Carbohydrate Research, 190 (1989) 267–277
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

THE SUBSITES OF MONOCLONAL ANTI-DEXTRAN IgA W3129

CORNELIS P. J. GLAUDEMANS, PAVOL KOVÁČ, AND AREPALLI S. RAO

National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892 (U.S.A.)

(Received September 6th, 1988; accepted for publication, December 27th, 1988)

ABSTRACT

Synthetic deoxyfluoro derivatives of methyl α-D-glucopyranoside, as well as methyl α-glycosides of isomalto-oligosaccharides, some having fluorine substituted for hydroxyl groups at selected positions, have been evaluated for their binding with a myeloma monoclonal IgA known to bind only to an oligosaccharide sequence at the nonreducing end of α-(1→6)-linked D-glucopyranans (dextrans). The results are compatible with the antibody's possessing one subsite of high affinity for its D-glucosyl group, the remaining three subsites having low affinities for their respective D-glucosyl residues. The high-affinity antibody-subsite occurs at the interior end of the sequence of four subsites, appears to be relatively accessible, and binds the (terminal) nonreducing D-glucosyl group of the oligosaccharidic determinant using two, and possibly three, hydroxyl groups in hydrogen bonding.

INTRODUCTION

We have reported on the subsite location of β-(1→6)-D-galactan-binding monoclonal antibodies in the past[1,2]. Then we used D-galacto-oligosaccharides having fluorine substituted for hydroxyl at specific positions in order to cause shifts in binding patterns due to hydrogen-bond interactions between protein and ligand. The D-galactan-binding, monoclonal antibodies investigated by us are capable of binding to internal, sequential residues of the polysaccharide chain[3]. In fact, most immunoglobulins are probably capable of doing so. This is not true, however, in the case of IgA W3129, first described by Weigert *et al.*[4a] and Cisar *et al.*[4b]. This myeloma immunoglobulin was shown[5] to be precipitated by branched, but not by linear, α-(1→6)-dextrans. The interpretation that this was due to the monoclonal antibody's having a binding area complementary *only* to the nonreducing, terminal epitope of the dextran chain was correct, as we showed later[6] by physical measurements on the same system. The cDNA of W3129 has been cloned[7] and from the amino acid sequence derived, a modeled three-dimensional structure has been proposed[8]. This antibody is the only well characterized immunoglobulin of which we are aware that exclusively binds the chain-terminal end of its polysaccharide antigen. It was, therefore, attractive to examine the IgA W3129–dextran system by

using an approach similar to that used by us for the X24-X44 IgA family of antigalactans[2,9]. We here report the mode of binding of mono- and oligo-saccharide derivatives of methyl α-D-glucopyranoside to IgA W3129. By using derivatives of methyl α-D-glucopyranoside, as well as of methyl α-glycosides of isomalto-oligosaccharides in which selected hydroxyl groups had been replaced by fluorine, we have been able to elucidate the occurrence of hydrogen-bonding in the binding to the subsite of highest affinity. This has also revealed the particular D-glucosyl unit, in the tetraose antigenic determinant, that binds in that protein subsite. In addition, the affinities of the other subsites with their D-glucosyl residues could be ascertained.

RESULTS AND DISCUSSION

In Table I are listed the association constants (K_a) and maximal ligand-induced fluorescence change (ΔF_{max}) for the ligands **1–18** with monoclonal IgA W3129. It may be seen that, of the monosaccharides (**1–5**), methyl 4-deoxy-4-fluoro- and methyl 6-deoxy-6-fluoro-α-D-glucopyranoside showed no measurable binding. Of the disaccharides **6–9**, only **7** showed binding (see later for the reasons for measuring these compounds), whereas all of the α-(1→6)-linked oligosaccharides tested (**10–18**) showed binding.

The interaction of immunodeterminants with their antibodies could, *a priori*, be mediated by hydrogen bonding, hydrophobic bonding, van der Waals or electrostatic interactions, or any combination of any of these. It appears likely that carbohydrates interact by hydrogen bonding with the proteins that bind them, at least to a significant extent. Structural data[10] on the arabinose-binding protein of *Escherichia coli*, and solution measurements on monoclonal antigalactan antibodies[1,2], as well as work on the D-glucose–phosphorylase complex[11], strongly suggest this to be so. The last case confirmed the solution occurrence of hydrogen bonds first observed in crystallographic analysis.

In the case of IgA W3129, the first question that arises is: given our finding (see later) that there are four subsites binding to D-glucosyl residues of the dextran, which one of these shows the strongest binding? It must be realized that, when an antibody shows that a disproportionately high fraction of its binding energy for a homo-oligosaccharidic determinant is due to the binding of *one* glycoside, it could, but *need not*, mean that the highest affinity subsite is filled by the (terminal)non-reducing end-group[12,13]. Immunization with *p*-diazophenyl β-D-galactopyranoside–bovine serum albumin (Gal-BSA) had led to hybridoma antigalactan monoclonal antibodies possessing a D-galactose-binding subsite of highest affinity which is located *centrally* in a series of four of these subsites[2]. We have here found that, in the case of IgA W3129, it so happens that the highest-binding subsite *is* in fact located at the inner terminus of the series of protein subsites. We could show this as follows: methyl α-D-glucopyranoside (**1**) causes a ligand-induced fluorescence change in W3129, and quantitation of this change[14–16] led to a K_a for that D-glucoside of 1.8×10^3 M^{-1} (see Table I). The K_a for methyl α-isomaltotetraoside

TABLE I

BINDING CONSTANTS (K_a) AND PERCENTAGE MAXIMAL FLUORESCENCE CHANGE (ΔF_{max}) FOR MONOCLONAL IgA W3129 WITH GLUCOPYRANOSE DERIVATIVES

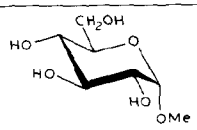
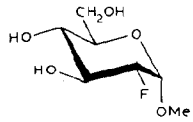
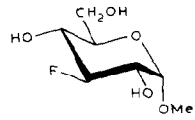
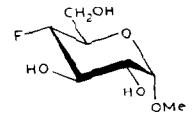
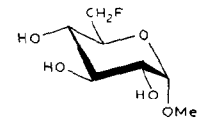
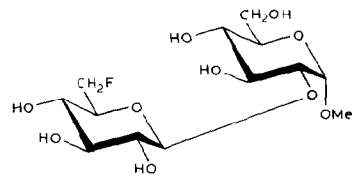
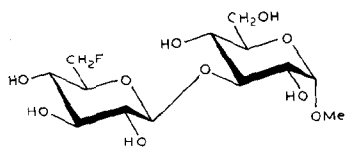
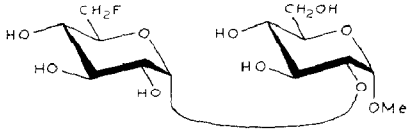
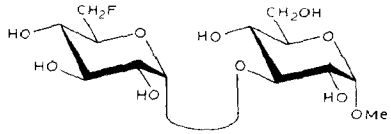
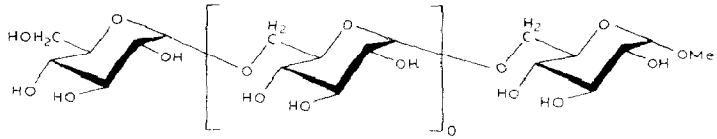
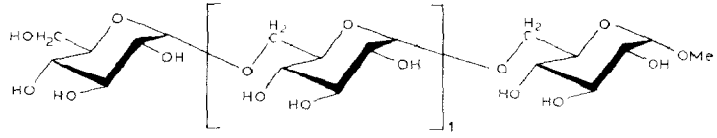
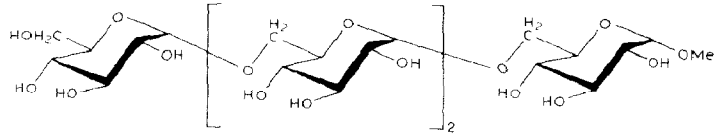
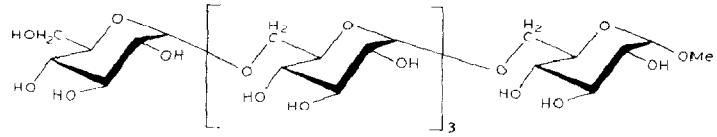
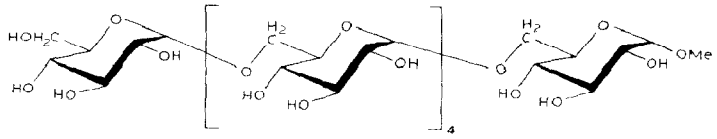
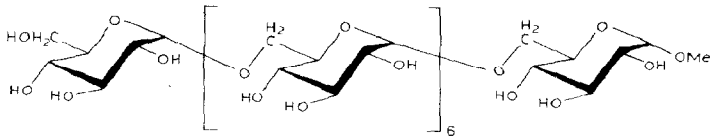
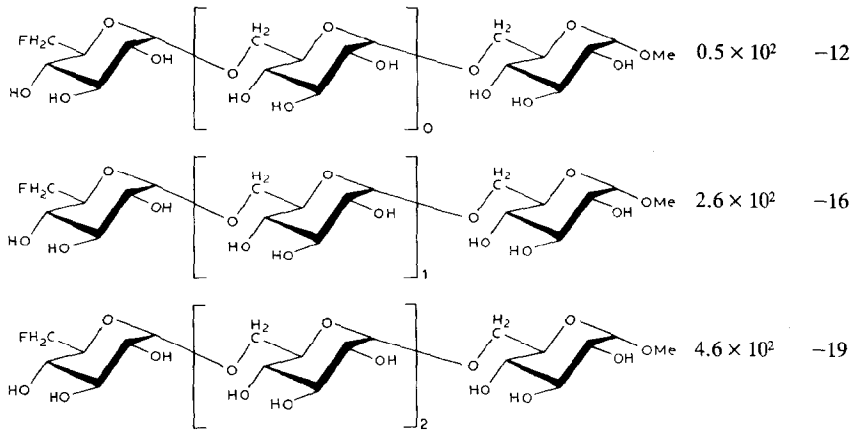
Compound number		K_a	ΔF_{max} (%)
1		1.8×10^3	−19
2		1.8×10^3	−15
3		8.7×10^3	−15
4		0	—
5		0	—
6		0	—
7		2.1×10^2	−18

Table I (continued)

Compound number		K_a	ΔF_{max} (%)
8		0	—
9		0	—
10		1.6×10^4	−16
11		6.7×10^4	−16
12		1.8×10^5	−13
13		1.9×10^5	−14
14		1.8×10^5	−15
15		1.7×10^5	−14



16	0.5×10^2	−12
17	2.6×10^2	−16
18	4.6×10^2	−19

(**12**) is 1.8×10^5 M^{-1}. Thus, the highest-affinity D-glucosyl-binding substite (called **A**) has a free energy (ΔG) of binding that amounts to some 62% of the total free-energy of binding that the antibody has for the maximally binding (see later) tetrasaccharide. That same subsite is *in*capable of significantly binding methyl 6-deoxy-6-fluoro-α-D-glucopyranoside (**5**), as **5** showed no measurable ligand-induced fluorescence change when added to the antibody. Actually, ligand **5** possesses a very weak affinity (K_a less than 7) for the highest-binding subsite. This can only be observed by attaching **5** to O-6 of methyl α-D-glucopyranoside or the methyl α-glycoside of an isomalto-oligosaccharide, and measuring the K_a, thus using the neighboring subsite(s) to help anchor the ligand to the antibody (see later). Thus, the foregoing observation shows that the strongest-binding subsite, in order to bind its D-glucosyl residue significantly, requires a free OH-6 group which apparently hydrogen-bonds to the protein by hydrogen donation, and the *only* D-glucosyl unit possessing a free OH-6 in the entire α-(1→6)-dextran molecule is the nonreducing end-group. Thus, that unit must be the one binding in the highest-binding subsite, as originally suggested by Kabat[12].

What about the influence of the other hydroxyl groups of the D-glucosyl residue seeking the strongest-binding subsite? We found that methyl 4-deoxy-4-fluoro-α-D-glucopyranoside (**4**) reveals no measurable, ligand-induced, fluorescence change either, showing a lack of significant binding to the highest-affinity subsite as well. Therefore, that subsite appears to require hydrogen-bond donation from both the OH-4 and -6 groups. On the other hand, the corresponding methyl 2-deoxy-2-fluoro-α-D-glucopyranoside (**2**) showed a binding constant equal to that of the non-fluorinated D-glucoside **1** (see Table I), suggesting that the 2-hydroxyl group of **1** is not involved in hydrogen-bond donation to the protein when binding. Methyl 3-deoxy-3-fluoro-α-D-glucopyranoside (**3**) showed an affinity some four times that of

1. It might be argued that, when **1** binds to W3129, there is a hydrogen bond in the strongest-binding subsite from the protein to O-3 of **1**. Then, when the 3-hydroxyl group is replaced by fluorine (to give **3**), this could result in a higher affinity of the hydrogen bond from the antibody to that ligand (**3**) due to the presence of the (highly electronegative) fluorine atom. It has been suggested, however, that the C–F---HO bond may be weak[17,18]. Alternatively, it may be that a positive charge on the protein surface in subsite **A** near OH-3 of **1** could cause the stronger binding of **3**; or the fluorine atom on C-3 in **3** may induce a higher acidity for the proton of the hydroxyl group at C-4, thus causing it to hydrogen-bond more strongly to the protein.

The caloric value of hydrogen bonds in the binding of substrates to proteins has been the subject of recent researches. For instance, site-specific mutagenesis of tyrosyl-tRNA synthetase has yielded enzymes having specifically altered amino acid side-chains, so that particular contributions to known hydrogen bonds could be scrutinized by binding measurements[19]. However, some of these changes could very well affect the globular structure of the enzyme, and thus, the measured binding-energy between the resulting mutant and the substrate. To the best of our knowledge, the only time the contribution of a single, overall hydrogen bond has been unambiguously measured is in the case[20] of the binding of benzyloxycarbonylglycyl-leucylleucine to thermolysin. There, a value of 17.2 kJ per mol was found. The binding energy of the highest-binding subsite with its D-glucosyl residue that is reported here is 18.4 kJ, indicating that it may arise entirely from one hydrogen-bond, in this case from the OH-4 and -6 groups to, apparently, the same recipient atom of the protein. Single atoms involved in multiple hydrogen-bonds have been observed before, notably in the complex[10] of arabinose with the arabinose-binding protein of *E. coli*.

Replacement of a hydroxyl group by a fluorine atom causes[21] a decrease in the van der Waals radius and, thus, in these substitutions, no increase in molecular volume occurs that could prevent binding. In addition, in our routine examination of the n.m.r. spectra of some of our ligands, both bearing and not bearing fluorine atoms, we have not seen conformational changes due to the introduction of the latter atom[22].

IgA W3129 has[7] at position 33H a tryptophanyl (Trp) residue which also occurs in the antigalactan immunoglobulins[2], and this Trp is solvent-exposed[23]. In W3129, this is the only solvent-exposed Trp, as it lacks[7] the one at 91L which occurs in all the antigalactans. The simplest ligand, **1**, causes a ΔF_{max} in IgA W3129 which is the same as that caused by the higher oligosaccharides **10–18** (see Table I); this indicates that the subsite with the highest affinity occurs near the ligand-perturbable Trp 33H of the antibody. Because we had already shown that the terminal D-glucosyl group in the dextran antigen seeks the highest-affinity subsite in IgA W3129, the immediate question that arises is: is this subsite **A** deeply buried inside the protein, or does it remain fairly accessible? In order to answer that question, we have measured whether binding occurs with any of the four disaccharides **6–9**. The

rationale for this is as follows: we found (see earlier) that the presence of a 6-fluorine atom in methyl 6-deoxy-6-fluoro-α-D-glucopyranoside lessens the binding of that D-glucoside in subsite **A** to an insignificant level. To probe the space around that subsite, the binding of a methyl α-D-glucopyranoside derivative bearing a large (bulky) substituent (attached at the permissible O-2 or -3), *but itself essentially incapable of binding* (such as a 6-deoxy-6-fluoro-D-glucopyranosyl group), had to be evaluated. Thus, the binding of disaccharides **6–9** was measured. Of these, only methyl 3-*O*-(6-deoxy-6-fluoro-β-D-glucopyranosyl)-α-D-glucopyranoside (**7**) showed binding, with a K_a of 2.1×10^2 M^{-1}. This means a free energy of binding of 13.4 kJ, and this shows that **7**, containing a bulky substituent, can still bind with 70% of the free energy exhibited by the binding of the unsubstituted **1**. Speculation as to why **7**, and none of the three other disaccharides **6, 8,** and **9** show binding, appears idle. This is more than likely due to unknown spatial restriction around subsite **A**, such as could be due to actual obstruction by peptide moieties of the IgA interfering with the space around O-2 (preventing **6** and **8** from binding) and above O-3 (preventing the binding of **9**). However, the observation that **7** does bind suggests that the highest-affinity subsite does not tightly encompass the antigenic terminus of the dextran.

Next, we evaluated the affinities that each of the other subsites of the immunoglobulin has for its D-glucosyl residues. It may be seen in Table I that the maximally binding oligosaccharide in the methyl α-isomalto series is methyl α-isomaltotetraoside (**12**), showing that the IgA possesses four subsites. Cisar *et al.*[5] proposed five subsites, but they used reducing oligosaccharides whose (terminal) reducing D-glucose residue, when in solution, equilibrates between the α/β-pyranose/furanose structures, thus introducing an element of uncertainty for the binding of that D-glucose residue. Here we did control the α-pyranoside structure of that terminal residue.

As already pointed out, in IgA W3129, the solvent-exposed Trp (33H) occurs near the highest-affinity subsite **A**, which binds the terminus of the antigen. Thus, if only the nonreducing D-glucosyl group of the tetraoside determinant in the dextran is capable of tryptophanyl perturbation, we can still measure the K_a of *all* ligands (including the monosaccharide **1**) by ligand-induced fluorescence change, as the highest-affinity subsite is, of necessity, filled first in all cases. From the K_a measurements of the IgA with **1** and **10–15**, it may be computed ($\Delta G_{\text{site B}} = \Delta G_{10} - \Delta G_1$, and $\Delta G_{\text{site C}} = \Delta G_{11} - \Delta G_{10}$, *etc.*) that the relative affinity-constants of each subsite for its D-glucosyl residue decreases as **A** > **B** > **C** > **D** (1.8×10^3, 8.9, 4.2, and 2.7 respectively; see Table I and Fig. 1). Individual free energies of binding that each subsite has for its D-glucosyl unit are additive to yield the free energy of binding for the entire epitope, as is shown later (see discussion of compounds **16–18**).

Kabat *et al.* partially mapped an IgM to lacto-*N*-tetraose[24]. By measuring affinities by inhibition of the precipitation of this antibody by oligo-, and some mono-, saccharides, *some* information was obtained about the relative binding in

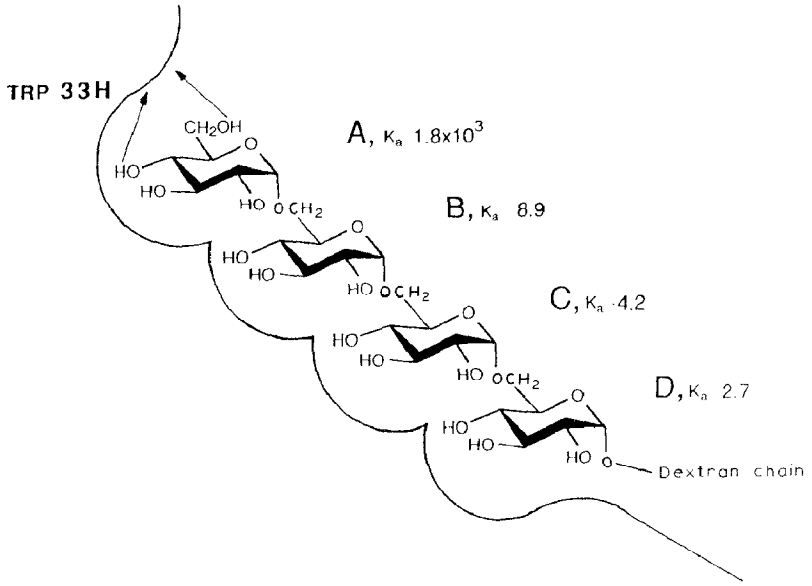


Fig. 1. Schematic representation of the subsites of IgA W3129, showing the tetrasaccharide epitope as well as the affinity constants each subsite possesses for its D-glucosyl residue. The approximate location of the tryptophanyl residue 33H is also indicated.

subsites. Young *et al.* obtained monoclonal antibodies to ganglio-*N*-triosylceramide[25], and studied the binding of *N*-acyl derivatives of the determinant as well as oligosaccharides of different length by hemagglutination–inhibition. In other investigations[26,27], work has been reported on monoclonal antibodies against human Le^a and Le^b blood-group antigens. All these determinants are *hetero*-oligosaccharides. We are unaware of systematic binding studies conducted on monoclonal antibodies and the appropriate anomeric glycosides of the individual component sugars of *hetero*saccharidic determinants. In the case of *homo*saccharidic determinants, as in the case of our galactan work[1,2] and the dextran investigation reported here, it is more difficult to assess subsite-affinity.

The position of the perturbable Trp also made it possible to evaluate whether *weak* binding of the 6-deoxy-6-fluoro-D-glucoside can take place at all in subsite **A**: if the disaccharide **16** is to bind, it could, *a priori*, do so by occupying subsites **B** and **C**, **C** and **D**, or **A** and **B**. The last possibility could occur only if the 6-deoxy-6-fluoro-D-glucosyl group of **16** could at least bind weakly in subsite **A** (as no noticeable binding is observed when **5** is added to IgA W3129). The former two modes (**B**–**C**, or **C**–**D**, or both) may occur simultaneously (because we have no evidence that the 6-deoxy-6-fluoro-D-glucosyl group cannot bind in subsites **B**, **C**, or **D**), but would not be observed by monitoring the changes in tryptophanyl fluorescence, because ligand-induced Trp-perturbation would not be expected in those remote sites. Compound **16** showed a K_a of 50 M^{-1} ($\Delta F_{max} = -12\%$). Since subsite **B** has a K_a of 8.9 M^{-1} for its D-glucosyl moiety, the additional binding observed should be

the result of binding to subsite **A**, because Trp-perturbation was observed. Therefore, subsite **A** must have a K_a of 5.6 M^{-1} for the 6-deoxy-6-fluoro-D-glucosyl group in compound **16**. [It can be computed that, if binding of **16** takes place simultaneously in subsites **B–C** and **C–D**, it would not influence the outcome of the experimentally measured K_a of **16** in subsites **A–B**. For a substance with $K_a = 50$ M^{-1} (measured at a concentration of antibody of 3.57×10^{-7} M; see Experimental section), the ligand concentration exceeds the concentration of antibody sites by a factor of 10^4 when only 3% of the sites are occupied by ligand, *i.e.* at the very initial stage of the titration. Thus, even depletion of the ligand concentration by simultaneous low-affinity binding of **16** at other subsites (**B–C** and **C–D** have affinities of 36 and 12, respectively) would not significantly alter the ligand concentration here used to compute the observed binding in the **A–B** sites.] Next, the binding of **17** was determined as $K_a = 2.6 \times 10^2$ M^{-1} (see Table I). Again, the 6-deoxy-6-fluoro-D-glucosyl group of **17** would bind weakly in subsite **A** (but sufficiently to cause ligand-induced fluorescence change), while the other two residues of **17** bound in subsites **B** and **C**. Since the latter subsites have affinities of 8.9 and 4.2 M^{-1}, respectively, the affinity that subsite **A** has for the terminal deoxyfluoro unit in **17** is now found to be 6.9 M^{-1}. Similarly, the K_a of **18**, found to be 4.6×10^2 M^{-1}, taking into account the affinities of subsites **B, C,** and **D** as 8.9, 4.2 and 2.7 M^{-1}, respectively, leads to an affinity of subsite **A** for the deoxyfluoro-D-glucosyl terminal group in **18** of 4.6 M^{-1}. These three independently measured values, namely 5.6, 6.9, and 4.6 M^{-1}, are in remarkable agreement with each other.

The general mode of binding of saccharides to IgA W3129 was thermodynamically mapped in this laboratory years ago[28]. We showed that the entropy of binding of the mono- and oligo-saccharides is quite negative, binding being driven by enthalpy. It is our interpretation that, if binding were due to non-charged interactions only, *i.e.* the return of protein- and ligand-bound water of hydration to the bulk solvent, it would have resulted in an overall increase in entropy, as it would be expected that that contribution would overwhelm the entropy decrease caused by immobilization of the bound ligand. Our finding[28] that the entropy change associated with binding is highly negative (*i.e.*, $T\Delta S$ for **1** and W3129 is ~ -23 kJ/mol, and ~ -37 kJ/mol for the entire carbohydrate epitope) indicates an alternative way of binding, and this is compatible with there being a strong contribution of hydrogen bonding to the binding energy. We remain nevertheless apprehensive about using thermodynamic data to describe *isolated* events at the molecular level.

The cDNA sequence[7] reveals that the antibody possesses a five-amino acid insert in the first hypervariable region of the light (L) chain. Padlan and Kabat[8] proposed that this loop projects out from the general combining area, to divide that area into two compartments. This would make it virtually impossible for a dextran chain to traverse the area, and would make binding by insertion of the chain end almost the only alternative. That would explain the exclusive end-binding characteristic of IgA W3129.

In conclusion, dextran-binding IgA W3129 has four D-glucosyl-binding subsites. The one with the highest affinity (subsite **A**) is located most internally in the protein, and binds only to the nonreducing-end D-glucosyl group of the polysaccharide chain, but does not tightly encompass that D-glucosyl moiety. Its interaction with the terminal D-glucosyl moiety is mediated mainly by hydrogen bonding of two hydroxyl groups to the protein, and possibly by a third one from the protein to OH-3. Its binding free-energy accounts for some 62% of the total free-energy of binding that the antibody has for the tetrasaccharide determinant. The binding energy that the other three subsites (**B, C,** and **D**) have for their D-glucosyl residues is far less than that of subsite **A**.

EXPERIMENTAL

Materials. — IgA W3129 was originally obtained at the Salk Institute. The immunoglobulin was purified by precipitation in 40% ammonium sulfate, followed by affinity chromatography over Sephadex, specific elution with pre-dialyzed methyl α-D-glucopyranoside, and extensive dialysis against phosphate-buffered saline (PBS) to remove all ligand. The preparation of methyl 2-deoxy-2-fluoro- (**2**), 3-deoxy-3-fluoro- (**3**), 4-deoxy-4-fluoro- (**4**), and 6-deoxy-6-fluoro-α-D-glucopyranoside (**5**) has been reported[29,30]. Also, the preparation of methyl 2-*O*- (**6**) and 3-*O*-(6-deoxy-6-fluoro-β-D-glucopyranosyl)-α-D-glucopyranosides (**7**), and methyl 2-*O*- (**8**), and 3-*O*-(6-deoxy-6-fluoro-α-D-glucopyranosyl)-α-D-glucopyranoside (**9**) has been reported[31]. The preparation of methyl α-isomalto- (**10**), isomaltotrio- (**11**), and isomalto-tetra-oside (**12**), as well as their respective 6'- (**16**), 6''- (**17**) and 6'''-deoxyfluoro (**18**) derivatives, and methyl α-isomalto-penta- (**13**), -hexa- (**14**), and -octa-oside (**15**) has been reported[32,33].

Methods. — Affinity constants were measured by using ligand-induced tryptophanyl fluorescence change as described before[14–16]. All measurements were performed at 25° in 0.01M PBS, using an antibody concentration of 357nM. Excitation and emission wavelengths were 295 and 334 nm, respectively. The instrument used was a Perkin–Elmer MPF-3L fluorimeter.

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