Binding Site of a Dextran-Specific Homogeneous IgM: Thermodynamic and Spectroscopic Mapping by Dansylated Oligosaccharides†

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ABSTRACT: The hapten binding properties of the homogeneous mouse IgM secreted by MOPC-104E were investigated. Hapten-association constants were determined either by equilibrium and displacement equilibrium dialysis or by fluorometric titrations of the protein with the fluorescent derivatives of the haptens. For the latter type of measurements, several oligosaccharides were derivatized to the corresponding dansylhydrazones. The synthesis, generally applicable to oligosaccharides with free reducing ends, is described. Analysis of the thermodynamic parameters for the binding of 18 haptens forms the basis for proposing a model of the binding site of MOPC-104E. This model is supported and refined by results of the measurements of linear and circular polarization of the fluorescence of the dansylated haptens. The binding site is proposed to consist of a cavity with about 12-Å depth, complementary to a terminal nonreducing nigerosyl group. At the entrance to this cavity, a further subsite is identified forming interactions of lower specificity with an additional glucose unit.

Only very few homogeneous immunoglobulins of the IgM class with known specificity for antigens or haptens were described among the large number of homogeneous antibodies and myeloma proteins which have been reported during the last years (Metzger, 1969; Potter, 1971; Riesen et al., 1975; Tolleshaug and Hannestad, 1975). One interesting example of this class is the murine IgM produced by MOPC-104E plasmacytoma which was first described and characterized by McIntire et al. (1965). A more detailed analysis of the binding properties of this IgM was carried out by Leon et al. (1970) and Young et al. (1971). They investigated the binding of a series of poly- and oligosaccharides mostly by indirect measurements such as precipitation and its inhibition with various ligands, passive hemagglutination inhibition, and microcomplement fixation. The results led to the conclusion that MOPC-104E binds most specifically carbohydrate moieties containing α-(1-3)-linked glucose units. Independent support for the specificity toward α-(1-3)-linked glucose units comes from the observation of Carson and Weigert (1973) that the reaction of MOPC-104E IgM with its antiidiotype antibodies is inhibited by serum of BALB/c mice immunized with a polyglucan which contains a high percentage of α-(1-3) linkages. Oligosaccharides prepared from either poly[α-D-Glcp-(1-3)-α-D-Glcp-(1-4)][1] (nigeran) or poly[α-D-Glcp-(1-3)] both produced by *Aspergillus niger* (Johnston, 1965) have been found to serve as haptens in studies of the binding properties of MOPC-104E (Leon et al., 1970; Young et al., 1971; this paper).

The present knowledge of the binding site of immunoglobulins has markedly advanced due to the direct structural analysis obtained through x-ray diffraction crystallography (Amzel et al., 1974; Segal et al., 1974; Poljak et al., 1976; Davies and Padlan, 1977). Other physicochemical studies using thermodynamic (Kabat, 1976; Karush, 1962; Schechter, 1972; Cisar et al., 1975), kinetic (Pecht et al., 1972; Haselkorn et al., 1974), and spectroscopic methods (Hsia and Piette, 1969; Schlessinger et al., 1974) have illuminated both structural and dynamic aspects of the antibody binding site–hapten interactions. Still, binding sites of immunoglobulins specific for oligosaccharide haptens are the least known, as no direct structural analysis is yet available. Also, none of the immunoglobulins investigated by the above methods belong to the IgM class which deserves special attention because of its biological function on humoral as well as on cellular levels.

In this paper, we describe a systematic analysis of the binding site of MOPC-104E by thermodynamic and spectroscopic methods. Direct binding measurements of a series of oligosaccharides were carried out by equilibrium dialysis to confirm at least a part of the published data, obtained by indirect binding measurements. Oligosaccharides, dansylated at their reducing end, were synthesized to investigate binding properties by fluorescence methods. From the analysis of all data available, coarse mapping of the binding site is presented. The detailed kinetic scheme of the interactions between the series of derivatized oligosaccharides and MOPC-104E is currently being attained. The above model has been used as a basis for the kinetic study.

Materials and Methods

Protein. MOPC 104E bearing mice were kindly donated by Dr. M. Potter, National Cancer Institute, Bethesda, Md., and the tumor was perpetuated subcutaneously in BALB/c mice. The IgM produced by this tumor was purified from the serum by affinity chromatography according to Hiramoto et al. (1972) and stored at 4 °C. Immunoelectrophoretic analysis of the protein shows a single precipitation arc either with goat

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[1] Abbreviations used: Glcp, glucopyranos(yl)ide; N2-CHO, α-D-Glcp-(1-3)-α-D-Glcp, nigerose; N3-, N4-, and N5-CHO, the corresponding tri-, tetra-, and pentasaccharide, respectively; N4A-CHO, α-D-Glcp-(1-3)-α-D-Glcp(1-4)-α-D-Glcp-(1-3)-α-D-Glcp; N3-, N4-, and N4A-CH_2OH, the corresponding alditols; N3-, N4-, N5-, and N4A-Dns, the corresponding 5-dimethylaminonaphthalene-1-sulfonylhydrazones; PBS, phosphate-buffered saline (pH 7.2), 0.02 M sodium phosphate, 0.15 M NaCl; DMF, *N,N*-dimethylformamide; IgM, immunoglobulin M.

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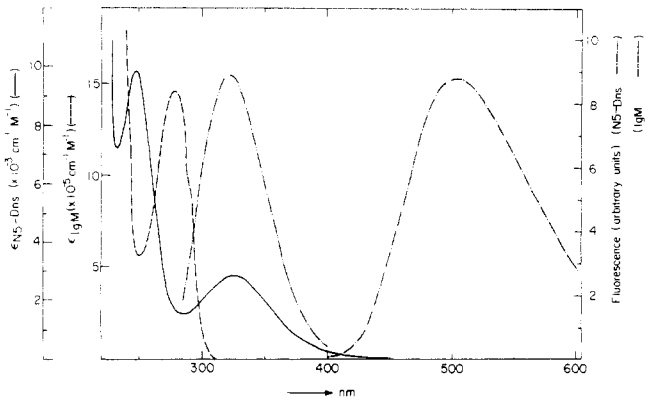


FIGURE 1: Absorption and emission spectra of MOPC-104E (- - -; • • •) and N5-Dns (—; • • •) both free in solution recorded at 25 °C. The emission spectra excited at 280 ± 1 nm (IgM) and 325 ± 2 nm (N5-Dns) are not corrected. No change in the hapten emission properties occurs upon binding (cf. Results).

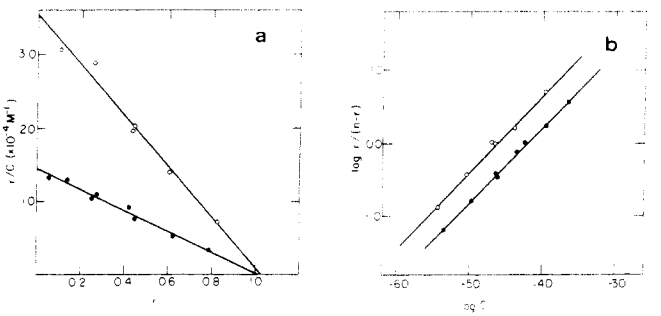
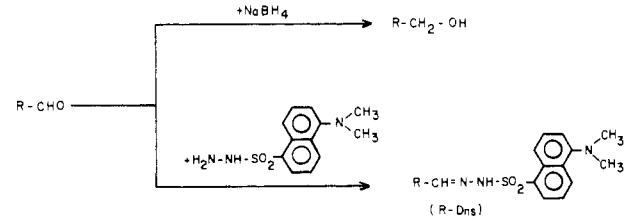


FIGURE 2: Scatchard (a) and Sips plots (b) obtained from equilibrium dialysis of MOPC-104E IgM with tritium-labeled N4A-CH_2OH at 4 (○) and 24 °C (●). Protein concentration was 2.2×10^{-5} M sites and the hapten concentration from 5×10^{-6} to 2.5×10^{-4} M. The solid lines were drawn using the least mean squares method.

anti-mouse serum or with dextran fraction S from *Leuconostoc mesenteroides* NRRL B-1355 (10 mg/mL) (Jeanes et al., 1954). This dextran was a kind gift of Dr. A. Jeanes, U.S. Department of Agriculture, Peoria, Ill. In microdisc electrophoresis (Rüchel et al., 1974), the protein migrates as a single band into the 1–30% polyacrylamide gel. Protein solution was dialyzed extensively against PBS and, before performing the measurements, concentrated in Selectron ultrathimbles (Schleicher and Schuell, Dassel, FRG). All further dilutions were made in PBS and the protein concentrations were determined spectrophotometrically using $OD_{280nm}^{1\%} = 16.0$ (Leon et al., 1970). The absorption and emission spectra of MOPC-104E IgM, recorded on a Cary 118 spectrophotometer and a Perkin-Elmer MPF 44A spectrofluorometer, respectively, are shown in Figure 1.

Haptens. The haptens used for the investigations were oligosaccharides or their derivatives in which the reducing end of the carbohydrate chain was modified by reduction to the alditol or by hydrazonation to the corresponding sugar hydrazone (Scheme I).

SCHEME I



The reducing oligosaccharides RCHO were prepared from either poly[α-D-Glcp-(1–3)] (isolated from *Aspergillus niger*) by partial acid hydrolysis according to Johnston (1965) (N3-, N4-, and N5-CHO) or by enzymatic degradation of nigeran as described by Reese and Mandels (1964) (N4A-CHO). (Mycodextranase and nigeran were kindly supplied by Dr. E. T. Reese, U.S. Army Natick Laboratories, Natick, Mass.) Oligosaccharides were purified by chromatography on washed Whatman 3MM paper in 1-butanol/pyridine/water (6:4:3, v/v). The oligosaccharides were detected by staining with periodate/alkaline silver nitrate (Osborn, 1963), eluted with water, and rechromatographed on Sephadex G-15 in water. Sugar moieties were detected with anthrone reagent (Kabat, 1961). The pooled fractions containing the oligosaccharides were lyophilized.

Alditols of the oligosaccharides were prepared by reduction with $NaBH_4$ or, for the tritium-labeled alditols, with sodium boro[^{3}H]hydride (661 mCi/mmol, Amersham U.K.) according to Young et al. (1971). Before applying the alditols to a Sephadex G-15 column, the reaction mixtures were evaporated to dryness five times with slightly acidified methanol to remove the borate as its methyl ester. The specific activity for the labeled N4A-CH_2OH was 5.46×10^{13} cpm/mol. Concentrations of all the oligosaccharide solutions were determined by using the anthrone test (Kabat, 1961).

Dansylation of Oligosaccharides. Thirty micromoles of the oligosaccharide was dissolved in 2 mL of absolute DMF containing 10 μL of concentrated hydrochloric acid. A solution of 50 mg of dansylhydrazine (Fluka, Buchs CH) in 500 μL of absolute DMF was added, and the mixture was allowed to react in a sealed vial for 75 min at 80 °C. After cooling, the mixture was subjected to preparative high-voltage electrophoresis on Whatman 3MM paper in pyridine/acetic acid/water (10:4:86, v/v, pH 5.3). After drying the paper, the yellow fluorescent zone with cathodal mobility was cut out and eluted with water. The concentrated eluate was chromatographed for 16 h on washed Whatman 3MM paper in 1-butanol/pyridine/water (6:4:3, v/v). The yellow fluorescent zone with the lowest mobility contained the sugar hydrazone and was eluted with water, evaporated to dryness, redissolved in water and evaporated three times to dryness to remove all residues of pyridine. Finally, the product was redissolved in water, lyophilized, and stored at −20 °C. All manipulations were performed in the dark so as to minimize photochemical decomposition. The obtained products showed in thin-layer chromatography only a single fluorescent spot in all solvents investigated. The yields were about 40% of the theoretical. The absorption and emission spectra of N5-Dns are shown in Figure 1.

Equilibrium dialysis was performed in lucite cells of 150-μL volume per half cell. Boiled cellulose dialysis tubings (Union Carbide Co., Chicago, Ill.) were used as membranes. The cell compartments were filled with 100 μL of a solution of the tritium-labeled hapten (1×10^{5}–5×10^{-4} M) or with 100 μL of protein solution (2.22×10^{-5} M, calculated for 90 000 daltons per site). The dialysis cells were left without shaking, and after reaching equilibrium (36 h at 4 °C and 24 h at 24 °C) 25-μL samples were withdrawn from each compartment and counted in 10 mL of Bray's solution (Bray, 1960). The equilibrium constants were evaluated by using the Sips (Karush, 1962) and Scatchard equation (Scatchard, 1949) (Figure 2). The association constants of various nonradioactive haptens were determined at 4 °C by displacement of the tritium-labeled N4A-CH_2OH with the respective nonlabeled haptens, using the method and equations described by Licht et al. (1977).

Fluorescence Titrations. The determination of the association constants of MOPC-104E IgM with various dansyl-labeled haptens was carried out by automatic titrations of the protein (2.14×10^{-6} M sites) with concentrated hapten solutions (about 5×10^{-3} M) on a Perkin-Elmer MPF 44A spectrofluorometer in the ratio mode. The intrinsic fluorescence of the protein (excitation at 280 ± 1 nm and emission at 340 ± 5 nm) was quenched upon binding the haptens. The quartz cuvette with a 7 × 7 mm light path (Hellma, Muellheim, FRG) containing the stirred protein solution was thermostated, and the hapten was added continuously by a motor-driven Hamilton syringe. The fluorescence intensity was recorded synchronously. To correct for nonspecific quenching, titrations with each of the haptens were repeated under the same conditions, using a solution of bovine γ-globulin at the same optical density. The recorded data were corrected for dilution and nonspecific quenching, and the saturation curve was computed. A minimization program based on the Powell algorithm (Powell, 1971) was then applied to the experimental data of the saturation curve. The best fitting values of the association constant K_0 and the maximal quenching Q_{max} were calculated, assuming that the heterogeneity index α and the number of sites n are both constant and equal to 1. Using the fitted Q_{max}, the Sips and Scatchard plots were also calculated from the experimental data. The computer programs used for this analysis will be described elsewhere (D. Lancet, in preparation). A typical set of graphs obtained by analyzing the experimental data is shown in Figure 3.

Linear Polarization of Fluorescence. The degree of linear polarization of the dansylated oligosaccharides free, bound to MOPC 104E, and in glycerol was measured at 4 and 25 °C on an instrument built by M. Shinitzky (Weber and Bablouzian, 1966). The freshly prepared thermostated samples were excited with linearly polarized light at 365 nm (mercury lamp). The ratio of the intensities of the emitted, parallel, and perpendicular polarized light ($I_\parallel/I_\perp$) was measured after passing a 418-nm cutoff filter. The fluorescence anisotropy factor r was calculated using the following equation:

$$r = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp} = \frac{I_\parallel/I_\perp - 1}{I_\parallel/I_\perp + 2}$$

From the observed anisotropy factor for the hapten solution in the absence (r_{free}) and presence of the antibody (r_{ob}) the fluorescence anisotropy of the bound hapten (r_{bound}) was calculated by the equation

$$r_{bound} = (r_{ob} - (C/H)r_{free})(H/X)$$

H, C, and X are the concentrations of the total, free, and bound hapten, respectively, calculated by using the total hapten and protein concentrations and the binding constants determined by the fluorometric titrations.

Circular Polarization of Fluorescence (CPL). CPL measurements were carried out on an instrument built at the Weizmann Institute of Science (Steinberg and Gafni, 1972; Gafni et al., 1973). The samples were excited at 365 nm with a high-pressure mercury arc lamp and the CPL spectrum was measured between 450 and 600 nm at room temperature. Protein was used at a concentration of 1.5×10^{-4} M (sites) and hapten concentration at 9×10^{-5} M. Under these conditions, 45-80% of the hapten was bound.

Results

Binding Measurements. The binding parameters obtained by equilibrium dialysis (direct and displacement measurements) are shown in Table II. All values observed at 4 °C agreed well with those reported earlier by Young et al. (1971), although the association constants determined by displacement equilibrium dialysis are all slightly higher than those obtained by quantitative inhibition of precipitation of MOPC-104E IgM with dextran B 1254 L. For N4A-CH_2OH (**14**), the association constant was also determined at 24 °C and was found to be $1.45 \pm 0.05 \times 10^4$ M^{-1} (see Figure 2). From the values of K_0 at both 4 and 24 °C, $\Delta H = -7.4$ kcal/mol and $\Delta S_u = +2.0$ eu were calculated, compared with −9.6 kcal/mol and −5.9

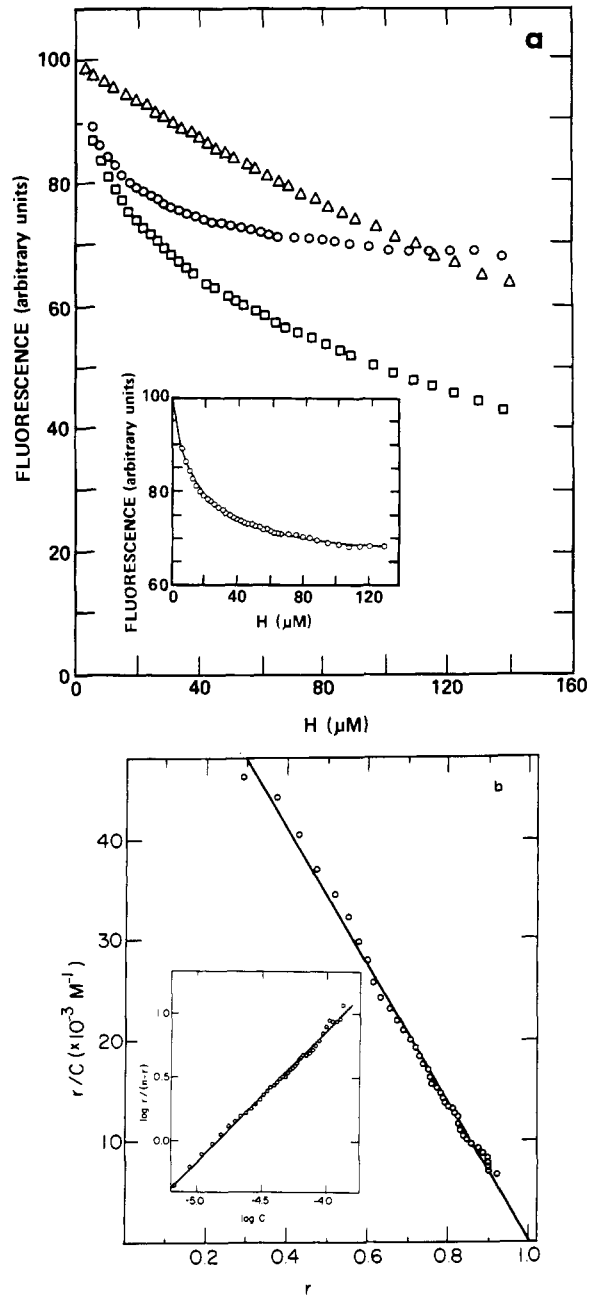


FIGURE 3: Data analysis of fluorometric titration of MOPC-104E IgM with N4A-Dns at 25 °C. Initial protein concentration was 2.12×10^{-6} M sites, the starting volume was 1.0 mL, and the titration was performed by continuously adding 45 μL of 3.31×10^{-3} M N4A-Dns solution. (a) Volume-corrected titration curve of bovine γ-globulin (△) and MOPC-104E IgM (□). The saturation curve (○) based on the specific quenching was calculated for both upper curves. Insert: Nonlinear iterative least-squares fitting of the experimental data of the saturation curve (○) to a theoretical curve (drawn line). For details, see Methods. (b) Scatchard and Sips plot (insert), calculated from the experimental data (○) and the fitted Q_{max}. The lines were drawn using the least mean squares method.

TABLE I: Association Constants and Fluorescence Anisotropy Factors of the Dansylated Oligosaccharides Bound to MOPC-104E IgM.

Hapten	K_0[a] × 10^{-4} M^{-1}		r_{free}[b]		r_{bound}[c]		r_{gly},[d]
	4 °C	25 °C	4 °C	25 °C	4 °C	25 °C	4 °C
N3-Dns	2.63 ± 0.01	0.85 ± 0.02	0.038	0.022	0.276	0.275	0.332
N4-Dns	2.19 ± 0.01	1.09 ± 0.01	0.038	0.023	0.291	0.244	0.336
N5-Dns	3.95 ± 0.10	1.79 ± 0.04	0.044	0.025	0.174	0.126	0.323
N4A-Dns	14.2 ± 0.5	6.98 ± 0.05	0.034	0.021	0.157	0.123	0.319

[a] The overall association constant is a mean of the values obtained from fluorometric titrations by three methods: fitting of the saturation curve, Sips plot, and Scatchard plot (see Materials and Methods). Heterogeneity index (α) and number of sites (n) in the Sips and Scatchard plots were found to be equal to 1.00 ± 0.02 for all haptens. Fluorescence anisotropy factor r of the oligosaccharides. [b] In buffer. [c] Bound to MOPC 104E IgM (see Materials and Methods). [d] In glycerol.

TABLE II: Binding Properties of Various Haptens Bound to MOPC 104E IgM at 4 °C.

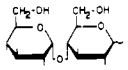
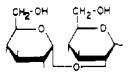
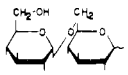
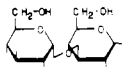
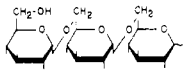
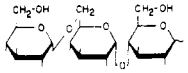
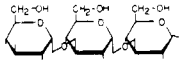
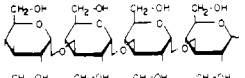
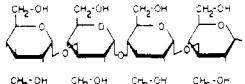
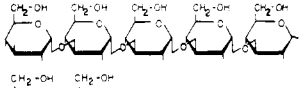
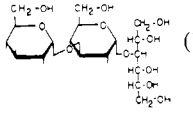
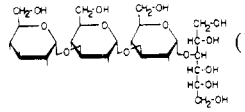
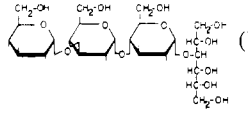
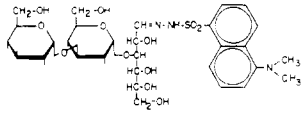
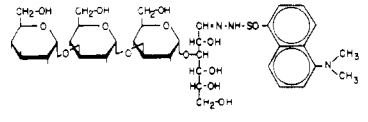
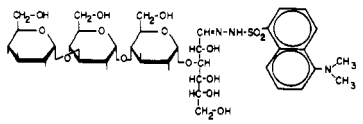
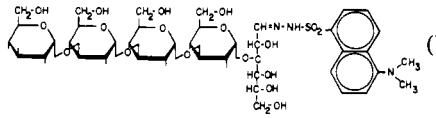
	Hapten	K_0 (× 10^{-4} M^{-1})	ΔG^e (kcal/mol)	% contribution to free energy	ΔH^f (kcal/mol)	$\Delta S_u{}^h$ (eu)	r_{bound}[i]
1		0.0054[a]	−2.19	37			
2		0.018[a]	−2.85	48			
3		0.028[a]	−3.09	53			
4		0.047[a]	−3.37	57			
5	 (N2-CHO)	1.2[a]	−5.15	88			
6		0.10[a]	−3.79	64			
7		0.13[a]	−3.93	67			
8	 (N3-CHO)	4.2[b]	−5.83	99			
9	 (N4-CHO)	3.5[b]	−5.74	98	−6.9[g]	+3.8[g]	
10	 (N4A-CHO)	4.6[b]	−5.88	100			
11	 (N5-CHO)	4.0[b]	−5.81	99			
12	 (N3-CH_2OH)	2.4[b,c]	−5.53	94			
13	 (N4-CH_2OH)	2.7[a]	−5.60	95			
14	 (N4A-CH_2OH)	3.6[b,c]	−5.75	98	−7.4 −6.8[g]	+2.0 +4.2[g]	
15	 (N3-Dns)	2.6[d]	−5.58	95	−8.8	−3.7	0.276
16	 (N4-Dns)	2.2[d]	−5.48	93	−5.4	+8.3	0.291

TABLE II (*Continued*)

	Hapten	K_0 ($\times 10^{-4}$ M^{-1})	ΔG^e (kcal/mol)	% contribution to free energy	ΔH^f (kcal/mol)	$\Delta S_u{}^h$ (eu)	$r_{bound}{}^i$
17	 (N4A-Dns)	14.2[d]	−6.51	111	−5.5	+11.7	0.157
18	 (N5-Dns)	4.0[d]	−5.80	99	−6.2	+6.7	0.174

[a] The overall association constant K_0 was determined by quantitative inhibition of precipitation (Leon et al., 1970; Young et al., 1971). [b] The overall association constant K_0 was determined by displacement equilibrium dialysis. [c] The overall association constant K_0 was determined by equilibrium dialysis with the tritium-labeled alditol. [d] The overall association constant K_0 was determined by fluorometric titrations. [e] The standard deviation of ΔG was less than 0.1 kcal/mol in all cases where the equilibrium constant was determined by the methods described in footnotes *b*, *c*, and *d*. [f] The enthalpy change was evaluated from the association constants at 4 and 25 °C (24 °C for hapten **14**). [g] Enthalpy and entropy values from microcalorimetric measurements (R. Zidovezki et al., unpublished results). [h] Entropy values were converted to the unitary scale (Kauzmann, 1959). [i] Fluorescence anisotropy of the dansylated haptens bound to MOPC 104E IgM.

eu respectively, reported by Young et al. (1971), based on measurements at 4 and 30 °C. The entropy values are corrected to the unitary scale according to Kauzmann (1959). For equilibrium dialysis at 24 °C, the protein was stabilized with 0.05% gelatine. Even in the presence of gelatine the protein began to aggregate in the lucite cells *after* 24 h at 24 °C, whereas in a reference solution kept in a glass tube under the same conditions for the same amount of time no aggregation was observed. Preliminary experiments showed that only ~10% quenching of the protein fluorescence occurs on saturation of MOPC-104E sites by N4A-CHO (**10**). This limited the possible use of the intrinsic fluorescence of the antibody to monitor binding. Thus, in order to improve the sensitivity, a series of dansylated derivatives of the oligosaccharides was synthesized as described above. This synthesis is generally applicable to reducing oligosaccharides (G. Schepers et al., unpublished results). Upon binding to MOPC-104E, these haptens show no change in their maxima of absorption or emission spectra. Also the emission intensity of the dansyl fluorophor (excited at 324 nm) of all conjugates did not undergo any change upon binding. However, a pronounced quenching of the protein intrinsic fluorescence was observed on binding those haptens. For binding the N4A-Dns (**17**), a maximal quenching Q_{max} of 35% was found and 29, 25, and 23% for N3-, N4-, and N5-Dns (**15, 16, 18**), respectively. The quenching of the protein (excited at 280 nm) was not accompanied by a fluorescence enhancement of the bound dansylated oligosaccharide expected if nonradiative energy transfer occurred as observed with other dansylated hapten–antibody systems (Pecht et al., 1971). Using the fluorescence quenching, the association constants of the dansylated haptens to MOPC-104E were measured at 4 and 25 °C by continuous fluorometric titrations. The binding parameters found for the dansylated oligosaccharides are reported in Table I. The influence of the dansyl group attached to the oligosaccharides on their binding properties to MOPC-104E is expressed in the differences between the affinities of the haptens as well as in their fluorescence properties. It is remarkable that at 4 °C there is almost no difference in the free energy of binding to the protein between the alditols and the corresponding dansyl derivatives of N3- and N4-CHO (**12**, 15 and **13**, 16) (Table II). On the other hand, it is noteworthy that the respective nonderivatized oligosaccharides show higher affinities. The dansylated pentasaccharide (**18**) is similar in its free energy of binding to the series of the nonlabeled α-(1-3)-linked oligosaccharides (**8–11**) and has the same association constant as N5-CHO (**11**). This set includes also N4A-CHO, the tetrasaccharide with one α-(1-4) linkage as well as its alditol N4A-CH_2OH (**14**). However, N4A-Dns (**17**) exhibits the highest affinity to MOPC-104E of all haptens investigated and has also the highest binding entropy value (ΔS_u = +11.7 eu) among the dansylated oligosaccharides. The corresponding value calculated for the N4A-CH_2OH (**14**) is remarkably lower (+2.0 eu). The lowest binding entropy for a dansylated derivative was obtained with N3-Dns (**15**) (ΔS_u = −3.7 eu), whereas in this case the enthalpy change (calculated from the association constants at 4 and 25 °C by using the van't Hoff equation) is the highest value (ΔH = −8.8 kcal/mol). The enthalpy changes for the other dansylated oligosaccharides (**16–18**) caused on binding to the protein are all in the same order of magnitude (−5.4 to −6.2 kcal/mol; cf. Table II).

Fluorescence Linear Polarization. The fluorescence anisotropy factors obtained for the various dansylated haptens at 4 and 25 °C in glycerol or in buffer and when bound to MOPC-104E IgM are listed in Table I. Comparing the values obtained in buffer and in the high-viscosity solvent (glycerol) with those of the bound haptens shows that the highest restriction in rotational freedom occurs upon binding the N3- and N4-Dns (**15, 16**). The anisotropy factors observed for the antibody bound N5- and N4A-Dns (**18, 17**) indicate that in these complexes the fluorophore is positioned so that limited interaction between the combining site and the dansyl residue takes place. The pronounced difference between the two tetrasaccharides N4- and N4A-Dns (**16, 17**) is especially noteworthy because these two haptens have the same length and differ only in one glucosidic linkage.

Circular Polarization of Luminescence (CPL). No circularly polarized component could be detected in the emission of any of the different dansyloligosaccharide–MOPC-104E complexes. Thus, in all cases studied the circularly polarized fraction of emitted light is beyond the experimental error of the instrument, i.e., smaller than $g_{em} = 5 \times 10^{-5}$.

Discussion

The binding properties to MOPC-104E of all the haptens investigated by us or those reported previously by Leon et al. (1970) and Young et al. (1971) are summarized in Table II. These data allow the delineation of a model for the binding site

of this immunoglobulin. This is essentially a thermodynamic mapping of the binding sites in analogy to studies done on other immunoglobulins (Kabat, 1976; Schechter, 1972; Cisar et al., 1975) or enzymes (Berger and Schechter, 1970; Sharon and Chipman, 1969). We used the spectroscopic measurements to substantiate the latter approach.

Comparison of the free energies of binding of the various haptens shows that already the monosaccharide methyl-α-D-glucopyranoside (**1**) binds with 37% of the free energy of the strongest binding (nonderivatized) hapten, the tetrasaccharide N4A-CHO (**10**) (≡100%). The disaccharides with α-(1-2)-α-(1-4)- and α-(1-6)-linked glucose units (**3, 2,** and **4**) achieve 48-57% and the disaccharide nigerose (N2-CHO) (**5**) reaches 88% of the maximal binding energy. A third glucopyranose unit increases the free energy of binding on going from isomaltose (**4**) to isomaltotriose (**6**) or to panose (**7**) only by −0.42 and −0.56 kcal/mol, respectively. In the series of the α-(1-3)-linked oligosaccharides, going from N2-CHO (**5**) to N3-CHO (**8**) the free-energy increase is of a similar magnitude (−0.68 kcal/mol). No difference is found between the free energy of binding of the two tetrasaccharides N4-CHO (**9**) and N4A-CHO (**10**) or among the trisaccharide N3-CHO (**8**) and the corresponding tetra- and pentasaccharides N4-CHO (**9**) and N5-CHO (**11**). The fact that for binding the disaccharide N2-CHO 88% of the maximal free energy of binding is already achieved and that high specificity toward α-(1-3)-linked glucose pyranosides is found lead to the conclusion that the two glucopyranose rings of N2-CHO have an optimal three-dimensional contact with the complementary residues in the binding site of MOPC-104E. An additional, third glucose unit also has contact with the protein but of much lower specificity and binding energy. It may therefore be assumed that, whereas the complementary residues for the first two glucopyranose rings on the nonreducing end of the haptens form a cavity, those residues interacting with the third glucose are located in a more open part of the binding site: The small cavity (~12-Å depth) is specific for the terminal nonreducing nigerosyl group. Any further glucose unit forms only loose contact with the antibody and this is expressed in relatively small contributions to the binding free energy (<1 kcal/mol). The subsite interacting with the third glucose unit shows no discrimination of the type of linkage to the disaccharide. The fact that modifications of the reducing end of N3- and N4-CHO (**12, 13, 15,** and **16**) decrease the free energy of binding may be a result of steric hindrance of the modified reducing end of these oligosaccharides. On the other hand, the respective derivatives of N5- and N4A-CHO (**14, 17,** and **18**) did not show such lower binding.

Further understanding of the interactions between the saccharides and the binding site is obtained from measurements of the fluorescence properties of the fluorophore attached to their reducing end, primarily from the linear and (the lack of) circular polarization of the fluorescence emitted by their MOPC 104E complexes.

The effect of binding to the slowly tumbling IgM (Holowka and Cathou, 1976) is apparent for the fluorophores in complexes with all the haptens (Table I). However, a marked difference in the rotational freedom of the fluorophore is apparent between the complexes N3- (**15**) and N4-Dns as compared with the N5- and N4-Dns (cf. **18**) complexes. This, combined with the observation that there is no effect on the spectral shape or intensity of the emission upon binding of the fluorophore to MOPC 104E (nor any induced CPL) suggests that the dansyl group in all bound haptens does not undergo a marked change in its environment, forms limited yet varying degrees of contact with the protein residues, and protrudes out into the solvent to different extents. (a) The exclusively α-(1-3)-linked tri- and tetrasaccharides are causing the fluorophore to be restrained in rotation probably by residues forming a groove beyond the main binding cavity. (b) The pentasaccharide derivative is long enough so that the Dns is freely rotating. (c) The N4A-Dns also allows the fluorophore higher mobility. This must be due to the α-(1-4) linkage as the terminal nonreducing nigerosyl binds to the cavity in all cases. The influence of the dansyl group on the binding energy of the respective haptens is expressed not only in their different free energy of binding but even more so in the different values of the enthalpy and entropy of binding. The two dansylated tetrasaccharides (**16, 17**) show the same binding enthalpy, and the markedly higher binding energy in the case of the N4A-Dns (**17**) is caused mainly by the higher attribute of the entropy. In the case of the trisaccharide N3-Dns (**15**), though its binding enthalpy is higher than those observed for the binding of other members of this series, the entropy change of its binding is small and negative, which results in a lower binding energy. In this case, the dansyl group may be affecting the binding directly. This is indicated primarily by the immobilization of the dansyl group as expressed in the linear fluorescence polarization but probably also in the negative value of binding entropy. The high positive binding entropy of N4A-Dns (**17**) (+11.7 eu) compared with that of N4A-CH_2OH (14) (+2.0 eu) and the different binding enthalpies (−5.5 and −7.4 kcal/mol, respectively) suggest that either interaction with the sulfohydrazine group or with the dimethylaminonaphthyl ring becomes significant. Still, it should be stressed that there is no immobilization of the fluorophore, which implies that it forms no stable contact with the protein and exerts its entropic contribution by causing water molecules to be liberated from the binding site and/or from the hapten. The slightly positive entropic contribution to the binding of N4A-CH_2OH, that we found in contrast to Young et al. (1971), was also confirmed by direct measurements of the reaction enthalpy (on a LKB batch microcalorimeter, ΔH = −6.8 kcal/mol and ΔS_u = +4.1 eu; R. Zidovezki, G. Schepers and I. Pecht, unpublished results).

Cisar et al. (1975) have shown that the binding sites of antidextran antibodies can generally be described either in terms of a groove or a cavity. In the case of MOPC-104E all the results support the model of a cavity-like binding site, which binds terminal, nonreducing nigerosyl groups. It also binds with lower affinity the terminal nonreducing end of non-α-(1-3)-linked glucans. This model is also supported by the data of Leon et al. (1970), who found that some polyglucans, which lack any α-(1-3) linkage, precipitate with MOPC-104E, whereas polysaccharides with significant amounts of α-(1-3) linked glucose do not precipitate. The conflicting results obtained by several groups who investigated the structure of some of these dextrans (Jeanes et al., 1954; Suzuki and Hehre, 1964; Bourne et al., 1974; Seymour et al., 1977; and the review by Sidebotham, 1974), especially their α-(1-3)-linkages content, allowed no conclusive decision concerning the binding properties of MOPC-104E to these antigens.

In conclusion, evidence was presented that the binding site of MOPC-104E constitutes a 12-Å deep cavity with subsites for two pyranose rings and a further subsite located in a more open groove following the cavity. The dimensions of this site are within the range expected on the basis of the extensive studies of Kabat and his colleagues on saccharide binding sites and the direct, crystallographic structures determined for immunoglobulin sites. It has previously been pointed out (Pecht et al., 1972) that the structural interpretation of the variation in affinity of a series of ligands to a given binding site is limited and that a more detailed and conclusive analysis can be

achieved by the kinetic mapping approach. We are currently investigating the kinetics of interaction between protein 104E and the series of dansylated oligosaccharides. The results clearly demonstrate that a conformational transition is induced in the immunoglobulin upon hapten binding. A detailed report of this study is in preparation.

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