Interaction of egg-white glycoproteins and their oligosaccharides with the monomer and the hexamer of chicken liver lectin

A multivalent oligosaccharide-combining site exists within the carbohydrate-recognition domain*

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Binding of egg-white glycoproteins and their oligosaccharides to hexameric solubilized form of the chicken hepatic lectin and the monomeric soluble fragment containing the carbohydrate-recognition domain has been investigated by several techniques. Ligand blotting revealed significant differences in binding to two forms of the lectin only for glycoproteins bearing multiple *N*-linked oligosaccharide moieties in their molecule (riboflavin-binding glycoprotein, avidin or ovomucoid). Inhibition studies indicated that inhibitory potency in a series of linear and branched *N*-acetyl-D-glucosamine-terminated oligosaccharides is critically dependent on the number and spatial arrangement of the terminal monosaccharide residues for both forms of the lectin. Direct binding of 4-hydroxyphenyl-derivatized radioiodinated oligosaccharides measured by equilibrium dialysis and frontal affinity chromatography points to the existence of two *N*-acetyl-D-glucosamine-combining sites per one subunit of the lectin, as has been recently reported for the rabbit and rat liver lectin [Lee & Lee (1988) *Biochem. Biophys. Res. Commun.* **155**, 1444–1452]. Highly branched (penta-antennary) oligosaccharides interact with more than one subunit of the hexameric form of the lectin and thus resemble the more complex interaction of the whole glycoprotein.

INTRODUCTION

Vertebrate liver lectins are among the best-studied cell-surface receptors mediating endocytosis (reviewed by Ashwell & Harford, 1982; Schwartz, 1984; Breitfeld *et al.*, 1985; Drickamer, 1988). They were isolated and characterized from chicken (Kawasaki & Ashwell, 1977), rabbit (Hudgin *et al.*, 1974), rat (Pricer & Ashwell, 1976), porcine (Bezouška *et al.*, 1985b) and human (Baenziger & Maynard, 1980) liver and studied with regard to many different aspects by a wide variety of methods. For mammalian liver lectins, two genes and three cell-surface polypeptide chains have been detected (Spiess & Lodish, 1985; McPhaul & Berg, 1987; Halberg *et al.*, 1987), but both their cell-surface arrangement and the occurrence of various functional states remain unsettled (Weigel *et al.*, 1986; Clarke *et al.*, 1987; Sawyer *et al.*, 1988; Bischoff *et al.*, 1988). Chicken hepatic lectin (CHL), on the other hand, is composed of a single 26 kDa polypeptide with a known amino acid sequence (Drickamer, 1981), which make it a particularly suitable model for studies on ligand binding and internalization.

CHL is an example of a type 2 integral membrane protein with the *N*-terminus facing the cytoplasm and the major part of polypeptide, containing the C-terminal carbohydrate-recognition domain oriented extracellularly (Drickamer & Mamon, 1982; Chiacchia & Drickamer, 1984). The chicken hepatic receptor specific for *N*-acetyl-D-glucosamine-terminated glycoproteins is arranged in the membrane probably as an oligomer containing a cluster of carbohydrate-binding sites; this form of the molecule is preserved during the solubilization of the receptor from the membrane (Loeb & Drickamer, 1987). Another advantage for

studies of binding properties and function of the lectin stems from the possibility of obtaining the extracellular 16 kDa carbohydrate-binding domain of the lectin under mild conditions (Chiacchia & Drickamer, 1984).

Although the carbohydrate-combining site of CHL has been examined in detail (Sikder *et al.*, 1983), studies aimed at determining the functional importance of the multivalent interaction between several carbohydrate moieties of a glycoprotein and the hexameric form of the lectin or its soluble fragment have only recently emerged (Loeb & Drickamer, 1988).

In the present study we have employed two series of structurally related oligosaccharides with the terminal *N*-acetyl-D-glucosamine to examine the carbohydrate-combining site of CHL in its native hexameric form as well as in the form of a monomeric soluble fragment. For direct binding studies a new type of 3-(4-hydroxyphenyl)propionyl-modified derivatives of oligosaccharides from *N*-glycoproteins has been employed (Piskarev *et al.*, 1990b). It was found that, similarly to other mammalian lectins, the carbohydrate-binding site of the CHL can discriminate between ligands having various type of branching. Interestingly there seems to be evidence that highly branched oligosaccharides (tetra- or penta-antennary) can interact simultaneously with two subunits of the lectin and thus resemble the complex interaction observed with the whole glycoprotein molecule.

EXPERIMENTAL

Materials

Sodium periodate, h.p.l.c.-grade acetonitrile, LiChrosolv, PEG

Abbreviations used: CHL and subCHL, chicken hepatic lectin in hexameric and monomeric form respectively; NC, nitrocellulose; PEG 6000, poly(ethylene glycol) 6000; RBG, riboflavin-binding glycoprotein.

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6000 and Fractogel TSK HW-40S were from Merck, Darmstadt, Germany; 3-(4-hydroxyphenyl)propionic acid *N*-hydroxysuccinimide ester (Bolton–Hunter reagent), *N*-acetyl-D-glucosamine and D-mannose were from Serva, Heidelberg, Germany; (+)-biotin *N*-hydroxysuccinimide ester was from Calbiochem, La Jolla, CA, U.S.A.; Sephadex G-15, Sephacryl S-200, Sepharose 4B and CNBr-activated Sepharose 4B were from Pharmacia–LKB Biotechnology, Uppsala, Sweden; BSA (20% solution) was from Sevac, Prague, Czechoslovakia; horseradish peroxidase (about 1000 units/mg) was from Boehringer–Mannheim, Germany; β -D-galactosidase and α -D-mannosidase were prepared from jack-bean (*Canavalia ensiformis*) meal, with chromatography on DEAE-Sepharose and phenyl-Sepharose as the major purification steps for β -D-galactosidase and α -D-mannosidase respectively (Li & Li, 1972; Einhoff & Rüdiger, 1986). IgG was prepared from outdated human plasma as described previously (Piskarev *et al.*, 1988a). All other chemicals were analytical-grade reagents.

Buffers

Buffer compositions were as follows: buffer A, 0.02 M-Tris/HCl/0.15 M-NaCl pH 7.8; buffer B, 0.02 M-Tris/HCl/0.15 M-NaCl/10 mM-CaCl₂ pH 7.8; buffer C, 0.02 M-Tris/HCl/0.15 M-NaCl/10 mM-CaCl₂/BSA (1 mg/ml), pH 7.8.

Preparation of egg-white glycoproteins

Ovalbumin, RBG and ovomucoid were isolated from hen's-egg white by preparative h.p.l.c. (Piskarev *et al.*, 1988a). Avidin (binding capacity for biotin 13.5 μ g/mg) was prepared in the Research and Development Laboratory of Poultry Industry).

Preparation of oligosaccharides

Glycoasparagines N1 and N2 were a gift of Dr. O. S. Novikova (N. D. Zelinsky Institute of Organic Chemistry). Oligomers of *N*-acetyl-D-glucosamine N3 and N4 were prepared from chitin hydrolysate by gel chromatography on a column of Fractogel TSK HW-40(S) (5 cm $\times$ 95 cm) eluted with 0.1 M-pyridine/acetate buffer, pH 5.0, followed by preparative h.p.l.c. on a Zorbax C₁₈ column (22 mm $\times$ 250 mm; du Pont de Nemours and Co., Wilmington, DE, U.S.A.) eluted with the linear acetonitrile/water gradient (V. E. Piskarev, unpublished work; full details are available from V.E.P. on request).

Complex oligosaccharides were released from glycoproteins by LiBH₄/LiOH treatment (Likhoshesterov *et al.*, 1988). Neutral oligosaccharides were either reduced to alditols or modified with the Bolton–Hunter reagent (Piskarev *et al.*, 1988b, 1990b). Oligosaccharide N(N)R was prepared from ovalbumin glycopeptide IIIA (Huang *et al.*, 1970) by exhaustive digestion with jack-bean α -D-mannosidase, whereas oligosaccharides N₃R and N₄R were prepared from triantennary and tetra-antennary oligosaccharides of orosomucoid. The final purification of all oligosaccharides used in the study was achieved by preparative chromatography on octadecyl- and amino-silica h.p.l.c. columns (Piskarev *et al.*, 1988b, 1989).

Analysis of oligosaccharides

Quantitative carbohydrate analysis of neutral sugars and amino sugars was done as described previously (Likhoshesterov *et al.*, 1987). The structures of all oligosaccharides used in the study were confirmed by ¹H-n.m.r. 500 MHz spectroscopy on a Bruker WM-500 spectrometer equipped with a Bruker Aspect-2000 computer (Piskarev *et al.*, 1988b, 1989).

Preparation of CHL and its subtilisin fragment (subCHL)

CHL was isolated as described previously (Kawasaki & Ashwell, 1977) from the pooled livers of freshly killed chickens

(Poultry Industry). GlcNAc–Sepharose 4B prepared by the method of Fornstedt & Porath (1975) has been used as the sorbent for affinity chromatography. The second affinity-chromatography step was performed in 0.05% Triton X-100. Eluted lectin was concentrated, extensively dialysed against buffer B with 0.05% Triton X-100, adjusted to a final protein concentration of 1 mg/ml and stored in the detergent at 4 °C.

SubCHL was prepared by a published procedure (Chiacchia & Drickamer, 1984). For analytical purposes samples were incubated for 45 min at 37 °C at different enzyme/protein ratios in a final volume of 50 μ l. The reaction was stopped by the addition of twice-concentrated SDS sample buffer and samples were analysed by SDS/PAGE. For preparative purposes, CHL (~1 mg/ml) was incubated with subtilisin at enzyme/protein ratio 1:100. Reaction was terminated by the addition of phenylmethanesulphonyl fluoride (5 mM) and cooling and the subCHL preparation stored at 4 °C.

Ligand blotting (dot-blot)

Egg-white glycoproteins were biotinylated with *N*-hydroxysuccinimide ester derivative dissolved in 0.1 M-NaHCO₃ and 10% (v/v) dimethylformamide. Horseradish peroxidase was conjugated with egg-white avidin by the periodate-oxidation method (Tijssen & Kurstak, 1984), and the conjugate was purified by gel filtration on a column of Sephacryl S-200 (1 cm $\times$ 120 cm) eluted with buffer C containing 10% (v/v) glycerol.

For dot-blot analysis, 0.5–2.0 μ g of CHL or subCHL was applied on to nitrocellulose (NC) membrane. All subsequent incubations were done at room temperature. The membrane was blocked for 1 h with 2% (w/v) BSA in buffer A, transferred to buffer B, incubated for 1 h with biotinylated glycoproteins, washed three times for 20 min with buffer B, incubated for 1 h with the avidin–peroxidase conjugate and washed three times for 20 min with buffer B. Detection of enzymic activity was done in a solution containing 50 ml of buffer B, 25 mg of 3,3'-diaminobenzidine, 68 mg of NiSO₄·7H₂O and 20 μ l of concentrated H₂O₂ (this solution was filtered before use and peroxide was added immediately before the detection). The formation of the coloured precipitate was stopped by washing the NC membrane in distilled water.

Inhibition and binding experiments

Egg-white ovomucoid and 4-hydroxyphenylated oligosaccharides were labelled with carrier-free Na¹²⁵I (Greenwood *et al.*, 1963) and purified by gel filtration on a column of Sephadex G-15 (1 cm $\times$ 50 cm) eluted with buffer C. The specific radioactivity of the product was about 2000 Bq/pmol, and for the binding test it was further diluted with the unlabelled ligand.

Inhibition experiments were performed using the poly(ethylene glycol) precipitation assay of Lehrman & Hill (1983) as modified by Grant & Kaderbhai (1986).

Binding studies were done by equilibrium dialysis, employing the multiple-chamber apparatus with rotating head (Englund *et al.*, 1969), concentration of the ligand being varied according to the results of inhibition experiments. In some experiments, frontal affinity chromatography was employed essentially as described by Ohyama *et al.* (1985).

Analytical methods

The concentration of proteins was determined by the bicinchoninic acid method (Smith *et al.*, 1985) with BSA as standard. The concentration of complex oligosaccharides was measured by the phenol/H₂SO₄ assay (Dubois *et al.*, 1956) with D-mannose as standard. SDS/PAGE was performed as described by Laemmli (1970).

Table 1. Summary of the basic data for oligosaccharides used in the study

Abbreviations of complex oligosaccharides are those assigned by Schachter (1986); N stands for *N*-acetyl-D-glucosamine. Oligosaccharides were used as alditols (-ol) or modified with Bolton-Hunter reagent (-BH). For details, see the Experimental section.

Oligosaccharide abbreviation	Structure	Source
N1	GlcNAc β 1→Asn	Synthesis
N2	GlcNAc(β 1→4)GlcNAc β 1→Asn	Synthesis
N3	[GlcNAc(β 1→4)] ₂ GlcNAc	Chitin
N4	[GlcNAc(β 1→4)] ₃ GlcNAc	Chitin
N(N)BH	$\begin{array}{c} \text{Man}(\beta 1 \rightarrow 4) \text{GlcNAc}(\beta 1 \rightarrow 4) \text{GlcNAc} \beta 1 \rightarrow \text{BH} \\ \swarrow \\ \text{GlcNAc}(\beta 1 \rightarrow 2) \text{Man}(\alpha 1 \rightarrow 3) \end{array}$	Ovalbumin
N ₂ BH	$\begin{array}{c} \text{GlcNAc}(\beta 1 \rightarrow 2) \text{Man}(\alpha 1 \rightarrow 6) \\ \swarrow \\ \text{Man}(\beta 1 \rightarrow 4) \text{GlcNAc}(\beta 1 \rightarrow 4) \text{GlcNAc} \beta 1 \rightarrow \text{BH} \\ \swarrow \\ \text{GlcNAc}(\beta 1 \rightarrow 2) \text{Man}(\alpha 1 \rightarrow 3) \end{array}$	IgG
N ₂ (N)ol	$\begin{array}{c} \text{GlcNAc}(\beta 1 \rightarrow 2) \text{Man}(\alpha 1 \rightarrow 6) \\ \swarrow \\ \text{GlcNAc}(\beta 1 \rightarrow 4) \text{Man}(\beta 1 \rightarrow 4) \text{GlcNAc}(\beta 1 \rightarrow 4) \text{GlcNAc} \beta 1 \rightarrow \text{ol} \\ \swarrow \\ \text{GlcNAc}(\beta 1 \rightarrow 2) \text{Man}(\alpha 1 \rightarrow 3) \end{array}$	RBG
N ₃ BH	$\begin{array}{c} \text{GlcNAc}(\beta 1 \rightarrow 2) \text{Man}(\alpha 1 \rightarrow 6) \\ \swarrow \\ \text{Man}(\beta 1 \rightarrow 4) \text{GlcNAc}(\beta 1 \rightarrow 4) \text{GlcNAc} \beta 1 \rightarrow \text{BH} \\ \swarrow \\ \text{GlcNAc}(\beta 1 \rightarrow 2) \text{Man}(\alpha 1 \rightarrow 3) \\ \swarrow \\ \text{GlcNAc}(\beta 1 \rightarrow 4) \end{array}$	Orosomucoid
N ₃ (N)ol	$\begin{array}{c} \text{GlcNAc}(\beta 1 \rightarrow 2) \text{Man}(\alpha 1 \rightarrow 6) \\ \swarrow \\ \text{GlcNAc}(\beta 1 \rightarrow 4) \text{Man}(\beta 1 \rightarrow 4) \text{GlcNAc}(\beta 1 \rightarrow 4) \text{GlcNAc} \beta 1 \rightarrow \text{ol} \\ \swarrow \\ \text{GlcNAc}(\beta 1 \rightarrow 2) \text{Man}(\alpha 1 \rightarrow 3) \\ \swarrow \\ \text{GlcNAc}(\beta 1 \rightarrow 4) \end{array}$	RBG
N ₃ (N)BH	$\begin{array}{c} \text{GlcNAc}(\beta 1 \rightarrow 2) \text{Man}(\beta 1 \rightarrow 6) \\ \swarrow \\ \text{GlcNAc}(\beta 1 \rightarrow 4) \text{Man}(\beta 1 \rightarrow 4) \text{GlcNAc}(\beta 1 \rightarrow 4) \text{GlcNAc} \beta 1 \rightarrow \text{BH} \\ \swarrow \\ \text{GlcNAc}(\beta 1 \rightarrow 2) \text{Man}(\alpha 1 \rightarrow 3) \\ \swarrow \\ \text{GlcNAc}(\beta 1 \rightarrow 4) \end{array}$	RBG
N ₄ BH	$\begin{array}{c} \text{GlcNAc}(\beta 1 \rightarrow 6) \\ \swarrow \\ \text{GlcNAc}(\beta 1 \rightarrow 2) \text{Man}(\alpha 1 \rightarrow 6) \\ \swarrow \\ \text{Man}(\beta 1 \rightarrow 4) \text{GlcNAc}(\beta 1 \rightarrow 4) \text{GlcNAc} \beta 1 \rightarrow \text{BH} \\ \swarrow \\ \text{GlcNAc}(\beta 1 \rightarrow 2) \text{Man}(\alpha 1 \rightarrow 3) \\ \swarrow \\ \text{GlcNAc}(\beta 1 \rightarrow 4) \end{array}$	Orosomucoid
N ₄ (N)ol	$\begin{array}{c} \text{GlcNAc}(\beta 1 \rightarrow 6) \\ \swarrow \\ \text{GlcNAc}(\beta 1 \rightarrow 2) \text{Man}(\alpha 1 \rightarrow 6) \\ \swarrow \\ \text{GlcNAc}(\beta 1 \rightarrow 4) \text{Man}(\beta 1 \rightarrow 4) \text{GlcNAc}(\beta 1 \rightarrow 4) \text{GlcNAc} \beta 1 \rightarrow \text{ol} \\ \swarrow \\ \text{GlcNAc}(\beta 1 \rightarrow 2) \text{Man}(\alpha 1 \rightarrow 3) \\ \swarrow \\ \text{GlcNAc}(\beta 1 \rightarrow 4) \end{array}$	RBG
N ₅ (N)ol	$\begin{array}{c} \text{GlcNAc}(\beta 1 \rightarrow 4) \\ \swarrow \\ \text{GlcNAc}(\beta 1 \rightarrow 6) \\ \swarrow \\ \text{GlcNAc}(\beta 1 \rightarrow 2) \text{Man}(\alpha 1 \rightarrow 6) \\ \swarrow \\ \text{GlcNAc}(\beta 1 \rightarrow 4) \text{Man}(\beta 1 \rightarrow 4) \text{GlcNAc}(\beta 1 \rightarrow 4) \text{GlcNAc} \beta 1 \rightarrow \text{ol} \\ \swarrow \\ \text{GlcNAc}(\beta 1 \rightarrow 2) \text{Man}(\alpha 1 \rightarrow 3) \\ \swarrow \\ \text{GlcNAc}(\beta 1 \rightarrow 4) \end{array}$	Ovomucoid
N ₅ (N)BH	$\begin{array}{c} \text{GlcNAc}(\beta 1 \rightarrow 4) \\ \swarrow \\ \text{GlcNAc}(\beta 1 \rightarrow 6) \\ \swarrow \\ \text{GlcNAc}(\beta 1 \rightarrow 2) \text{Man}(\alpha 1 \rightarrow 6) \\ \swarrow \\ \text{GlcNAc}(\beta 1 \rightarrow 4) \text{Man}(\beta 1 \rightarrow 4) \text{GlcNAc}(\beta 1 \rightarrow 4) \text{GlcNAc} \beta 1 \rightarrow \text{BH} \\ \swarrow \\ \text{GlcNAc}(\beta 1 \rightarrow 2) \text{Man}(\alpha 1 \rightarrow 3) \\ \swarrow \\ \text{GlcNAc}(\beta 1 \rightarrow 4) \end{array}$	Ovomucoid

Fig. 1. Analysis of the interaction of egg-white glycoproteins with the CHL by ligand blotting (dot-blot)

CHL (upper row) and subCHL (lower row) (0.5, 1.0 and 2.0 μ g each) applied to NC membrane and processed as described in the Experimental section. Detection was done with biotinylated ovalbumin (a), RBG (b), ovomucoid (d) and avidin-peroxidase (a-d).

Fig. 2. Inhibition by linear oligomers of GlcNAc of 125 I-ovomucoid binding to subCHL (a) and CHL (b)

Symbols and abbreviations for the inhibitors (see Table 1) are as follows: $\circ$, N1; $\blacksquare$, N2; $\triangle$, N3; and $\square$, N4. Other details are described in the Experimental section.

Fig. 3. Inhibition by branched oligosaccharides containing terminal GlcNAc of 125 I-ovomucoid binding to subCHL (a) and CHL (b)

Symbols and abbreviations for the inhibitors (see Table 1) are as follows: $\bullet$, $N_2(N)ol$; $\square$, $N_3(N)ol$; $\blacktriangle$, $N_4(N)ol$; and $\blacksquare$, $N_5(N)ol$. Other details are given in the Experimental section.

RESULTS

Table 1 summarizes the characteristics of oligosaccharides used as ligands to examine the carbohydrate-combining site of the CHL. Included are two series of oligosaccharides having a terminal *N*-acetyl-D-glucosamine residue: linear oligosaccharides N1–N4 and complex branched oligosaccharides N(N)R– $N_5(N)$ R prepared from egg-white glycoproteins. In all examples, h.p.l.c. on preparative columns was employed as a major separation step to resolve the complex mixtures of oligosaccharides found in egg-white glycoproteins (Piskarev *et al.*, 1988b, 1989; Alonso *et al.*, 1988); an efficient combination of h.p.l.c. on octadecyl- and amino-silica packings is usually required. For oligosaccharides prepared by partial enzymic hydrolysis, e.g. N(N)R, N_3 R or N_4 R, the separation by h.p.l.c. seems to be essential to obtain the desired product, as the enzymic cleavage always leads to complex mixtures of oligosaccharides, even when purified compounds are treated with a high excess of enzymic activity. For the final evaluation of structure, high resolution 1H -n.m.r. has proved to be very useful (Vliegthart *et al.*, 1983).

To evaluate the interaction of CHL and subCHL with various egg-white glycoproteins we have adopted the technique of ligand blotting with lectin molecules immobilized on NC membrane. Although this method can only provide semi-quantitative data, it is apparent from Fig. 1 that great differences in binding exist between glycoproteins bearing only one oligosaccharide per molecule (ovalbumin; Fig. 1a) when compared with other glycoproteins possessing two (RBG; Fig. 1b) or even four or five (avidin, ovomucoid) carbohydrate side chains in the molecule (Piskarev *et al.*, 1990a; Zheng *et al.*, 1988). These differences are observed only for the oligomeric form of the lectin.

Figs. 2 and 3 present inhibition data from experiments using both series of oligosaccharides. Several conclusions can be drawn from the evaluation of these results.

(1) Branched oligosaccharides are better inhibitors than linear oligosaccharides for both forms of the lectin (cf. Fig. 2 and Fig. 3).

(2) Highly branched oligosaccharides are better inhibitors than the less-branched oligosaccharides (Figs. 3a and 3b).

Table 2. Binding data for the interaction between radioiodinated oligosaccharides and monomeric and hexameric form of CHL

Equilibrium dialysis (E) or frontal affinity chromatography (F) were employed to measure apparent association constants (K_a) at 4 °C and the number of binding sites per monomer ($n-M_r$ 16000) or hexamer ($N-M_r$ 240000) of the chicken liver lectin. A description of the binding assays is given in the Experimental section. See Table 1 for abbreviations of oligosaccharides. Abbreviation: n.d., not determined.

Compound		subCHL		CHL	
		$10^{-6} \times K_a$ (l·mol ⁻¹)	n	$10^{-6} \times K_a$ (l·mol ⁻¹)	N
N(N)BH	(E)	0.041 ± 0.003 (5)	1.80 ± 0.12 (5)	0.047 ± 0.005 (5)	11.90 ± 0.26 (5)
	(F)	0.024 ± 0.002 (3)	n.d.	0.036 ± 0.003 (3)	n.d.
N ₂ BH	(E)	0.31 ± 0.02 (5)	0.88 ± 0.15 (5)	1.5 ± 0.1 (5)	5.78 ± 0.45 (5)
N ₃ BH	(E)	1.20 ± 0.05 (5)	1.05 ± 0.06 (5)	16.0 ± 0.92 (5)	4.91 ± 0.34 (5)
N ₃ (N)BH	(E)	1.40 ± 0.14 (5)	1.14 ± 0.06 (5)	25.0 ± 0.58 (5)	4.10 ± 0.56 (5)
N ₄ BH	(E)	3.60 ± 0.27 (4)	0.97 ± 0.21 (4)	84.0 ± 3.70 (4)	3.50 ± 0.24 (4)
N ₄ (N)BH	(E)	7.40 ± 0.14 (4)	1.23 ± 0.17 (4)	150.0 ± 4.80 (4)	3.01 ± 0.28 (4)
Ovomucoid	(F)	10.3 ± 0.25 (5)	n.d.	620.0 ± 17.3 (5)	n.d.

(3) Approximate values of apparent inhibition constants (I_{50} ; defined as the concentrations of inhibitor necessary for 50 % inhibition) for the highly branched oligosaccharides (tetra- and penta-antennary) are substantially higher than for the less-branched oligosaccharides. This non-linear increase in the inhibitory potency is observed only for the oligomeric form of the lectin (cf. Fig. 3a and Fig. 3b).

Quantitative evaluation of the binding data (Table 2) supported the conclusions of inhibition studies. The binding of mono-antennary ligand N(N)BH to both forms of the lectin was quite weak and allowed one to assess the total number of carbohydrate-combining sites, which is close to two for the monomeric fragment (or 12 for the CHL containing predominantly hexamers of the lectin). Increasing the number of terminal *N*-acetyl-D-glucosamine residues leads to a significant increase in the value of the association constant. The first notable increase occurs for the interaction of biantennary oligosaccharides with both forms of the lectin; the other remarkable increase is observed only for the oligomeric form of the lectin and concerns the interaction with highly branched tetra- or penta-antennary oligosaccharides. The value of the apparent association constant for the latter oligosaccharide approaches that found for the interaction of the whole glycoprotein.

DISCUSSION

The aim of the present study was to investigate the carbohydrate-binding site of CHL using defined oligosaccharides with terminal *N*-acetyl-D-glucosamine residues. Although the carbohydrate specificity for smaller ligands has been described (Sikder *et al.*, 1983), this is, as far as we know, the first time that such large series of structurally defined oligosaccharides have been applied. When compared with the related mammalian lectins, CHL provides a great advantage for this kind of study, since it has only one type of subunit. Moreover, the monomeric fragment of the lectin containing the carbohydrate-recognition domain can be prepared by mild proteolytic digestion (Chiacchia & Drickamer, 1984).

We have adopted ligand blotting [a technique that has recently become very popular for monitoring biological interactions (Drickamer, 1989)] to observe the binding of egg-white glycoproteins to both forms of the lectin. Interaction of various glycoproteins with subCHL was too weak to produce reproducible staining, as was the case of the interaction of ovalbumin, which has only one oligosaccharide per molecule. For highly glycosylated proteins, multiple interactions of several of their

carbohydrate moieties with the binding sites of the lectin have probably resulted in quite a sensitive detection.

The evaluation of the quantitative binding data revealed several unexpected findings. First, it seems that every carbohydrate-recognition domain of CHL has two monosaccharide-combining sites, a finding that is in accordance with those published by Lee & Lee (1988) for the rabbit and rat liver lectin. Moreover, the inhibition data obtained in our experiments seem to correspond quite well to those reported by Loeb & Drickamer (1988), who used different ligand (agalacto-orosomucoid). The existence of multiple carbohydrate-binding sites within the subunit of CHL can probably explain most of the co-operative effects observed when comparing uni- and multi-valent ligands (Kuhlen Schmidt *et al.*, 1984; Lee *et al.*, 1983). Another interesting finding is that the highly branched (tetra-antennary or penta-antennary) oligosaccharides can probably interact with several subunits of the lectin. Detailed study of this type of interaction can lead to important conclusions as to the flexibility of the individual subunits of the lectin.

In our previous study (Bezouška *et al.*, 1985a) we suggested that the striking increase in the value of the association constant for the interaction of the rat hepatic lectin in the series of monoantennary ligand – multiantennary ligand – whole glycoprotein is primarily mediated by the oligomeric structure of the lectin. Although this conclusion remains generally true, it is necessary to introduce a principal correction into this model owing to the existence of multivalent carbohydrate-recognition site within a single subunit of the lectin.

It seems that several features of the current molecular model suggested for the mammalian lectin receptor (Hardy *et al.*, 1985; Braiterman *et al.*, 1989; Herzig & Weigel, 1989) are also acceptable for CHL, including the hexameric arrangement in the plasma membrane and in solution. As is apparent from the results presented here, both the increase in oligosaccharide branching and the existence of preformed oligomers of the lectin contribute significantly to their highly specific interaction. Moreover, no principal differences could be seen in the values for apparent association constants of this interaction if measured with the highly branched oligosaccharides or glycopeptides as against the whole complex glycoproteins.

CHL and mammalian hepatic lectins are important examples of cell-surface receptors for endocytosis having a constitutive rate of recycling among various cellular compartments (Schwartz *et al.*, 1984). Therefore the functional role of the cross-linking of several carbohydrate-recognition domains within one hexameric molecule, or several such hexamers, by the interacting carbohydrate ligand, is unclear.

In spite of the enormous recent increase of our knowledge on the chemistry, biochemistry and molecular biology of vertebrate liver lectins, the question of the physiological importance of receptor-mediated endocytosis of glycoproteins has remained largely unanswered (Mellow *et al.*, 1988). It is interesting to note here that birds represent a very suitable model for such studies because of the existence of two main sites of glycoprotein biosynthesis in birds, resulting in completely different arrays of carbohydrate structures: the liver and the oviduct. Future studies on glycoprotein transport between these tissues and the role lectin receptors may play in it may thus reveal new physiologically important metabolic pathways.

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