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BINDING STUDIES ON A MOUSE-MYELOMA IMMUNOGLOBULIN A HAVING SPECIFICITY FOR β-D-(1→6)-LINKED D-GALACTOPYRANOSYL RESIDUES*

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ABSTRACT

The free energies of binding between immunoglobulin A J539 (Fab') and methyl 6-*O*-acetyl-β-D-galactopyranoside (**1**) and 6-*O*-β-D-galactopyranosyl-1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose (**2**) have been measured. The values found suggest that bulky substitution on O'-6 or O-1, O-2, O-3, and O-4 in the hapten 6-*O*-β-D-galactopyranosyl-D-galactose (**3**) does not interfere with effective binding of that ligand and the immunoglobulin. This conclusion supports the postulations that (*a*) the ligand **3** binds only on one side of the molecule, and (*b*) the combining site of the immunoglobulin J539 appears to be located on an exposed surface area.

INTRODUCTION

We have reported on a number of immunoglobulins having specificity for antigens containing the repetitively linked β-D-(1→6)-linked D-galactopyranosyl residue[1–4]. We proposed in a recent paper[3] that the combining regions of immunoglobulins J539 and X-24 involve a surface area on these proteins, rather than a cleft. By high-resolution X-ray analysis of a homogeneous, human immunoglobulin, Poljak *et al.*[5] have shown that the hypervariable positions in the variable regions of both light and heavy chains are located at one end of the Fab' fragment of the immunoglobulin molecule, thus indicating that the site is in a fairly shallow depression. It is not surprising that anti-idiotypic antibodies specific for the variable region of an immunoglobulin can be inhibited in their interactions with the immunoglobulin by the binding of a specific hapten covering the shallow, reactive area of the combining site[6,7]. The very fact that anti-idiotypic antibodies to immunoglobulins can be relatively readily obtained argues against the combining site's being deeply buried inside an immunoglobulin. Also, most antigens are polymeric and, in order that an antibody may interact effectively with the antigen, it should not have the combining site inaccessible to the antigenic determinants that are located on a large, polymeric molecule.

*Dedicated to Professor Michael Heidelberger in honor of his 87th birthday.

In mapping the combining regions of haptens with immunoglobulins A J539 and X24, we concluded that both proteins apparently interact with that region of the hapten 6-*O*-*β*-D-galactopyranosyl-D-galactose (Gal_2, **3**) which is located to one side of the molecule[3] (see Fig. 1). We assumed that conformation from considerations of least orbital-overlap of the oxygen atoms of **3** near the intersaccharidic linkage[2]. We have looked further into the interaction of D-galactopyranosyl derivatives with immunoglobulin J539, in order to find whether this assumption is justified.

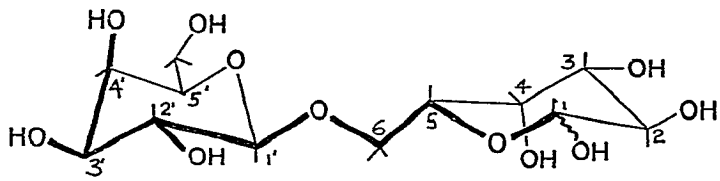

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Fig. 1. The postulated conformation of 6-*O*-*β*-D-galactopyranosyl-D-galactose (**3**). [The heavy bonds indicate the area of strongest binding to the immunoglobulin A J539 (Fab').]

Methyl 6-*O*-acetyl-*β*-D-galactopyranoside (**1**) was prepared according to a known procedure[8] and obtained crystalline. Its binding with IgA J539 Fab' was studied by using tryptophanyl fluorescence[9], and it was compared to the binding between methyl *β*-D-galactopyranoside and IgA J539 Fab' (see Table I). Next, it was decided that, if Gal_2 (**3**) has the conformation depicted in Fig. 1, and if the binding of the latter hapten and IgA J539 Fab' is, indeed, mediated mostly through interaction of the one side of the disaccharide molecule (see the heavy segment in Fig. 1), substitution at O-1, O-2, O-3, and O-4 in **3** should not greatly affect binding with the immunoglobulin. Consequently, the 1,2:3,4-diisopropylidene acetal (**2**) of **3** was prepared[10], and its affinity for IgA J539 Fab' was determined by fluorescence titration[9].

TABLE I

THE BINDING CONSTANTS OF HAPTENS CONTAINING D-GALACTOPYRANOSE DERIVATIVES WITH MURINE IMMUNOGLOBULIN A J539 (Fab')

Hapten	$K_d \times 10^{-3}$ (*liter. mol*$^{-1}$)
Methyl *β*-D-galactopyranoside[3]	1.0
Methyl 6-*O*-acetyl-*β*-D-galactopyranoside	1.5
6-*O*-*β*-D-Galactopyranosyl-D-galactose[3]	11.4
6-*O*-*β*-D-Galactopyranosyl-1,2:3,4-di-*O*-isopropylidene-*α*-D-galactose	26.0

RESULTS AND DISCUSSION

Methyl 6-*O*-acetyl-β-D-galactopyranoside (**1**) was prepared by detritylation of methyl 2,3,4-tri-*O*-benzyl-6-*O*-trityl-β-D-galactopyranoside followed by acetylation and reductive debenzylation[8]. The crystalline material enhanced the tryptophanyl fluorescence of IgA J539 Fab′ by 26.14%. Quantitative, fluorescence titration as already described[9] yielded an association constant (K_a) of 1.50×10^3 (see Table I). By using the expression $\Delta G = -RT \ln K_a$, it was found that the free energy of binding is 5.9% higher than that of methyl β-D-galactopyranoside with IgA J539 Fab′, whose binding constant was found[3] to be 1.01×10^3 (see Table I). It is possible that the binding of carbohydrate haptens with immunoglobulins is mediated by electrophilic groupings. The presence of an acetyl group at O-6 of **1** could cause a slightly higher, positive charge on the glycosidic oxygen atom through an inductive effect, and this could, perhaps, account for the somewhat higher binding-energy of **1** compared to that of methyl β-D-galactopyranoside. Previously, we have shown[3] that the 5-(hydroxymethyl) group of methyl β-D-galactopyranoside does not make a large contribution to the binding of that ligand with IgA J539 and X24 (Fab′). We have now shown that a relatively bulky group, such as the acetyl group, in no way hinders the binding of **1** with the immunoglobulin J539 Fab′ fragment. This fact lends strength not only to our earlier postulation that D-galactopyranose-derived ligands bind only on one side of the molecule, but also to the assumption that the immunoglobulin combining-site may be on an exposed surface rather than in a deep cleft.

In addition to these results, we have already shown[3] that, in **3**, the intersaccharidic sequence from O-1′ to C-6–C-5–O-5 is important for binding to the globulin fragment. From comparison with the data on the binding of epoxypropyl β-D-galactopyranoside[3] with IgA J539 Fab′, it could be inferred that O-4, O-3, O-2, and O-1 of the reducing moiety in **3** are of relatively little importance in contributing to the binding energy. A region of a molecule may not contribute to the binding, but bulky substitution could still alter the capacity of the ligand to enter a combining site if the space in that site is restricted. If, however, binding is not diminished following substitution by bulky groupings, this would indicate that the combining site may be on an exposed surface of the immunoglobulin A molecule. It thus became of interest to verify this for the disaccharide **3**, and to prepare a derivative in which would be blocked (by bulky substituents) those oxygen atoms that we had previously postulated to be oriented away from the actual binding area of the immunoglobulin combining-site[3].

The well known synthesis of the D-galactobiose **3** actually employs[10] the condensation of tetra-*O*-acetyl-α-D-galactopyranosyl bromide[11] and 1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose[12], so that deacetylation of the protected disaccharide would conveniently yield a suitably protected D-galactobiose derivative. The 6-*O*-β-D-galactopyranosyl-1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose, **2**, was obtained as a levorotatory, amorphous powder. It increased the tryptophanyl fluorescence of IgA J539 Fab′ by 42.3%. Its n.m.r. spectrum showed two, well

separated, anomeric protons and twelve methyl protons, six of which had identical chemical-shifts. The n.m.r. spectrum of vacuum-distilled 1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose also shows two isopropylidene methyl groups in an identical environment. Thus, the substitution of the D-galactopyranosyl group in 1,2:3,4-di-*O*-isopropylidene-α-D-galactose to form **2** does not cause asymmetry in the partially protected disaccharide so as to split the chemical shifts of the two identical isopropylidene methyl groups. Although several conformations can accommodate this observation, it is nevertheless true that, applied to **2**, the conformation that we proposed earlier[3] would also put the D-galactosyl group in **2** far enough away from the two isopropylidene groups (which appear to be in an almost identical environment; see Fig. 2b) so as not to disturb this near-identity. A conformation such as that depicted in Fig. 2a would place one of these two methyl groups much nearer to the D-galactosyl group, thus making indistinguishable chemical-shifts more unlikely.

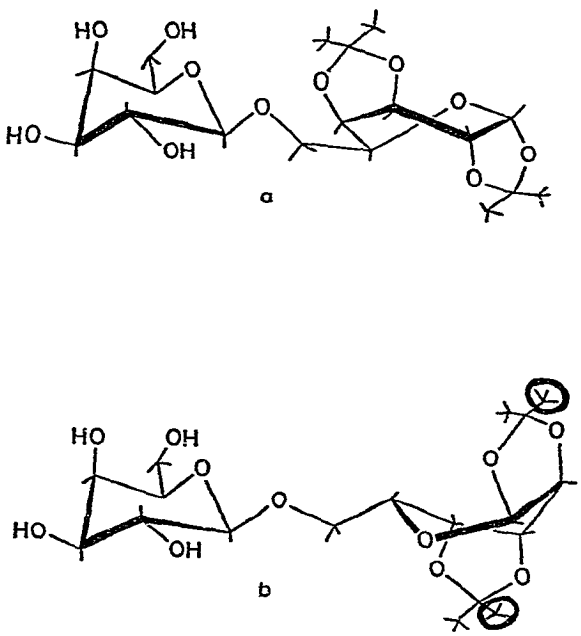

Fig. 2. Possible conformations of 6-*O*-β-D-galactopyranosyl-1,2:3,4-di-*O*-isopropylidene-α-D-galactose. [The circled methyl groups in b are approximately equidistant from the D-galactopyranosyl group.]

However, the major observation of interest is that the affinity constant for binding between **2** and IgA J539 Fab′ is 2.6×10^4 (see Table I). When the free energy of binding is again calculated from this value by the equation $\Delta G = -RT \ln K_a$, it is seen that the partially protected disaccharide **2** has a binding energy 8.8% higher than that of the free disaccharide **3**; this result is not unreasonable. If substitution at O-1, O-2, O-3, and O-4 of **2** is to have little bearing on its binding with immunoglobulin,

it should not be supposed that **2** should of necessity bind less strongly than does **3**. Compound **3** is a reducing disaccharide, and the specificity of IgA J539 is for multiple D-galactosyl residues; hence, although the (reducing) D-galactose residue of **3** will be mostly in a ring form, it is undoubtedly in equilibrium with its acyclic form to some small extent, and the latter would not bind so well to the immunoglobulin. In compound **2**, the 1,2-*O*-isopropylidene group locks the D-galactose residue in the pyranoid form, and this would contribute to an overall higher binding-energy.

EXPERIMENTAL

Methyl 6-O-acetyl-β-D-galactopyranoside (**1**). — This compound was prepared by a known procedure[8], and obtained crystalline, m.p. 150–152°, $[\alpha]_D^{20}$ −13° (*c* 0.7, EtOH) (lit.[10] m.p. 142°, $[\alpha]_D^{20}$ −14°).

Anal. Calc. for $C_9H_{16}O_7$: C, 45.74; H, 6.82. Found: C, 45.47; H, 6.89.

6-O-β-D-Galactopyranosyl-1,2:3,4-di-O-isopropylidene-α-D-galactopyranose (**2**). — Tetra-*O*-acetyl-α-D-galactopyranosyl bromide[11] (15 g) and distilled 1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose[12] (9.9 g) were condensed in the presence of silver carbonate (9.9 g) and Drierite (39 g) in ether (240 ml) as described[10]. The suspension was filtered, and the filtrate was evaporated *in vacuo* to a syrup which was placed on a column of silica gel 60 (E. Merck, Darmstadt, Germany) and eluted with 3:7 ethyl acetate–benzene. Appropriate fractions were pooled, and evaporated to a syrup (12.1 g) showing a single spot in thin-layer chromatography (t.l.c.) on silica gel plates with the same solvent. This 1,2:3,4-di-*O*-isopropylidene-6-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-galactopyranose was dissolved in methanol (100 ml), and treated with 1M barium methoxide (2.2 ml) overnight at room temperature. The base was neutralized by the addition of Dry Ice, and the solution was evaporated to a syrup. T.l.c. (9:1-dichloromethane–methanol) showed a few minor impurities, and the product was purified by repeated chromatography on a column of silica gel, first with the t.l.c. solvent, and then with 4:1 chloroform–methanol. Appropriate fractions were pooled, and evaporated to yield a pure product. Ethanol was removed by evaporation *in vacuo*, and the product was dried *in vacuo* for 3 h at 90° to yield **2** as a white, amorphous powder, $[\alpha]_D^{20}$ −49.7° (*c* 2, aqueous 0.05M Tris, pH 7.4). The material showed two anomeric protons in its n.m.r. spectrum: one doublet at τ ~4.44, and the intersaccharidic, anomeric proton at ~5.39. The twelve protons of the four methyl groups appeared in three peaks: two had a 3-proton intensity (τ 8.44 and 8.56), and a third one, a 6-proton intensity at τ 8.67.

Anal. Calc. for $C_{18}H_{30}O_{11}$: C, 51.17; H, 7.16. Found: C, 51.32; H, 7.38.

Tryptophanyl fluorescence titration. — The Fab' fragment of J539 was used[3] in aqueous Tris buffer (0.05M) at pH 7.4. The absorbance of the protein solution was 0.05 to avoid internal quenching. A Perkin–Elmer MPF 3 fluorometer was used as described[2,3,9]. Compound **1** was used at 135.0mM concentration, and **2** at 9.8mM. All procedures were conducted as reported previously[9]. For Scatchard plots of the titrations, see Fig. 3.

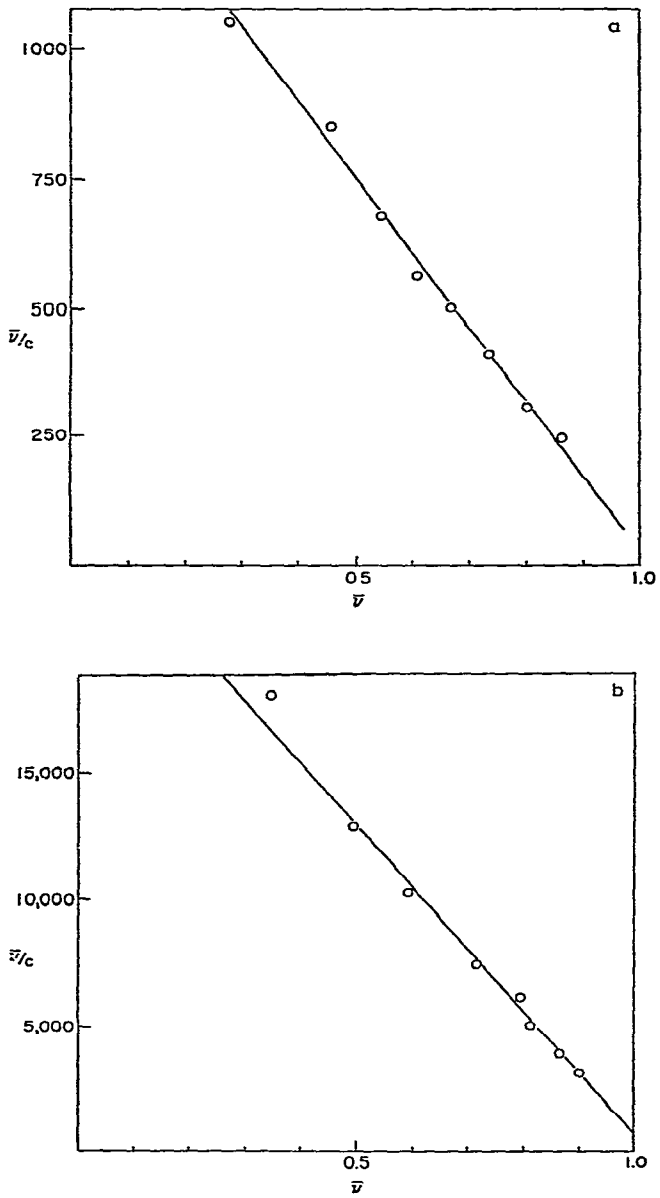

Fig. 3. Scatchard plots for the binding between the Fab' fragment of J539 and (*a*) methyl 6-*O*-acetyl-*β*-D-galactopyranoside and (*b*) 6-*O*-*β*-D-galactopyranosyl-1,2:3,4-di-*O*-isopropylidene-*α*-D-galactopyranose.

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