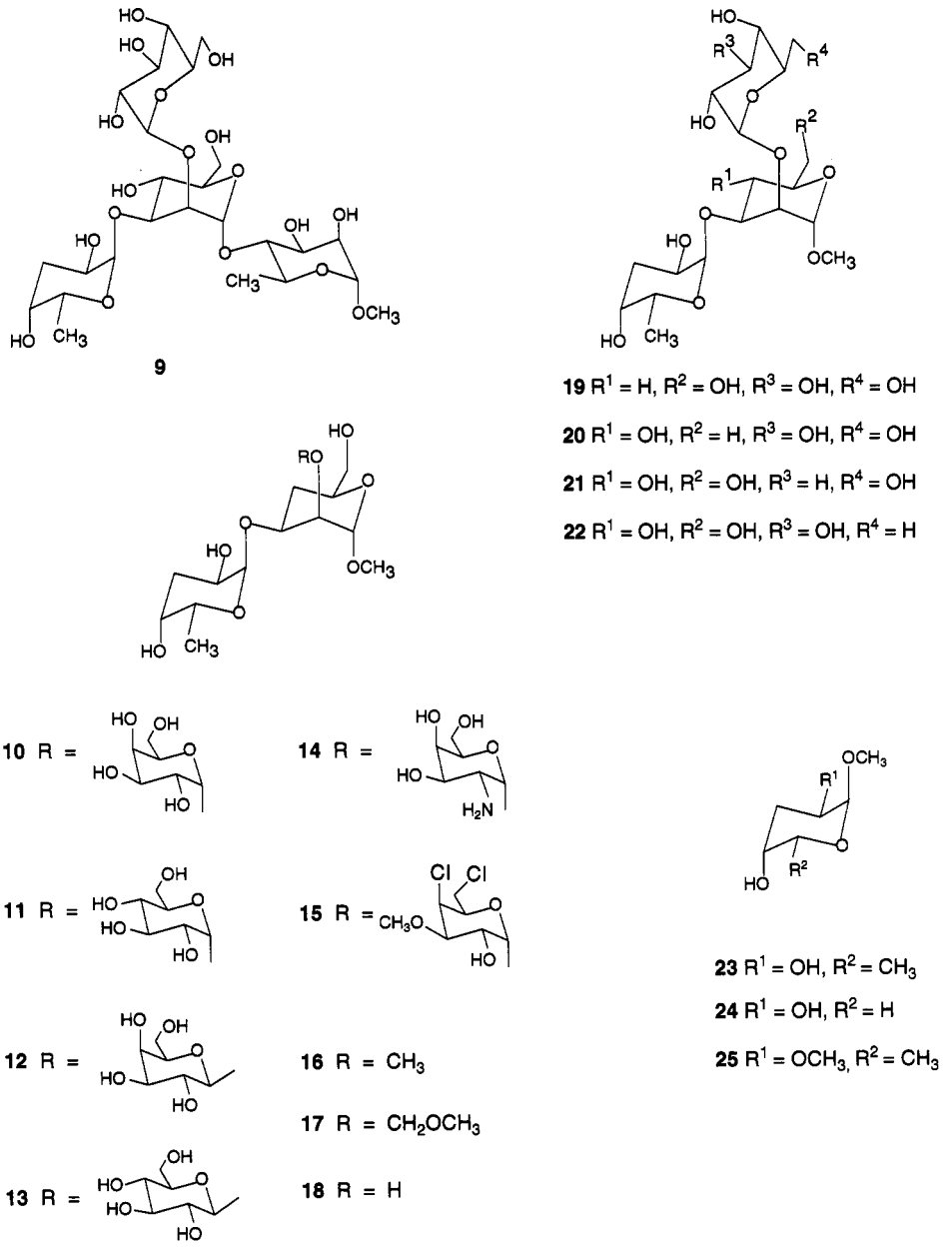


FIGURE 6: Structures of the synthetic oligosaccharide used as inhibitors in EIA and microcalorimetry experiments.

Table 5: Thermodynamics of the Binding of *Salmonella* Serogroup B O-Antigen Analogues by Sel55-4

methyl glycoside ligands	K (M^{-1})	$\Delta G°$ (kJ mol^{-1})	$\Delta H°$ (kJ mol^{-1})	$-T\Delta S°$ (kJ mol^{-1})
(**10**) Galα[Abe]Man	2.1 ● 0.2[a] × 10^5	−30.5 ± 0.4	−20.5 ± 0.5	−10.0 ± 0.6
(**11**) Glcα[Abe]Man	1.3 ± 0.2 × 10^5	−29.2 ± 0.3	−37.6 ± 1.1	8.4 ± 1.1
(**12**) Galβ[Abe]Man	7.9 ± 1.4 × 10^2	−16.5 ± 0.4	−103.2 ± 4.2	86.6 ± 4.2
(**13**) Glcβ[Abe]Man	3.0 ± 1.4 × 10^4	−25.6 ± 1.2	−12.1 ± 3.4	−13.5 ± 3.6
(**14**) (2-amino-2-deoxy-Gal)[Abe]Man	1.3 ± 0.3 × 10^5	−29.3 ± 0.5	−26.9 ± 1.2	−2.4 ± 1.3
(**15**) (3-*O*-CH_3-4,6-dichloro-Gal)[Abe]Man	3.0 ± 0.8 × 10^5	−31.2 ± 0.6	−34.0 ± 2.3	2.8 ± 2.4
(**16**) Abe(2-*O*-CH_3-Man)	1.2 ± 0.2 × 10^5	−29.0 ± 0.3	−32.1 ± 0.7	3.1 ± 0.9
(**17**) Abe (2-*O*-CH_2OCH_3-Man)	6.9 ± 0.6 × 10^4	−27.6 ± 0.2	−26.6 ± 0.6	−1.0 ± 0.7
(**18**) AbeMan	2.4 ± 0.2 × 10^4	−25.0 ± 0.2	−28.8 ± 0.7	3.8 ± 0.9
(**19**) Gal[Abe](4-deoxy-Man)	5.1 ± 1.7 × 10^4	−26.9 ± 0.8	−27.7 ± 3.8	0.8 ± 3.9
(**20**) Gal[Abe](6-deoxy-Man)	8.6 ± 1.2 × 10^4	−28.2 ± 0.3	−26.5 ± 1.2	−1.7 ± 1.3
(**21**) (3-deoxy-Gal)[Abe]Man	8.8 ± 1.4 × 10^4	−28.3 ± 0.4	−33.0 ± 2.2	4.7 ± 2.2
(**22**) (6-deoxy-Gal)[Abe]Man	9.0 ± 2.3 × 10^4	−28.3 ± 0.6	−20.4 ± 1.6	−7.9 ± 1.7
(**23**) Abe	1.5 ± 0.4 × 10^3	−18.1 ± 0.6	−33.0 ± 4.5	14.9 ± 4.8
(**24**) 3-deoxy-Ara	1.2 ± 0.5 × 10^2	−11.9 ± 2.8	−20.3 ± 4.9	8.4 ± 5.7
(**25**) 2-*O*-CH_3-Abe	1.9 ± 0.1 × 10^3	−18.7 ± 0.2	−24.6 ± 5.8	5.8 ± 5.8

[a] Uncertainties represent three standard deviations obtained from regression analysis.

While binding-site interactions are unchanged for the larger saccharides **26** and **27**, changes in enthalpy may arise from additional protein–saccharide interactions with offsetting entropy costs. Intriguingly, these effects may also contain a component for the increased saccharide–water interactions that can occur at the periphery of the binding site.

Table 6: Thermodynamics of the Binding of Fragments of the *Salmonella* Serogroup B O-Antigen to Sel55-4 at 25 °C[a]

ligand	K (10^5 M^{-1})	$\Delta G°$ (kJ mol^{-1})	$\Delta H°$ (kJ mol^{-1})	$-T\Delta S°$ (kJ mol^{-1})
(**10**) Gal[Abe]Man→OMe	2.1 ± 0.2	−30.5 ± 0.4	−20.5 ± 0.5	−10.0 ± 0.6
(**26**) $(Gal[Abe]ManRha)_2$	4.9 ± 1.3	−32.6 ± 2.0	−34.1 ± 4.6	1.7 ± 2.3
(**27**) $(Gal[Abe]ManRha)_3$	4.7 ± 1.0	−32.2 ± 1.6	−44.1 ± 7.1	11.7 ± 2.3
(**28**) GalManRhaGal[Abe]ManRha	24 ± 6	−36.4 ± 0.6	−37.4 ± 0.4	0.9 ± 0.7
(**29**) Gal[Abe]ManRhaGalManRha	0.4 ± 0.1	−26.5 ± 0.6	−37.9 ± 3.1	11.4 ● 3.1

[a] Data for compounds **26** and **27** are from Sigurskjold *et al.* (1991).

Table 7: Contributions from Each of the Pyranose Residues to the Overall Binding of the Trisaccharide Epitope to Sel55-4[a]

residue	$\Delta(\Delta G°)$ (kJ mol^{-1})	$\Delta(\Delta H°)$ (kJ mol^{-1})	$-T\Delta S°$ (kJ mol^{-1})
Abe	−18.1 ± 0.6	−33.0 ± 4.5	14.9 ± 4.8
Man[b]	−10.9 ± 0.7	0.9 ± 4.6	−11.8 ± 4.9
Man[c]	−6.9 ± 0.6	4.2 ± 4.6	−11.1 ± 4.9
Gal[b]	−1.5 ± 0.5	11.6 ± 0.9	−13.1 ± 1.1
Gal[c]	−5.5 ± 0.4	8.3 ± 0.9	−13.8 ± 1.1

[a] Uncertainties have been calculated according to the law of error propagation. [b] Mannose and galactose contributions calculated by comparing **23** with **16** and **16** with **10**. [c] Mannose and galactose contributions calculated by comparing **23** with **18** and **18** with **10**.

Two heptasaccharides (**28** and **29**), each with only one abequose residue, were prepared by mild acid hydrolysis of octasaccharide **26** (two repeating units) which was obtained from the polysaccharide by cleavage with a phage-associated rhamnosidase (Baumann *et al.*, 1993). The heptasaccharides lack an abequose residue at either the reducing or the nonreducing terminus, respectively, and bind in two distinct modes. Compound **29** binds an order of magnitude weaker than the octasaccharide, while **28** binds a factor of 5 stronger. This represents a remarkable example in which destruction of a possible binding epitope actually leads to enhanced binding. Most interestingly, the observed effects appear to be entropic—the two heptasaccharides and the octasaccharide bind with virtually identical enthalpy contributions but with relatively large variations in their entropy terms. Molecular modeling of **29** into the binding site in its global minimum-energy conformation showed that the terminal galactose collides with amino acids at the surface of the antibody but beyond the immediate vicinity of the binding site.

Contributions to binding by the different pyranose rings were estimated from the thermodynamic data of compounds **10**, **16**, **18**, and **23**, and these relative energies are summarized in Table 7. All of the enthalpy is provided by the binding of the abequose unit, whereas mannose and galactose contribute only modestly to the free energy, mainly via favorable entropy and small unfavorable enthalpy components. In estimating the relative contribution of the galactose residue, it may be argued that the 2-*O*-methyl analogue **16** is a more approriate reference compound than disaccharide **18**, since the latter displays a naked OH group rather than the ether oxygen atom that occurs in the native trisaccharide. A free-energy difference of 4.0 kJ mol^{-1} attributable to less favorable enthalpic interactions by **18** relative to **16** appears to support the choice of **16** as reference. A similar effect is seen when a methoxymethyl group (**17**) replaces galactose as a mimic of the anomeric linkage and the endocyclic oxygen and C-5 atoms (Table 5). Without reference to the crystal structure, the most plausible inference is that of the three saccharide residues comprising the antigenic determinant: abequose which provides all the enthalpy of binding is the most buried in the antibody-binding site, while galactose and, to a lesser extent, mannose are exposed to bulk water, since their favorable entropy terms suggest that the major driving force for their binding is solvent displacement.

Binding of methyl 3-deoxyarabinose (**24**) relative to that of **23** provides an estimate of the contribution from the abequose 6-CH_3 group, and its replacement by H lowers ΔG by 6 kJ mol^{-1}. Significantly less enthalpy reflects the lost van der Waals interactions, while a relatively more favorable entropy term, $-T\Delta S$, suggests that the peptide chain of the binding pocket has gained motional freedom. Methylation of the 2-OH of the abequose methyl glycoside (**25**) provides an indication that there are no major steric constraints to the introduction of the *O*-methyl group in the vicinity of this portion of the epitope and also suggests that this OH group may act as a hydrogen-bond acceptor but not a hydrogen-bond donor to protein.

The four monodeoxy congeners **19–22** all display slightly weaker binding than the native trisaccharide **10**. Most significant is the 3.6 kJ mol^{-1} smaller free energy of the 4-deoxymannose analogue **19** which is consistent with this OH group's involvement in a neutral acceptor–neutral donor hydrogen bond, which has been estimated at 4 kJ mol^{-1} (Street *et al.*, 1986). Compound **14**, in which the 2-OH of galactose has been substituted by an amino group, retains its activity. Since the amino group is partly unprotonated at pH 8.0, it may still participate in a hydrogen bond, but the approximately 2 kJ mol^{-1} loss of activity for the introduction of a 2-deoxy-galactose residue (see EIA section) indicates only weak hydrogen bonds at this site. The binding of the deoxy congeners **20–22** suggests that none of these OH groups (Man 6-OH and Gal 3-OH and 6-OH) forms a hydrogen bond with the antibody. Consistent with these data, the trisaccharide **15**, in which the galactose residue bears a 3-*O*-methyl group to simulate the ether oxygen of the native polysaccharide as well as two chlorine atoms at C-4 and C-6 which are largely isosteric with hydroxyl groups, is the only analogue to bind more strongly than trisaccharide **10**.

Ancillary evidence of the importance to binding of the galactose anomeric configuration and its stereochemistry at C-4 were probed by examination of the congeners **10–13**. Epimerization of the 4-OH group to form the *α-gluco* analogue **11** only weakly reduces the binding, whereas inversion of the anomeric configuration of the galactose residue to the *β*-anomer **12** reduces binding by more than 3 orders of magnitude. This weak inhibitor possesses an extremely large enthalpy change and a comparable large and positive entropy term, changes that are indicative of binding site interactions that differ substantially from all other analogues tested. Surprisingly, incorporation of both the *β*-anomeric and C-4 epimer modifications in compound **13** restores much of the free energy of binding and more typical enthalpy and entropy values.

DISCUSSION

In this molecular recognition study, strong enthalpic interactions originating with a single monosaccharide dominate oligosaccharide–antibody interactions, even though the antibody-binding site is complementary to a trisaccharide-sized epitope. In fact, its cavity- or pocket-type site is close to the previously envisioned lower limit for carbohydrate antibodies

```
                1
VH              EVQVQQSGTVLARPGASVKMSCKASGYTFTNYWMHWIKQR
VGAM 3-0        ---L------------------T------S-----V---

               41
VH              PGQGLEWIGAIYPGNSATFYNHKFRAKTKLTAVTSTITAY
VGAM 3-0        ----------------D-S--Q--KG-A-------AS---

               81
VH              MELSSLTNEDSAVYYCTR GGHGY YGDYWGQGASLTVSS
VGAM 3-0        ----------------- P-N-- -F------TT-----
 D, JH2
```

```
                1
VL              QAVVTQESALTTSPGETVTLTCRSSTGTVTSGNHANWVQE
Vλ1             --------------------------A--TS-Y------

               41
VL              KPDHLFTGLIGDTNNRAPGVPARFSGSLIGDKAALTITGA
Vλ1             ----------G---------------------------

               81
VL              QPEDEAIYFCALWCNNH WIFGGGTKLTVLG
Vλ1,Jλ1         -T----------YS-- -V----------
```

FIGURE 7: Homologies of the V_H (upper diagram) and V_L (lower diagram) sequences. The sequences of the Vλ1, Jλ1, and JH2 genes are from Kabat *et al.* (1991), the V_H gene VGAM3-0 is from Winter *et al.* (1985), and the D segment is the closest match from the preferred reading-frame list of Abergel and Claverie (1991).

(Kabat, 1966; Arakatsu *et al.*, 1966). The well-characterized monoclonal $IgG_1\lambda_1$ antibody exhibits a specificity profile that resembles those of serotyping reagents used to identify and group *Salmonella* bacteria (Kauffmann, 1975; Lüderitz *et al.*, 1966), and these characteristics were deliberately secured via a hyperimmunization strategy coupled with the use of a screening panel of chemically defined glycoconjugates. In order to establish the atomic details of oligosaccharide–antibody recognition, the IgG isotype was particularly important, since structural studies of antibody-combining sites by crystallography require small antigen-binding fragments, Fab, that are barely accessible from IgM but readily obtained from immunoglobulins of the IgG or IgA class. Frequently, antibodies generated in response to carbohydrate antigens belong overwhelmingly to the IgM class, since carbohydrate antigens behave as T-independent antigens and often do not induce an amnestic response nor show class switching from IgM to IgG. However, when presented to the mouse immune system as an integral part of the bacterial cell wall, the humoral response to O-polysaccharides includes an IgG component (Elkins & Metcalf, 1984; Carlin *et al.*, 1987; Perlmutter *et al.*, 1978), often IgG_3. With the aid of glycoconjugates bearing the immunodominant sugar abequose and its stereoisomers, rigorous screening protocols ensured the identification of antibodies with the desired binding profile and relatively high affinity.

The protein sequences of the V_L and V_H domains suggest that several somatic mutations occurred during the immune response to this antigen and imply an affinity-driven maturation of the immune response, which is consistent with the observed isotype and, for carbohydrates, the relatively high association constant (Table 5). Se155-4 is a λ1 antibody, and in the mouse, the various Cλ light-chain genes are grouped, with only one Vλ gene per pair of Cλs (Sanchez *et al.*, 1991). Compared to the germ line Vλ1/Jλ1 sequences (Figure 7), there are a total of eight residue differences in the light chain arising from somatic mutation, four of which are in the first complementarity determining region CDR L1 and two in CDR L3. The other changes are in the third framework region and in the Jλ1 segment. The sequence of the Se155-4 V_H domain shows that it is derived from a gene in the J558 family, the largest mouse V_H family. Computer searches of the protein and gene data bases showed that the Se155-4 V_H had highest homology to a gene sequence termed VGAM3-0 (Winter *et al.*, 1985), with 86 identical residues out of 98 in the V_H (Figure 7). The number of differences is higher than for V_L, and given the number of genes in the J558 family, an origin from another gene cannot be ruled out.[2] Of the 12 differences, seven are in framework regions and are all conservative changes. In the CDRs, there is one difference in CDR H1 and four in CDR H2. All except one of the 12 differences (Phe59) could arise from single-base changes. Without the nucleotide sequence, the D segment could not be matched exactly to the known groups (Kabat *et al.*, 1991), but it is similar to the sequences commonly found in D segments of the SP2 family (Abergal & Claverie, 1991). Though the CDR H3 is similar in length to those of other anti-carbohydrate J558 antibodies, it has a particularly striking composition of four glycines, two tyrosines, and one histidine.

The Se155-4 antibody is unusual in having several histidine residues in the binding-site vicinity, five of its six CDRs bearing His residues. The crystal structure (Cygler *et al.*, 1991) shows that one, His 35H, is at the base of the pocket into which the abequose unit fits, and it helps to position a critical water molecule. Two more, His 34L and His 101H, are at the top of the site near the Gal and Man subsites, respectively. These histidine residues are the probable cause of the remarkable pH sensitivity shown by Se155-4. Antigen binding is lost when the pH is lowered to 5.0 or below (Figure 4), and this behavior made it possible to purify the protein by affinity chromatography under very mild conditions. Some of the histidines are close to each other in the three-dimensional structure, and it has been found that the Se155-4 Fab is capable of binding copper ions (Young *et al.*, unpublished observations). On the basis of the above homologies, three of the five His residues, 34L, 62H, and 101H, appear to have arisen by somatic mutation, and the other two, 97L and the key residue 35H, are germ line.

Another interesting change in Se155-4 is the somatic mutation of Tyr 94L in the CDR L3 to a cysteine residue. Cysteine is not common in CDRs, but site-directed mutagenesis (Anand *et al.*, 1992) has shown that Cys 94L is not essential for antigen binding, as it can be replaced with either serine or the germ line residue tyrosine (Brummell *et al.*, 1993). The change from a bulky aromatic side chain to the more compact methylthiol group does not affect binding, and in the crystal structure, this residue is positioned with its side chain on the outside of the CDR. In addition, Fab molecules alkylated during their preparation did not show any impairment in their reactivity with antigen. Hence, this residue could be very useful for introducing reporter groups into the binding-site region.

The X-ray diffraction data for the crystal structure of Se155-4 complexed with trisaccharide **10** and compound **27** (Cygler *et al.*, 1991) revealed electron density that defined the positions of the abequose, mannose, and galactose residues of one repeating unit but not that of the rhamnose residue, and neither of the flanking repeating units were seen. A schematic diagram of the binding site and its complexed trisaccharide epitope together with the inferred hydrogen-bonding map is presented in Figure 8. The calorimetry data which provided estimates of the size of the binding site (Sigurskjold *et al.*, 1991), the key polar contacts, the strong

[2] The sequence shown was obtained with ascites-produced IgG. Samples produced by cell culture were found to have Ala at residue 68 of the V_H rather than Thr and, hence, have one more homology to VGAM3-0.

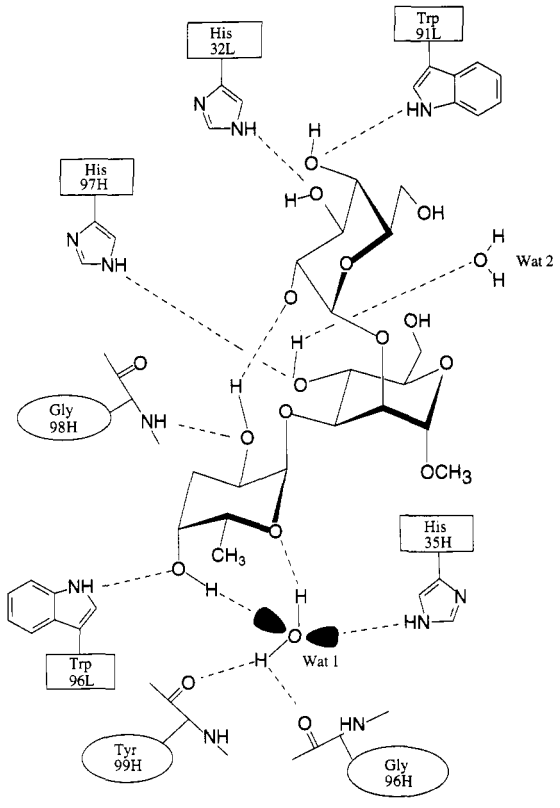


FIGURE 8: Hydrogen-bonding scheme for Se155-4, deduced from the crystal structures (Cygler *et al.*, 1991; Bundle *et al.*, 1994), numbered according to the Kabat scheme (Kabat *et al.*, 1991). In the consecutive numbering of the sequence figures above, this 32L, 35H, and 97H are 34L, 35H, and 101H, Trp 91L and 96L are 93L and 98L, Gly 96H is 100H, and Tyr 99H is 103H.

complementarity for abequose, and the peripheral location of the galactose residue can be seen to be consistent with this crystal structure. Thermodynamic data for binding of trisaccharide **10**, monosaccharides **23–25**, and disaccharide derivatives **16–18** show, in a striking fashion, that the abequose residue provides all the enthalpy of binding whereas galactose and mannose contribute only modestly to free energy (Table 7). Furthermore, the latter contributions arise mainly via favorable entropy, suggesting that an important driving force for binding of the mannose and galactose components is solvent displacement, since this is the only source of favorable entropy in such association processes (Finkelstein & Janin, 1989). Several ordered water molecules are observed in the crystal structure of the binding cavity of the native, ligand-free Fab (Cygler *et al.*, manuscript in preparation).

As with all other carbohydrate–protein complexes, hydrogen bonds play a crucial role in complex stability, and the modeling studies correctly identified the OH groups that participate in three antibody–oligosaccharide hydrogen bonds described in Figure 8: Abe O-4, Abe O-2, and Man O-4. From the relative magnitude of the (ΔG) values for **19** and **10** (Table 5), the hydrogen bond from Man O-4 to His 101H is inferred to be a neutral donor–acceptor pair (Street *et al.*, 1986), and this conclusion is supported by calorimetry measurements in two different buffer systems (Sigurskjold & Bundle, 1992). Attempts to replace His 101H by Asp, Glu, Asn, and Gln were made during mutagenesis studies in order to introduce a charged or neutral amino acid side chain that was capable of providing bidentate hydrogen binding to Man O-4 and O-6 (*cf.* Quiocho, 1986). All of these mutations were less active than the native Fab (Brummell *et al.*, 1993). Both hydroxyl groups of abequose are involved in conjugated networks of hydrogen bonds at the base of a binding pocket involving a bound water molecule and Trp 98L. The coordination of multiple hydrogen bonds between abequose and protein accounts for the absolute stereochemical requirements of the binding site and explains how the weak hydrogen-bond strength of a neutral donor–acceptor pair (Street *et al.*, 1986) has been amplified via the formation of conjugated hydrogen bonds (Quiocho, 1988, 1989).

Comparison of the methyl glycosides of abequose (**23**) and 3-deoxyarabinose (**24**) reveals that removal of the 6-CH_3 group reduces ΔG by a surprising 6 kJ mol^{-1}, with significantly less enthalpy reflecting lost van der Waals interactions, while a relatively more favorable entropy term, $-T\Delta S$, indicates that the residues forming the binding pocket have gained motional freedom.

Structural and stereochemical changes to the α-Gal residue are accompanied by large swings in the enthalpy and entropy of binding (Table 5), but even major changes in this residue provide active derivatives. Replacement of Gal by either methyl or methoxymethyl groups leads to discaccharides with activities resembling that of the native trisaccharide **10**, suggesting a marginal role in binding for the galactose residue. Nevertheless, the hydrogen-bonding map (Figure 8) indicates two or three hydrogen bonds between this residue and the protein. The relative strength of the intersaccharide hydrogen bond between Abe O-2 and Gal O-2 is particularly interesting, since its formation is made possibly by a conformational shift that occurs when the ligand is bound (Bundle *et al.*, accompanying paper). Compound **14** in which the Gal 2-OH has been replaced by an amino group retains its activity, which suggests that, since the amino group is partly uncharged at pH 8.0, this group may still participate in the postulated hydrogen bonds. On the other hand, preliminary EIA data for a 2-deoxy galactose derivative of the tetrasaccharide **9** (preliminary data for unstable compound) suggest that the absence of a hydrogen-bond acceptor causes 2 kJ mol^{-1} diminution of binding energy. The activities of the deoxy compounds **5**, **6**, and **8** suggest that neither the glycosylated Gal 3-O nor the Gal 6-OH groups form productive H-bonds with the antibody. This conclusion, as well as that predicting no H-bond to the Gal 4-OH, is supported by the activity of the dichloro compound **15**. A methyl group at O-3 of Gal mimics the anomeric carbon of the preceding rhamnose unit in the polysaccharide antigen, and the two chlorine atoms which are largely isosteric with hydroxyl groups replace the 4-OH and 6-OH of Gal. This is the only compound that binds as strongly, if not slightly stronger, than the natural epitope, αGal[Abe]Man. Thus, it seems that the hydroxyls at positions 4 and 6 of Gal are mainly involved in van der Waals interactions with the antibody. The increase in enthalpic interactions for compound **10** is consistent with this conclusion, since Cl produces stronger van der Waals interactions than OH. Consistent with these inferences, epimerization of the 4-OH of Gal produces a slightly weaker binding (compound **11**), although the changes in enthalpy and entropy are considerably larger than the overall change in free energy. This suggests that the postulated hydrogen bond involving this OH gorup is very weak in solution, consistent with its partial exposure to bulk water.

Compound **12** shows dramatically large changes in enthalpy and entropy compared to the other ligands. In fact, it is the very large magnitude of the enthalpy that enables the accurate measurement of such relatively weak binding by titration microcalorimetry. The experiment was reproduced with new batches of antibody and ligand and gave the same results

within experimental uncertainty. There is a clear overall trend from comparing the thermodynamic data for all the ligands that variations in ΔH and $T\Delta S$ compensate each other, rendering changes in ΔG much smaller. This trend also applies to compound **12**, so the binding thermodynamics of this ligand is not anomalous in this respect. However, the source of the huge enthalpy is puzzling. An enthalpy of this magnitude seems unlikely to stem from direct interactions with the neutral sugar moiety, and without structural information, definitive conclusions cannot be reached. Comparison with **13** indicates that the 4-OH group of **12** must play a crucial role, and it is probable that the significantly different orientation of a β-Gal as opposed to an α-Gal residue results in new saccharide–protein contacts. These could induce mutually unfavorable conformational changes which result in the formation of strong ionic interactions for the peptide chain in a microenvironment with a low dielectric constant. The very large entropy term signifies major losses of motional freedom.

The galactose residue occupies a position at the periphery of the binding site with some freedom of movement, and the thermodynamic parameters observed for functional group replacements (**10–18**) are typical of those reported for lectin–oligosaccharide interactions (Lemieux *et al.*, 1991; Sophr *et al.*, 1992). The enthalpy–entropy compensations seen by this group were ascribed to changes in water structure at the water–complex interface, and it seems equally probable that similar effects are operative here for replacement of those functional groups that are exposed to water.

Heptasaccharides **28** and **29** bind with identical enthalpies, but a 10 kJ mol^{-1} difference in entropy ($T\Delta S$) accounts for the 50-fold weaker binding of **29**. Molecular modeling of heptasaccharides in conformations that are close to the global potential energy minima shows that while **28** may be accepted in the binding pocket without serious steric clashes, the terminal reducing galactose (including subsequent residues in larger structures) would collide with the protein surface in such conformations. These clashes could be avoided for **29** by changes to some or all of the glycosidic torsion angles Man-Rha and Rha-Gal. The smaller favorable entropy when heptasaccharide **29** binds results from the loss of conformational entropy corresponding to the forbidden conformers about these two glycosidic linkages.

A crystal structure for Se155-4 complexed with **28** has recently been solved (Cygler *et al.*, unpublished observations) in which the position of all seven pyranose residues could be defined. An interesting feature of this structure (and the complex of **10** with a mutant single-chain Fv molecule; Zdanov *et al.*, 1992) is the absence of a Abe O-2 to Gal O-2 hydrogen bond. Transferred NOE experiments for antibody–trisaccharide complexes reported in the following paper (Bundle *et al.*, 1994) show that two unique bound conformations are consistent with the NOE data. One of these corresponds to a bound conformation containing a saccharide–saccharide hydrogen bond, while in the other, the Abe O-2 to Gal O-2 separation is too large to permit a direct hydrogen bond. Since replacement of Gal 2-OH by either NH_2 or H groups indicates this hydrogen bond to be weak ($\sim$2 kJ mol^{-1}), its formation does not appear to be crucial to complex stability.

CONCLUSION

Aromatic amino acid side chains play a major role in the definition of the specificity of a trisaccharide-sized antibody-binding site. The enthalpy of binding is derived almost exclusively from interaction of the most buried saccharide residue with the protein, while displacement of water molecules provides an additional and substantial source of binding energy. Although the crystal structure of the Fab–trisaccharide complex indicates two or three hydrogen bonds between amino acids and sugar residues that are close to the periphery of the binding site, functional group replacement studies show that these do not contribute substantially to the free energy of binding.

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