Structure of heparin fragments with high affinity for lipoprotein lipase and inhibition of lipoprotein lipase binding to α_2 -macroglobulin-receptor/low-density-lipoprotein-receptor-related protein by heparin fragments

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Heparin-derived deca- and octa-saccharides were subjected to affinity chromatography on lipoprotein lipase-Sepharose and the fractions eluted at high salt concentration were analysed by strong-anion-exchange chromatography. Two high-affinity deca-saccharides were isolated and the structure determined by one- and two-dimensional ^1H -n.m.r. spectroscopy. The affinities of ^3H -labelled low-molecular-mass heparin and size-fractionated deca-, octa-, and hexa-saccharides for lipoprotein lipase immobilized on microtitre plates were determined from saturation curves. From competition experiments the affinities of unlabelled heparins and pure deca- and hexa-saccharide fragments were determined. The binding was size- and charge-dependent, but

structural dependency was also indicated. Thus substitution of a 2-*O*-sulphated L-iduronic acid with D-glucuronic acid was less important than the sulphation pattern of the D-glucosamine residue for affinity for lipoprotein lipase. Heparin inhibits binding of lipoprotein lipase to α_2 -macroglobulin-receptor/low-density-lipoprotein receptor-related protein. The effects of size, charge and structure for this inhibition were studied. The ability of the heparin fragments to inhibit binding correlated with their affinity for lipoprotein lipase. This indicates that the inhibition of the binding of lipoprotein lipase to α_2 -macroglobulin-receptor/low-density-lipoprotein receptor-related protein by heparin is exclusively mediated by binding of heparin to lipoprotein lipase.

INTRODUCTION

Lipoprotein lipase (LPL, triacylglycerol-protein acylhydrolase, EC 3.1.1.34) resides on the capillary walls of most tissues, where it is activated to hydrolyse triacylglycerols in chylomicrons and very-low-density lipoproteins (VLDL) circulating in the plasma [1,2]. The binding to the vessel wall is mediated through heparan sulphate proteoglycans on the endothelial cells. The active enzyme is a dimer of two identical 55 kDa glycoprotein subunits that loses hydrolytic activity upon dissociation [3]. Heparin and lipid seem to stabilize the dimeric form [1,4]. Injection of heparin displaces LPL from its heparan-sulphate-proteoglycan-binding sites on the endothelium and releases it into the circulation as heparin-LPL complexes [4], resulting in increased lipolysis of plasma lipoproteins [5]. Previous studies which have investigated the structural features required for the interaction of various glycosaminoglycans with LPL both *in vivo* and *in vitro* [6–11] have shown that the interaction is related to size and sulphation as well as to structure. Merchant et al. [9] have reported that decasaccharides were the minimal size of oligosaccharides which released substantial amounts of LPL in rabbits, whereas *in vitro* experiments demonstrated that an oligosaccharide mixture mainly consisting of octasaccharides were the smallest heparin fraction capable of displacing LPL from immobilized heparin [7].

In the primary structure of LPL [12], there are three clusters of basic residues which conform to the heparin-binding consensus

sequences proposed by Cardin and Weintraub [13] (X-B-B-X-B-X) and (-X-B-B-X-X-B-X) where B and X denote basic and hydrophobic residues respectively. Five basic residues in two of these sequences (R279, R280, R282, R296 and R297) have been shown to be critical for the high affinity for heparin [14].

The α_2 -macroglobulin receptor ($\alpha_2\text{MR}$)/low-density-lipoprotein-receptor-related protein (LRP) is a large (4526-amino acid) integral membrane glycoprotein present on a variety of cell types such as hepatocytes, macrophages, monocytes, fibroblasts and smooth-muscle cells [15–18]. Recent findings have demonstrated that, besides the binding and endocytosis of α_2 -macroglobulin-proteinase complexes [19], $\alpha_2\text{MR}$ /LRP binds, e.g., apolipoprotein E-enriched β -migrating VLDL (β -VLDL) [20] and LPL [21]. In addition, attachment of LPL to the receptor resulted in enhanced binding of chylomicrons and β -VLDL, i.e., LPL can mediate binding of lipoprotein to $\alpha_2\text{MR}$ /LRP. However, addition of heparin abolishes the binding of LPL to $\alpha_2\text{MR}$ /LRP [21,22].

The aim of the present study was, by using size-fractionated and radiolabelled oligosaccharides, to determine the affinity of oligosaccharides for LPL and, by competition experiments, to test the influence of size, charge density and structure of different heparin fragments on their affinity for LPL. For this purpose we used hexasaccharides (previously isolated and characterized [23]) and decasaccharide, which, by affinity chromatography on LPL-Sepharose, showed high affinity for LPL. The isolation and

Abbreviations used: LPL, lipoprotein lipase; VLDL, very-low-density lipoprotein; β -VLDL, β -migrating VLDL; $\alpha_2\text{MR}$, α_2 -macroglobulin receptor; LRP, low-density-lipoprotein-receptor-related protein; LMM, low molecular mass; std, standard; SAX, strong anion exchange; 1D, one-dimensional; ROESY, rotating-frame nuclear-Overhauser-enhancement spectroscopy; IdoA, α -L-idopyranosyluronic acid; GlcA, β -D-glucopyranosyluronic acid; UA, 4-deoxy- α -L-threo-hex-4-enopyranosyluronic acid; p, pyranose; S, sulphate; LA, low affinity; HA, high affinity.

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structural characterization by ^1H -n.m.r. of two deca-saccharide fragments are described. In addition, the structural requirements of heparin for inhibition of the binding of LPL to $\alpha_2\text{MR}$ /LRP were investigated by determination of the relative inhibitory effect of heparin fractions and the pure heparin fragments on the binding of ^{125}I - $\alpha_2\text{MR}$ /LRP to LPL.

MATERIALS AND METHODS

Heparin fractions

All heparins were prepared from sodium heparin or side fractions from the manufacture of tinzaparin sodium. These starting materials were obtained from Novo Nordisk A/S. Tinzaparin sodium, a low-molecular-mass (LMM) heparin, and the side-fraction were obtained by enzymic depolymerization of sodium heparin. The side fraction (batch 894701) was fractionated to size-homogeneous deca-, octa-, hexa- and tetra-saccharides by gel filtration on Sephadex G-50 essentially as described by Larnkjær et al. [23]. The preparation and structural characterization of four pure ($> 94\%$) hexasaccharide components (Hexa 1, Hexa 2, Hexa 3 and Hexa 4) by strong-anion-exchange (SAX) HPLC and ^1H -n.m.r. spectroscopy respectively have been described elsewhere [23]. Three tetrasaccharide components were isolated essentially as described by Linker and Hovingh [24], and their structures were established from ^1H -n.m.r. analyses. Deca-saccharide components (Deca 1, Deca 2 and Deca 45) were also obtained by SAX-HPLC. A deca-saccharide fraction (150 mg) was applied to a Mono Q HP 16/10 column (1.6 cm $\times$ 10 cm; Pharmacia, Uppsala, Sweden) and eluted at a flow rate of 2 ml/min with a linear gradient of NaCl (0.95–1.44 M) at pH 3.5 over a 150 min time period. The elution profile was monitored by UV detection at 235 nm. Fractions (4 ml each) were collected at each run and the two last-eluted major peaks (Deca 1 and Deca 2) and the peak eluted at 45 min (Deca 45) were pooled, freeze-dried, desalted on a Sephadex PD 10 column (Pharmacia) and freeze-dried. Deca 1 and Deca 2 were rechromatographed. The size-homogeneous fractions and the pure fragments were analysed by analytical SAX-HPLC using a Mono Q HP 5/5 column (0.5 cm $\times$ 5 cm; Pharmacia). A 50 μl (10 mg/ml) portion of octa- and deca-saccharides were injected and eluted with a complex NaCl gradient at pH 3.5 (Table 1). Hexasaccharides were analysed using gradients as described previously [23]. Catalytic reduction of the double bond of LMM heparin and the side fraction (batch 894701) with tritium gas in solution was performed by Amersham International. The crude [^3H]LMM heparin was purified and analysed for distribution of the radioactivity as previously described [25], and the specific radioactivity was 8.5 mCi/mg. The radiolabelled side fraction was fractionated

into ^3H -labelled deca-, octa-, hexa- and tetra-saccharide of specific radioactivity 0.61, 0.69, 0.96 and 1.84 mCi/mg respectively, by gel filtration using the same method as for the preparation of non-labelled size-fractionated oligosaccharides. The distribution of the radioactivity was determined by a radiochromatography analyser (model A-100; Packard). Molecular mass was determined by high-performance size-exclusion chromatography as described by Kristensen et al. [26], and molar concentrations were calculated by using molecular mass values of 10.6, 4.6, 3, 2.4, 1.8 and 1.2 kDa for standard (std) heparin, LMM heparin, deca-, octa- hexa- and tetra-saccharides, respectively.

^1H -n.m.r. spectroscopy

Each deca-saccharide was freeze-dried twice from $^2\text{H}_2\text{O}$. One- and two-dimensional (1D and 2D) ^1H -n.m.r. spectra were acquired using a 400 MHz Bruker AMX spectrometer. The experiments were performed at 320 K on samples with a concentration of 7 mM. Standard parameters were applied in the experiments. The residual $^1\text{H}^2\text{HO}$ signal in the spectra was suppressed through presaturation. All chemical shifts are reported relative to the signal from the unsaturated 4-proton, which was assigned to 6.00 p.p.m. relative to tetramethylsilane.

$\alpha_2\text{MR}$ /LRP

$\alpha_2\text{MR}$ /LRP was produced by ligand-affinity chromatography of solubilized placental membranes and iodinated to a specific activity of about 13.3 mCi/mg according to previously described methods [22].

LPL

LPL was purified from bovine milk [27], radiolabelled with ^{125}I (about 0.4 mCi/mg) and repurified as previously described [28].

Affinity chromatography

Deca- and octa-saccharides were fractionated on an affinity column of LPL, which was coupled to CNBr-activated Sepharose according to the method described by Liu et al. [29]. The gel was equilibrated with 0.1 M NaCl/0.01 M Tris/HCl, pH 7.4. Deca- or octa-saccharide (10–30 mg in 5 ml of equilibration buffer) was applied to the column at a flow rate of 1.0 ml/min. After washing the column with equilibration buffer until the A_{250} was negligible, the bound saccharides were eluted with equilibration buffer containing 2.0 M NaCl. Fractions (4 ml each) were collected.

Table 1 Optimized linear NaCl gradients at pH 3.5 for analytical SAX-HPLC of octa- and deca-saccharide

The flow rate was 1 ml/min.

Octasaccharide		Decasaccharide	
Time intervals for linear gradient (min)	Concn. (M) in time interval	Time (min)	Concn. (M)
0 $\rightarrow$ 30.0	0.72 $\rightarrow$ 0.98	0 $\rightarrow$ 30.0	1.02 $\rightarrow$ 1.05
30.0 $\rightarrow$ 80.0	0.98 $\rightarrow$ 1.11	30.0 $\rightarrow$ 55.0	1.05 $\rightarrow$ 1.18
80.0 $\rightarrow$ 90.0	1.11 $\rightarrow$ 1.18	55.0 $\rightarrow$ 100.0	1.18 $\rightarrow$ 1.24
90.0 $\rightarrow$ 105.0	1.18 $\rightarrow$ 1.37	100.0 $\rightarrow$ 115.0	1.24 $\rightarrow$ 1.50
105.0 $\rightarrow$ 110.0	1.37 $\rightarrow$ 1.50	115.0 $\rightarrow$ 120.0	1.50 $\rightarrow$ 1.50
110.0 $\rightarrow$ 120.0	1.50 $\rightarrow$ 1.50		

Heparin-LPL solid-phase binding assay

LPL (100 μ l; 5 μ g/ml) in 50 mM NaHCO₃, pH 9.6, were applied to microtitre wells (Polysorb; Nunc) and incubated for 2 h at room temperature. In some experiments, trace amounts of ¹²⁵I-LPL were added to quantify the amounts of LPL adsorbed to the wells (~500 fmol/well). After blocking with 10% BSA for 2 h at room temperature, the wells were washed with incubation buffer (140 mM NaCl/10 mM Hepes/2 mM CaCl₂/1 mM MgCl₂/1% BSA, pH 7.4). Increasing concentrations of radio-labelled LMM heparin and size-fractionated oligosaccharides were added to the wells. In a parallel assay, the non-specific background binding was determined as the non-displaceable binding when 100-fold molar excess of the corresponding unlabelled heparin, relative to the concentration of the [³H]heparin, was added. The final incubation volume was 200 μ l. The BSA used in the experiments does not bind heparin (results not shown). After incubation overnight at 4 °C the wells were washed three times with 200 μ l of incubation buffer. The bound [³H]-heparin was solubilized by the addition of two 100 μ l portions of 10% SDS, and the bound radioactivity was measured in a liquid-scintillation counter (model 1900 TR; Packard). Specific binding was determined by subtracting non-specific binding from total binding. For competition with various heparin fragments, LPL-coated wells were incubated overnight with [³H]LMM heparin (100 μ l; 40 ng/ml) in the presence of increasing concentrations (100 μ l) of each of the various heparins. Each separate experiment was repeated at least three times, and each result is expressed as the mean $\pm$ S.D. for quadruplicate samples. The binding data were analysed by the best non-linear curve fit [30] using the computer program Enzfitter from Elsevier Biosoft (Cambridge, U.K.). For competition experiments the concentration of the unlabelled heparin resulting in 50% displacement (IC₅₀) of the labelled compound was evaluated by fitting logistic functions [31] with a computer program.

Heparin inhibition of α_2 MR/LRP binding to LPL

To LPL-coated wells, 50 μ l of ¹²⁵I- α_2 MR/LRP (80 c.p.m./ μ l) and 50 μ l of increasing concentrations of each of the various heparins were added. The same procedures as for the binding and competition experiments were applied. The radioactivity was determined in a γ -radiation counter (model A5005; Packard).

RESULTS

Affinity and SAX chromatography

'Overloading' amounts of octa- and deca-saccharides were applied to the LPL-Sepharose column and the fractions eluting at low salt concentration (low affinity, LA) and high salt concentration (high affinity, HA) were analysed by analytical SAX-HPLC. Whereas the LA fractions and the size-fractionated mixtures showed essentially identical elution profiles, the HA fractions consisted mainly of the highly negatively charged components (Figure 1). To study the structure and the binding affinities of the HA fractions, milligram amounts of the components were required. Only the two major highly sulphated deca-saccharides (Deca 1 and Deca 2) were purified by semi-preparative SAX-HPLC. For comparison, a less-sulphated deca-saccharide fraction (peak eluted at 45 min; Deca 45) was also isolated. The components were analysed by analytical SAX-HPLC (Figure 2), and Deca 1 and Deca 2 were estimated to be approx. 95 and 90% pure respectively.

Figure 1 Characterization of LPL affinity-fractionated deca- and octa-saccharides by SAX-HPLC

The ordinate shows the absorbance at 235 nm. Decasaccharides (a) and octasaccharides (b) eluted at high salt concentration (high-affinity fractions) from an LPL-Sepharose column were analysed for charge heterogeneity by analytical SAX-HPLC using the complex NaCl gradients defined in Table 1. For comparison, the profiles of the deca- and octa-saccharide mixtures are included as well.

Structure of Deca 1 and Deca 2

The structure of the two deca-saccharide components was established by analyses of 1D and 2D ¹H-n.m.r. including TOCSY, COSY and ROESY (rotating-frame nuclear-Overhauser-enhancement spectroscopy). The assignments for the hexuronic acids residues and D-glucosamine residues are given in Tables 2 and 3 respectively.

The presence of a triplet at 3.40 p.p.m. in 1D spectra of Deca 1 is characteristic for the D-glucuronate H-2 proton [32], and the rest of the protons from the D-glucuronate residue were assigned by analyses of the COSY and TOCSY spectra. The protons of the non-reducing-end sugar were easily assigned from the COSY and TOCSY spectrum as they resonate at unique positions due to the double bond in the ring [32–34]. The chemical shift of H-2 protons (4.63 p.p.m.) was in accordance with O-sulphation [24,35,36]. The three remaining internal hexuronic acids were 2-O-sulphated L-iduronic acid residues [33]. All the protons could be assigned from examination of COSY and TOCSY spectra. On

Figure 2 Characterization of Deca 1, Deca 2 and Deca 45 derived from size-fractionated deca-saccharides

Overlay plot of the elution profiles of the three deca-saccharide fraction: Deca 1 (trace a), Deca 2 (trace b) and Deca 45 (trace c), prepared by semi-preparative SAX-HPLC, and the deca-saccharide mixture (trace d) from analytical SAX-HPLC using NaCl gradients as defined in Table 1.

Table 2 ^1H -n.m.r. assignments for the protons of the hexuronic acid in Deca 1 (D1) and Deca 2 (D2)

Chemical shifts (δ) in p.p.m. at 320 K are shown.

Residue	H-1	H-2	H-3	H-4	H-5
D1: ΔUA	5.52	4.63	4.32	6.00	
IdoA*	5.22	4.33	4.19	4.11	4.85
†	5.22	4.36	4.22	4.11	4.87
‡	5.22	4.36	4.24	4.11	4.87
GlcA	4.62	3.40	3.68	4.80	4.82
D2: ΔUA	5.51	4.62	4.31	6.00	
IdoA*	5.21	4.32	4.21	4.13	4.83
†	5.21	4.34	4.22	4.13	4.90
‡	5.21	4.34	4.24	4.13	4.90
§	5.21	4.34	4.24	4.13	4.90

* Assignments for residue preceding the reducing end D-glucosamine residue.

† Position within the deca-saccharide not determined.

the TOCSY spectra the characteristic chemical shifts are depicted (Figure 3). Protons from one of the L-iduronic acid residues resonated at more upfield positions, which indicated a position adjacent to the reducing end D-glucosamine residue [23], and it was confirmed from an inter-residual cross-peak in the ROESY spectrum between the H-5 (GlcN) and H-2 (IdoA) protons (4.15; 4.33 p.p.m.) (Figure 4). The anomeric protons of the D-glucosamine residues showed resonances between 5.40 and 5.58 p.p.m.. The cross-peaks showing connectivity to the H-2 resonances at ~ 3.30 p.p.m. established the N-sulphation of all the D-glucosamine residues [34,37]. All other protons of the D-glucosamine residues were identified from the COSY and TOCSY spectra by starting from their respective anomeric signals. The resonances of the H-6 protons (4.24–4.41 p.p.m.) were in accordance 6-O-sulphation of all D-glucosamine rings. As the D-

Table 3 ^1H -n.m.r. assignments for the protons of the glucosamine residue in Deca 1 (D1) and Deca 2 (D2)

Chemical shifts (δ) in p.p.m. at 320 K are shown.

Residue	H-1	H-2	H-3	H-4	H-5	H-6,H-6
D1: GlcN*	5.58	3.31	3.67	3.75	3.98	4.24, 4.39
†	5.46	3.28	3.68	3.73	4.15	4.33, 4.38
‡	5.42	3.28	3.75	3.72	4.02	4.28, 4.39
§	5.40	3.30	3.67	3.83	4.03	4.28, 4.41
	5.40	3.30	3.69	3.75	4.03	4.28, 4.39
D2: GlcN†	5.43	3.26	3.70	3.72	4.13	4.31, 4.35
	5.41	3.26	3.69	3.73	4.00	4.29, 4.39
¶	5.39	3.28	3.67	3.73	4.00	4.26, 4.37
	5.39	3.28	3.67	3.74	4.00	4.29, 4.37
§	5.39	3.28	3.67	3.84	4.01	4.23, 4.33

* Assignments for D-glucosamine residue preceding the glucuronic acid.

† Assignments for the reducing-end D-glucosamine residue.

‡ Assignment for D-glucosamine residue following the glucuronic acid.

§ Assignment for the D-glucosamine residue adjacent to the 4,5-unsaturated residue.

|| Assignment for the D-glucosamine residue preceding the iduronic acid adjacent the reducing end.

¶ Position within deca-saccharide not determined.

glucosamine resonances are sensitive to the neighbouring hexuronic acids as well as to position within the oligosaccharide, much information about the sequence could be deduced from the assignments. The anomeric proton resonance at 5.58 p.p.m. is shifted downfield compared with the anomeric protons of the 4,5-unsaturated ring, which normally has the most downfield anomeric resonance (5.52 p.p.m.). This is characteristic for the D-glucosamine residue preceding a D-glucuronic acid residue [32]. The D-glucosamine unit following the D-glucuronic acid residue was identified by the reversed relative position of the H-3 and H-4 resonance [32]. The reversed relative position of H-3 and H-4 protons was distinguished from cross-peaks with H-2 and H-5 protons respectively in the COSY spectrum. This corresponded to the residue with the anomeric proton at 5.42 p.p.m.. The reducing-end D-glucosamine was easily identified by the ROESY spectra as only an intra-residual cross-peak between H-1 and H-2 protons was observed for the anomeric proton [23]. Thus the anomeric proton at 5.46 p.p.m. corresponded to the reducing end D-glucosamine residue. The H-5 proton of the reducing end residue resonates downfield (4.15 p.p.m.) compared with the H-5 proton of internal D-glucosamine residues (4.02 p.p.m.). Except for the anomeric proton of the D-glucosamine residue preceding the D-glucuronate residue, the anomeric protons of the reducing-end residues were shifted downfield by about 0.05 p.p.m.. The H-4 proton of D-glucosamine adjacent to the 4,5-unsaturated ring showed a downfield shift (3.83 p.p.m.) compared with other D-glucosamine units (3.72–3.75 p.p.m.). The D-glucosamine preceding the L-iduronic acid adjacent to the reducing-end D-glucosamine residue was identified by a cross-peak between the H-1 (GlcN) and the H-5 (IdoA) at 5.42 and 4.85 p.p.m. This corresponded to the D-glucosamine residue following the D-glucuronic acid and thus the sequence was established:

In Deca 2 all the internal hexuronic acids were 2-O-sulphated iduronic acids residues revealed by the anomeric resonance at 5.21 p.p.m., simplifying the structure of Deca 2. In addition, the

Figure 3 400 MHz ^1H -n.m.r. 2D TOCSY spectrum of decasaccharide Deca 1

The spectrum was recorded in $^2\text{H}_2\text{O}$ at 320 K. The subspectra for resolved residues are indicated: A, ΔUA ; B, GlcN preceding GlcA; C, reducing-end GlcN; D (shoulder), GlcN following GlcA; E, GlcN; F, IdoA; G, IdoA adjacent to reducing end; H, GlcA; *, H-4 proton of GlcN adjacent to ΔUA .

chemical-shift values of glucosamine residues were in accordance with 2,6-*O*-sulphation. Thus Deca 2 was derived from the repeating disaccharide unit having the structure:

i.e. Deca 2 differs from Deca 1 by one sugar unit (IdoA2S and GlcA respectively) and hence by one sulphate group. The assignments of the protons of reducing-end glucosamine, the iduronic acid adjacent to the reducing end unit, the glucosamine preceding this iduronic acid and finally the glucosamine adjacent to the non-reducing end were established in a manner similar to that used to assign the protons of Deca 1.

Binding of heparin and heparin fragments to LPL

The effect of the chain length of heparin on its binding to LPL was investigated by binding of [^3H]LMM heparin, deca-, octa- and hexa-saccharides to LPL immobilized on microtitre plates. The binding was saturable and specific for all heparins, although the non-specific binding increased as the chain length decreased (Figure 5). It was not possible to obtain specific binding of [^3H]tetrasaccharides to the adsorbed LPL. The binding data were

analysed by the Enzfit program, and Scatchard plots of the saturation curves are shown as insets. The shorter oligosaccharides showed lower affinity, demonstrating a correlation between the saccharide chain length and its affinity for LPL. The K_D varied from 13.1 ± 0.3 nM for LMM heparin binding to LPL to 1.88 ± 1.3 μM for the binding of hexasaccharides. The K_D values of the octa- and deca-saccharide were 187 ± 25 nM and 53.0 ± 1.61 nM respectively. The effect of the structure and the degree of sulphation was studied by competing purified heparin components with [^3H]LMM heparin. The three decasaccharides, Deca 1, Deca 2 and Deca 45, and four hexasaccharides differing in structure and degree of sulphation, were tested. The hexasaccharide Hexa 1 was a heptasulphated saccharide having the structure:

Hexa 2 and Hexa 3 were both octasulphated with the structures:

Figure 4 400 MHz ^1H -n.m.r. 2D ROESY spectrum of decasaccharide Deca 1

The spectrum was recorded in $^2\text{H}_2\text{O}$ at 320 K. Characteristic cross-peaks are indicated: GlcN_R , reducing-end GlcN , IdoA_R , IdoA adjacent to reducing-end GlcN .

and

$\Delta\text{UAp}2\text{S}(1 \rightarrow 4)\text{-}\alpha\text{-D-GlcNp}2\text{S}6\text{S}(1 \rightarrow 4)\text{-}\alpha\text{-L-IdoAp}2\text{S}(1 \rightarrow 4)\text{-}\alpha\text{-D-GlcNp}2\text{S}6\text{S}(1 \rightarrow 4)\text{-}\beta\text{-D-GlcAp}(1 \rightarrow 4)\text{-}\alpha\text{-D-GlcNp}2\text{S}6\text{S}$

respectively; Hexa 4 contained nine sulphate groups having the structure:

$\Delta\text{UAp}2\text{S}(1 \rightarrow 4)\text{-}\alpha\text{-D-GlcNp}2\text{S}6\text{S}(1 \rightarrow 4)\text{-}\alpha\text{-L-IdoAp}2\text{S}(1 \rightarrow 4)\text{-}\alpha\text{-D-GlcNp}2\text{S}6\text{S}(1 \rightarrow 4)\text{-}\alpha\text{-L-IdoAp}2\text{S}(1 \rightarrow 4)\text{-}\alpha\text{-D-GlcNp}2\text{S}6\text{S}$ [23]

In addition, std heparin, LMM heparin and size-fractionated deca- and hexa-saccharide mixtures were tested for comparison (Figure 6). The competition was dose-dependent, and the shorter oligosaccharides competed at progressively higher concentration (Table 4). On a weight basis, LMM heparin and standard heparin were equally effective, but in molar concentration the LMM heparin was about 2–3 times less potent. The higher sulphated decasaccharides Deca 1 and Deca 2 were as effective competitors as the decasaccharide mixture ($\text{IC}_{50} \sim 0.17\text{--}0.32 \mu\text{M}$), whereas the less sulphated decasaccharide Deca 45 showed a 10-fold lower affinity ($\text{IC}_{50} = 2.1 \mu\text{M}$). Also Hexa 3 and Hexa 4 showed the same affinity as the hexasaccharide mixture

($\text{IC}_{50} = 13\text{--}27 \mu\text{M}$) components. However, Hexa 2 ($\text{IC}_{50} = 140 \mu\text{M}$) and Hexa 3 ($\text{IC}_{50} = 27 \mu\text{M}$), both octasulphated hexasaccharides, exhibited different affinities, indicating structural dependency as well. The tetrasaccharide fraction competed very poorly ($\text{IC}_{50} = 470 \mu\text{M}$) with the binding of $[^3\text{H}]\text{LMM}$ heparin.

Inhibition of LPL binding to $\alpha_2\text{MR/LRP}$ by heparin

Heparin inhibits the binding of LPL to $\alpha_2\text{MR/LRP}$ [21,22] and the effect of the chain length and degree of sulphation on the inhibition was studied. The inhibition was found to be strongly dependent on the molecular mass, i.e., std heparin ($\text{IC}_{50} = 0.0028 \mu\text{M}$) and LMM heparin ($\text{IC}_{50} = 0.0092 \mu\text{M}$) were about 100-fold and 20-fold more active than decasaccharides ($\text{IC}_{50} = 0.24 \mu\text{M}$) (Figure 7; Table 5) respectively. The degree of sulphation was important as well. The less sulphated decasaccharide Deca 45 was less effective ($\text{IC}_{50} = 1.5 \mu\text{M}$) than Deca 1, Deca 2 and the decasaccharide mixture, which all had an IC_{50} of $\sim 0.25 \mu\text{M}$. The octasaccharide mixture was as effective as the low-sulphated decasaccharide Deca 45, and the hexasaccharides were even less active, although a difference between the hexasac-

Figure 5 Dose-dependent binding of ^3H -labelled heparin fractions to LPL

Immobilized LPL (500 fmol/well) was incubated for 16 h at 4 °C with the indicated concentration of ^3H -labelled LMM heparin (a), deca- (b), octa- (c) and hexa- (d) saccharide. The specific binding (●) was determined as the total binding corrected for the non-specific binding (○) measured in the presence of excess unlabelled compound. The results presented are representative of those obtained in experiments reproduced three times, and each data point is expressed as the mean $\pm$ S.D. for quadruplicate samples. The inset shows the Scatchard analysis of experimental data performed by the Enzfitter data program.

charides was noticed. Hexasaccharide Hexa 2 was as effective as the least-sulphated hexasaccharide, Hexa 1, and about 5 times less active than Hexa 3, which contained the same amount of sulphate groups. Three tetrasaccharides, namely Tetra 1:

$\Delta\text{UAp}2\text{S}-(1 \rightarrow 4)-\alpha\text{-D-GlcNp}2\text{S}6\text{S}-(1 \rightarrow 4)-\alpha\text{-L-IdoAp}2\text{S}-(1 \rightarrow 4)-\alpha\text{-D-GlcNp}2\text{S}$

Tetra 2:

$\Delta\text{UAp}2\text{S}-(1 \rightarrow 4)-\alpha\text{-D-GlcNp}2\text{S}6\text{S}-(1 \rightarrow 4)-\beta\text{-D-GlcAp}-(1 \rightarrow 4)-\alpha\text{-D-GlcNp}2\text{S}6\text{S}$

and Tetra 3:

$\Delta\text{UAp}2\text{S}-(1 \rightarrow 4)-\alpha\text{-D-GlcNp}2\text{S}6\text{S}-(1 \rightarrow 4)-\alpha\text{-L-IdoAp}2\text{S}-(1 \rightarrow 4)-\alpha\text{-D-GlcNp}2\text{S}6\text{S}$

and the tetrasaccharide mixture were tested as inhibitors and the structural difference did not seem to be important at this small chain length. All tetrasaccharides were equally poor inhibitors,

requiring high doses for inhibition ($\text{IC}_{50} = 300\text{--}340 \mu\text{M}$). For clarity, in Figure 7 only the inhibition curves for the tetrasaccharide mixture and Tetra 1 are depicted.

DISCUSSION

Previous studies had demonstrated that the interaction between heparin and LPL is of an ionic nature and relatively non-specific (for a review, see [4]). Chromatography of size-homogeneous deca- and octa-saccharides on immobilized LPL afforded the fractions with the highest affinity for this enzyme. Analysis by analytical SAX-HPLC established that these fractions were the most sulphated components, confirming the ionic nature of the interaction. Two major decasaccharide fragments (Deca 1 and Deca 2) with high affinity for LPL were isolated by SAX-HPLC and the structure determined by one- and two-dimensional ^1H -n.m.r. analysis. The decasaccharides Deca 1, Deca 2 and Deca 45 represent 10, 3 and 10 mol% of the decasaccharide mixture.

Figure 6 Competition of heparin fractions with $[^3\text{H}]$ LMM heparin for binding to adsorbed LPL

Wells coated with 500 fmol of LPL were incubated with $[^3\text{H}]$ LMM heparin (7.1 nM) and increasing amounts of standard heparin, LMM heparin, decasaccharides (a) and hexa- and tetra-saccharides (b) for 16 h at 4 °C. Binding of $[^3\text{H}]$ LMM heparin in absence of unlabelled heparins (control) was set at 100%. Each point represents the mean value for quadruplicate samples. The largest S.D./mean $\times 100$ value is < 15%.

Table 4 Competition of $[^3\text{H}]$ LMM heparin (LMM-hep) binding to LPL by heparin fractions

The IC_{50} is the concentration of the heparin/oligosaccharide required to inhibit $[^3\text{H}]$ LMM heparin binding to 50% of that determined in the absence of competitors.

	Std.hep	LMM-hep	Deca	Deca 2	Deca 1	Deca 45
IC_{50} ($\mu\text{g/ml}$)	0.047	0.053	0.82	0.51	0.95	6.30
IC_{50} (μM)	0.0044	0.012	0.27	0.17	0.32	2.1
	Hexa	Hexa 4	Hexa 3	Hexa 2	Hexa 1	Tetra
IC_{50} ($\mu\text{g/ml}$)	26	24	49	250	160	560
IC_{50} (μM)	14	13	27	140	87	470

However, the trisulphated disaccharide repeating unit that makes up the Deca 2 saccharide is the most frequent disaccharide in unfractionated heparin and heparin fractions with higher molecular masses. The bond in this major disaccharide unit is the one preferably cleaved when heparin is depolymerized by heparinase [35]. The high-affinity decasaccharides Deca 1 and Deca 2 do not contain the antithrombin-binding sequence [38,39]. This is in accordance with previous findings demonstrating different binding sites for antithrombin III and LPL [40].

The binding affinity of ^3H -labelled heparin fractions and LMM heparin to immobilized LPL decreased continuously with decreasing molecular size. Previous studies, using oligosaccharide mixtures, showed that a mixture mainly consisting of octasaccharides was the smallest oligosaccharide capable of dissociating LPL from immobilized conventional heparin [7]. However, in the present study LPL could bind hexasaccharides in a saturable manner. The affinity ($K_D = 13.1$ nM) of $[^3\text{H}]$ LMM heparin for LPL is in agreement with the affinity ($K_D = 43$ nM) from studies of the association of LPL and heparin (molecular mass 6.6–8.0 kDa) in solution using the technique of fluorescence-

polarization spectroscopy [8]. The difference in affinity might be due to the higher salt concentration (0.2 M NaCl) in that compared with our experiment (0.14 M NaCl), a difference in the molecular-mass distribution of the LMM heparins or in the methods used (solid-phase assay and solution assay). In the same study, Clarke et al. [8] reported a heparin/LPL-dimer ratio of 1:1 for larger heparins (molecular mass > 10 kDa), i.e., simultaneous binding to both LPL subunits, whereas shorter heparins (molecular mass 6.6–8.0 kDa) formed 2:1 complexes. In the present study, ratios of about 2:1 and 1:1 were found. However, such stoichiometry calculations are very sensitive to estimation of errors regarding the amount of the LPL immobilized on the plates, determination of LPL concentration and the state of LPL (monomer-dimer), and hence more accurate determination of the stoichiometry was not possible. Experiments performed so far have shown that most of the LPL is inactive on the plates (results not shown). The effects of the size-homogeneous oligosaccharides on LPL release *in vivo* [40a] corresponded with these *in vitro* results. Both the affinity and the releasing effect decreased continuously with decreasing chain length.

The effect of structure and degree of sulphation of heparin or oligosaccharides on binding to LPL was investigated by competition of heparin oligosaccharides and pure heparin components with $[^3\text{H}]$ LMM heparin. In addition to the expected decrease in affinity with decreasing chain length, the affinity was dependent on the degree of sulphation. Deca 1 and Deca 2 exhibited the same affinity as the decasaccharide mixture. However, the less sulphated decasaccharide fraction (Deca 45) showed an approx. 10-fold lower affinity. Competition of hexasaccharide fragments also indicated a dependence of structure on the binding to LPL. Besides the observed dependency of content of sulphate groups, two hexasaccharides of similar charge differed in affinity. Hexa 2, which lacks a 6-*O*-sulphate group on the reducing-end glucosamine residue, showed lower affinity than Hexa 3, which contained a GlcA residue instead of an IdoA2S residue. On the other hand, Hexa 3 had the same affinity as the hexasaccharide derived from the repeating disaccharide unit, Hexa 4, containing one sulphate group more. This is in accordance with the finding that the two decasaccharides Deca 1 and Deca 2 exhibited the

Figure 7 Inhibition of ^{125}I - $\alpha_2\text{MR}$ /LRP binding to adsorbed LPL by heparin fractions: effect of concentration

Wells coated with 500 fmol of LPL were incubated with ^{125}I - $\alpha_2\text{MR}$ /LRP (4000 c.p.m./well) and increasing amounts of standard heparin, LMM heparin, deca-saccharides (a) and octa-, hexa- and tetra-saccharides (b) for 16 h at 4 °C. Binding of ^{125}I - $\alpha_2\text{MR}$ /LRP in the absence of unlabelled heparins (control) was set at 100%. Each point represents the mean value for triplicate samples. The largest S.D./mean $\times$ 100 value is < 16%.

Table 5 Inhibition of ^{125}I - $\alpha_2\text{MR}$ /LRP binding to LPL by heparin fractions

The IC_{50} is the concentration of the heparin/oligosaccharide required to inhibit ^{125}I - $\alpha_2\text{MR}$ /LRP binding to 50% of that determined in absence of inhibitors. Abbreviation: std hep, std heparin.

	std hep	LMW-hep	Deca	Deca 2	Deca 1	Deca 45	Octa
IC_{50} ($\mu\text{g/ml}$)	0.030	0.058	0.71	0.88	0.70	4.2	2.5
IC_{50} (μM)	0.0028	0.0092	0.24	0.29	0.23	1.5	1.0
	Hexa	Hexa 4	Hexa 3	Hexa 2	Hexa 1	Tetra	Tetra 1
IC_{50} ($\mu\text{g/ml}$)	20	25	30	120	140	360	380
IC_{50} (μM)	11	14	17	70	81	300	340

same affinity for LPL. Likewise Hexa 1, containing a GlcNAc6S residue and a non-sulphated L-iduronic acid, showed a slightly, but not significantly, higher affinity for LPL than Hexa 2. Thus this indicates that the substitution of a 2-O-sulphated L-iduronic acid with D-glucuronic acid is not as important as the sulphation pattern of the D-glucosamine residue for the affinity for LPL. Tetrasaccharides were capable of competing, but 10000-fold excess was required to obtain displacement of the labelled LMM heparin. The IC_{50} (12 nM) of the unlabelled LMM heparin was in accordance with the apparent K_D (13.1 nM) of the $[^3\text{H}]$ LMM heparin. The IC_{50} (4.4 nM) of the standard heparin was of the same order of magnitude as the K_D (6.1 nM) reported by Clarke et al. [8]. As the IC_{50} of the deca- and hexa-saccharide mixtures were determined relative to the binding of $[^3\text{H}]$ LMM heparin the IC_{50} values were 10–20-times higher than the apparent K_D measured by addition of $[^3\text{H}]$ Deca and Hexa fractions.

Previous studies have demonstrated that LPL may act as ligand for $\alpha_2\text{MR}$ /LRP and mediate or enhance the binding of lipoprotein particles to the receptor. This interaction is blocked by heparin [21,22]. The minimal chain length and the structural requirements of heparin to inhibit LPL binding to the receptor was investigated. The ability of oligosaccharides to block the

binding of ^{125}I - $\alpha_2\text{MR}$ /LRP to LPL correlated with the ability to compete with ^3H -LMM binding to adsorbed LPL, i.e., the same dependency of chain length and differential inhibitory effects of different hexasaccharides were observed. For each tested heparin fraction or heparin fragment the IC_{50} values calculated from the competition and inhibition experiments were of the same order of magnitude. Since $\alpha_2\text{MR}$ /LRP does not interact with heparin [41], this indicates that inhibition of the binding of LPL to $\alpha_2\text{MR}$ /LRP by heparin is exclusively mediated by binding of heparin to LPL, involving the same binding sites as those used for attachment of LPL for cell surfaces. The binding of heparin to LPL might cause a steric hindrance for LPLs binding to the receptor or it might induce a change in conformation or in the electrostatic nature of LPL that reduces the affinity for the receptor.

LPL is cleared by the liver, in part by heparin-sensitive sites [42,43]. Studies have demonstrated that shorter heparins (LMM heparin and oligosaccharides) have reduced ability to prevent clearance of LPL by the liver, but the same releasing effect of LPL from extrahepatic tissue [10,29] as unfractionated heparin. This implies a difference between the binding sites in the liver that mediate uptake and degradation of LPL and the extrahepatic

endothelial binding sites. The inhibition of binding of LPL to α_2 MR/LRP was size-dependent, but the inhibitory effect of the oligosaccharides and heparin fractions corresponded very well to the affinity for LPL measured from competition experiments. This indicates that other heparin-sensitive receptors contribute to the hepatic clearance of LPL in addition to α_2 MR/LRP.

α_2 MR/LRP might play an important role in the development of foam cells in atherosclerotic lesions. Immunocytochemical analysis of cell composition of lesions of young adults demonstrated that the predominant cell type was smooth-muscle cells [44]. Recent findings have shown that the only lipoprotein receptor expressed in lesion smooth-muscle cells *in vivo* is α_2 MR/LRP [45] and LPL is expressed in subsets of macrophages and smooth-muscle cells in atherosclerotic lesion [46]. Thus the LPL-mediated binding and uptake of lipoprotein via the α_2 MR/LRP may contribute to atherogenesis. The inhibition of the binding of LPL to α_2 MR/LRP by heparin indicates one way in which heparin can affect the development of atherogenesis. The present study has demonstrated that the interaction is charge- and size-dependent. Although the position of the sulphate groups seems to play a role, no detailed structure required for LPL binding could be identified.

We thank Lene Hastrup and Lene Lund Clausen for excellent technical assistance, and Wita Davidsen for secretarial assistance. We also wish to thank Dr. Svend Hoime Hansen and Professor Jørgen Gliemann for their critical review of the manuscript before its submission. Anni Larnkjær is a recipient of a Fellowship from The Academy of Technical Sciences in Denmark.

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