Interaction of Lipoprotein Lipase with Heparin Fragments and with Heparan Sulfate: Stoichiometry, Stabilization, and Kinetics†

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ABSTRACT: The interaction of lipoprotein lipase (LPL) with heparan sulfate and with size-fractionated fragments of heparin was characterized by several approaches (stabilization, sedimentation, surface plasmon resonance, circular dichroism, fluorescence). The results show that heparin decasaccharides form a 1:1 complex with dimeric LPL and that decasaccharides are the shortest heparin fragments which can completely satisfy the heparin binding regions in dimeric LPL. Equimolar concentrations of octasaccharides also stabilized dimeric LPL, while shorter fragments (hexa- and tetrasaccharides) were less efficient. Binding of heparin did not induce major rearrangements in the conformation of LPL, supporting the view that the heparin binding region is preformed in the native structure. Interaction of LPL with heparan sulfate, as studied by surface plasmon resonance, was found to be a fast exchange process characterized by a high value for the association rate constant, 1.7×10^8 M^{-1} s^{-1}, a relatively high dissociation rate constant, 0.05 s^{-1}, and as a result a very low equilibrium dissociation constant equal to 0.3 nM at 0.15 M NaCl. The contribution of electrostatics was estimated to be 44% for the binding of LPL to heparan sulfate, 49% for the binding of LPL to unfractionated heparin, and 60% for the binding of LPL to affinity-purified heparin decasaccharides at 0.15 M NaCl. The number of ionic interactions between LPL and high-affinity decasaccharides was estimated to be 10. We propose an essential role of electrostatic steering in the association. Monomeric LPL had 6000-fold lower affinity for heparin than dimeric LPL had, expressed as a ratio of equilibrium dissociation constants. A model for binding of LPL to heparan sulfate-covered surfaces is proposed. Due to the fast rebinding, LPL is concentrated to the close proximity of the heparan sulfate surface. As the dissociation is also fast, the enzyme exchanges rapidly between specific binding sites on the immobilized heparan sulfate, without leaving the surface. This model may also apply to LPL at the endothelium of blood vessels.

Interaction of lipoprotein lipase (LPL)[1] with cell surface heparan sulfate proteoglycans is functionally important (Olivecrona & Bengtsson-Olivecrona, 1989). While attached to the vascular endothelium via interaction with heparan sulfate, LPL binds circulating lipoproteins and hydrolyzes their triglycerides and some of their phospholipids (Deckelbaum et al., 1992). Interaction with heparan sulfate also appears essential for the nonenzymatic function of LPL to mediate uptake of lipoproteins in cells by receptor-mediated endocytosis (Mulder et al., 1992; Eisenberg et al., 1992).

Heparan sulfate and heparin are glycosaminoglycans which consist of disaccharide units of uronic acid and D-glucosamine (Fransson, 1989). Heparan sulfate is found on cell surfaces and in the extracellular matrix covalently bound to core proteins (Ruoslahti, 1995). Heparin is synthesized in mast cells, is not bound to cell surfaces, and is more sulfated than heparan sulfate (Fransson, 1989). There are approximately 2−3 sulfate groups per disaccharide unit in heparin compared to 0.2−2 in heparan sulfate. Heparan sulfate is probably the physiologically relevant ligand for LPL, while the interaction with heparin is pharmacologically interesting due to its frequent use in antitrombotic therapy.

Active LPL is a metastable dimer of noncovalently bound, identical subunits (Osborne et al., 1985). Dissociation of the dimers into monomers leads to irreversible inactivation of the enzyme (Osborne et al., 1985). Heparin protects LPL from inactivation (Bengtsson-Olivecrona & Olivecrona, 1985). The LPL monomer has lower affinity for heparin and elutes at a lower salt concentration from heparin−Sepharose columns (Bengtsson-Olivecrona & Olivecrona, 1985). Modeling of the structure of LPL according to the X-ray structure of the evolutionary related pancreatic lipase (van Tilbeurgh et al., 1994) and also studies of LPL by site-directed mutagenesis (Ma et al., 1994; Hata et al., 1993) suggest that the putative heparin binding region in LPL consists of four spatially distinct clusters of positively charged amino acid residues.

The concentration of active LPL in blood is very low, 1.4 ± 0.9 milliunits/mL, corresponding to 4.2 ng/mL or 0.039 nM (Tornvall et al., 1995). Intravenous injection of heparin releases LPL from the endothelium and increases the plasma level more than 100-fold (Olivecrona & Bengtsson-

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[1] Abbreviations: LPL, lipoprotein lipase; NHS, *N*-hydroxysuccinimide; EDC; *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide; SDS, sodium dodecyl sulfate; LMW heparin, low molecular weight heparin; CD, circular dichroism.

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Olivecrona, 1989). The smallest heparin fragments which are able to efficiently release LPL into the circulation are octasaccharides (Liu et al., 1991, 1992). The blood concentration of inactive LPL monomers is higher, about 100 ng/mL (Tornvall et al., 1995). This concentration is not much influenced by the injection of free heparin (Vilella et al., 1993). LPL in blood is not in a true thermodynamic equilibrium with LPL bound to the endothelium due to avid uptake and degradation of LPL by the liver (Wallinder et al., 1984).

Recent studies have focused on isolation of heparin-like fragments which have the most optimal structure for interaction with LPL (Parthasarathy et al., 1994; Larnkjær et al., 1995). The affinity increases with increasing chain length and degree of sulfation (Larnkjær et al., 1995). Clarke et al. (1983) showed, by studies of fluorescence anisotropy, that LPL forms a 1:1 complex with heparin chains which have molecular weights above 10 000, whereas for shorter heparin fragments the stoichiometry to LPL is 2:1.

The aim of the present work was to study the kinetics and mechanism of the LPL−heparin interaction. For this, we have used stability assays, gradient ultracentrifugation, circular dichroism, fluorescence, and plasmon-based biosensor technology. Our data result in much higher affinity constants for heparin than that previously reported by Clarke et al. (1983) and a 1:1 stoichiometry with dimeric LPL even for decasaccharides. Our data show that LPL is concentrated to the close proximity of the heparan sulfate layer because of a fast association rate.

MATERIALS AND METHODS

Materials. LPL was purified from bovine milk as described (Bengtsson-Olivecrona & Olivecrona, 1991). Monomeric LPL was prepared by treatment with 1 M guanidine hydrochloride (Osborne et al., 1985), followed by purification on heparin−Sepharose (Bengtsson-Olivecrona & Olivecrona, 1985). Unfractionated heparin and low molecular weight (LMW) heparin were obtained from Novo Nordisk A/S. Size-fractionated tetra-, hexa-, octa-, and decasaccharides as well their tritium-labeled derivatives were obtained by gel filtration of an enzymatic depolymerization mixture on a Sephadex G-50 column (Larnkjær et al., 1995). Deca 1 (ΔUA2S-(1→4)-[α-D-GlcNS6S(1→4)-α-L-IdoA2S(1→4)]$_2$-α-D-GlcNS6S(1→4)-β-D-GlcA(1→4)-α-D-GlcNS6S(1→4)-α-L-IdoA2S(1→4)-α-D-GlcNS6S), Deca 2 (ΔUA2S(1→4)-[α-D-GlcNS6S(1→4)-α-L-IdoA2S(1→4)]$_4$-α-D-GlcNS6S), and Deca 45 (mixture of less sulfated decasaccharides) were purified as described by Larnkjær et al. (1995). Endothelial heparan sulfate was a kind gift from Prof. Lars-Åke Fransson, Department of Physiological Chemistry, University of Lund, Sweden. An amino-coupling kit containing *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide (EDC), and 1 M ethanolamine and CM5 sensorchips were obtained from Pharmacia Biosensor. Streptavidin and inositol hexasulfate were purchased from Sigma.

Enzyme Characterization. LPL activity was determined using assay systems with tributyrin and Intralipid (Lookene & Bengtsson-Olivecrona, 1993). Protein was determined by the bicinchoninic acid assay (Pierce) and by the absorbance at 280 nm.

Stability Studies. LPL (0.1 μM) was incubated alone or in the presence of different amounts of heparin fragments in 20 mM Tris, 0.15 M NaCl, pH 7.4 at 37 °C. At certain time points, aliquots of the mixtures were withdrawn for activity measurements in the tributyrin system.

Sedimentation Studies. Ultracentrifugation was carried out as previously described (Lookene & Bengtsson-Olivecrona, 1993). Linear sucrose gradients from 10% to 5% (by mass) in 20 mM Tris, 0.3 M NaCl, pH 7.4, were made in polypropylene tubes. The total volume was 4.8 mL. LPL was incubated with ^{3}H-labeled heparin fragments in 20 mM Tris, 0.3 M NaCl, pH 7.4 at 10 °C, for 10 min. The mixtures (0.1 mL) were then layered on top of sucrose gradients. Centrifugation was performed for 18 h at 10 °C in a Beckman ultracentrifuge (L5-65B) using an SWTi 50.1 rotor. After the run, the tubes were emptied through puncture with a syringe needle, and fractions of 0.25 mL were collected from the bottom. LPL distribution was determined by measurements of catalytic activity and of protein. The distribution of the heparin fragments was determined by radioactivity measurements.

Circular Dichroism and Fluorescence Studies. Circular dichroism (CD) measurements were carried out using a JASCO J-700 spectropolarimeter and cuvettes with 2 mm light paths. Spectra were base line corrected and also corrected for dilution during the titrations. Octasaccharide spectra were subtracted from the corresponding LPL/octasaccharide mixture spectra. The temperature was 10 °C, and it was controlled by a digital control device.

Fluorescence measurements were performed on a Shimadzu spectrofluorophotometer Model RF 500. Emission spectra of the fluorescence of tryptophans upon excitation at 280 nm were determined between 300 and 450 nm. The enzyme concentration was 3 μM, and octasaccharide was added up to 100 μM. The measurements were performed in 20 mM Hepes, 0.3 M NaCl at 10 °C.

Surface Plasmon Resonance Analysis. All measurements were performed using a BIAcore system (Pharmacia, Biosensor) using CM sensorchips. Heparin and heparan sulfate were biotinylated via their amino groups (Lee & Conrad, 1984). A solution of 5−10 mg/mL heparin or heparan sulfate in 0.2 M $NaCO_3$, pH 8.6, was incubated with a 10-fold molar excess of sulfo-NHS-biotin (Pierce) for 3 h at room temperature. The excess of nonreacted biotin was removed on a NAP-5 column (Pharmacia).

Immobilization of streptavidin to sensorchip CM5 was performed by methodology described previously (Johnsson et al., 1991). The surface was activated by injecting 30 μL of a mixture of EDC/NHS (0.2 M/0.05 M) at a flow rate of 5 μL/min. Then streptavidin (0.1 mg/mL in 10 mM acetate, pH 4.5) was injected. Nonreacted activated groups were blocked by injection of 30 μL of 1 M ethanolamine, pH 8.5. Biotin-labeled heparin or heparan sulfate was then coupled to the surface which had previously been derivatized by streptavidin.

Measurements of binding kinetics were performed in 20 mM Hepes, 3.4 mM EDTA, pH 7.4, at different concentrations of NaCl. In some cases, the running buffer contained also 1 mg of bovine serum albumin/mL or 0.1% (by mass) Triton X-100. Stock solutions of LPL in 10 mM Bistris, 1.1 M NaCl were diluted directly in the running buffer just before injection. For kinetic measurements at salt concentrations higher than 0.4 M NaCl, the stock solution of LPL was first dialyzed against the running buffer.

The surfaces of the sensorchips were in all cases regenerated by injection of 1.5 M NaCl or 0.1% (by mass) SDS. The chips were used for about 4 weeks in repetitive experiments.

Determination of Kinetic Parameters. (a) Calculation of Rate Constants. An integrated form of the rate equation was used for analysis of the association phase (O'Shannessy et al., 1993):

$$\Delta R = R_t - R_o = \frac{Lk_{ass}\Delta R_{max}[1 - e^{-(Lk_{ass}+k_{diss})t}]}{Lk_{ass} + k_{diss}} \quad (1)$$

where R_t is the signal observed during the association phase, R_t is proportional to the concentration of LPL at the surface, R_o defines the baseline (response at $t = 0$), ΔR_{max} is the capacity of the immobilized heparin to bind LPL expressed in response units, L is the concentration of LPL in the injected solution, and k_{ass} and k_{diss} are association and dissociation rate constants, respectively.

The dissociation phases were analyzed by the equation:

$$R_t = R_a e^{-k_{diss}t} + R_{(t\to\infty)} \quad (2)$$

where R_t is the response at time t in the dissociation phase, $R_{(t\to\infty)}$ is the response value after complete dissociation, and R_a is the response at $t = 0$. Because of bulk effects, the first 5 s of the association phase and of the dissociation phase was not used for calculations.

(b) Determination of Diffusion Coefficient (D). The diffusion coefficient (D) for LPL was calculated from the equation (Karlsson et al., 1993):

$$J = (1.47)Lb(Dl)^{2/3}(u/h)^{1/3} \quad (3)$$

where J is the flux of mass to the surface (mol·s^{-1}), b, l, and h are the width (0.5 mm), length (2.1 mm), and height (0.05 mm) of the flow cell, respectively, u is the linear flow rate, and L is the concentration of LPL.

(c) Determination of Equilibrium Dissociation Constants (K_d) for Size-Fractionated Heparin Fragments by Competition Studies. K_d values for the nonimmobilized heparin fragments were calculated using the equation:

$$\Delta R = R_0 - R_e = 0.5r(L + H + K_d - \sqrt{(L + H + K_d)^2 - 4LH}) \quad (4)$$

where ΔR is the difference between steady-state response values in the absence (R_0) and in the presence (R_e) of competing heparin fragments, L is the concentration of LPL, H is the concentration of competing fragment, and r is a constant. When $H \gg L$, a simplified equation was used:

$$\Delta R = R_0 - R_e = \frac{rLH}{K_d + H} \quad (5)$$

Data Analysis. The data were analyzed by nonlinear regression using the FIG.P program (Biosoft, Cambridge, U.K.) and the SAAM program (Resource Facility Analysis, University of Washington, Seattle).

RESULTS

Stabilization of LPL with Heparin Fragments of Different Lengths. It was previously shown that binding of heparin to LPL protects the enzyme from inactivation (Bengtsson-Olivecrona & Olivecrona, 1985). Here we used the stabilizing effect to determine the minimal size of a heparin fragment which was able to interact with LPL (Figure 1). In the absence of heparin, LPL lost most of its activity (>95%) when incubated for 5 min at 37 °C in a buffer containing 0.15 M NaCl (Figure 1A). Considerable stabilization, although not complete, was obtained by standard heparin. More than 80% of the enzyme activity remained after 20 min and about 50% remained after 100 min when the enzyme was incubated with 6.7 μg/mL or with 335 μg/mL standard heparin (Figure 1A). Thus, a 50-fold increase in heparin concentration did not cause higher stabilization. With decasaccharides, a molar ratio of 1:1 (decasaccharide/LPL) stabilized the enzyme to a level comparable to the maximal stabilization obtained by standard heparin (Figure 1B). Addition of a 100-fold excess of decasaccharide did not lead to further stabilization, indicating that already at a molar ratio of 1:1 most LPL molecules were complexed to decasaccharides.

Also with octasaccharides maximal stabilization was obtained already at an equimolar ratio (1:1), and the time-courses for inactivation were similar to those in the presence of decasaccharides or standard heparin (Figure 1C). These data show that octa- and decasaccharides form 1:1 complexes with dimeric LPL. A rough estimate from the inactivation curves suggested that at least 90% of the enzyme was complexed in the equimolar mixtures, assuming that the enzyme was fully stable in the complex. Thus, the values for the dissociation constants of the complexes must be much lower than the concentrations of heparin fragments used here ($\ll$100 nM).

Tetrasaccharides and hexasaccharides were less efficient in stabilizing the enzyme (Figure 1D,E). Higher concentrations of these fragments were needed, and the levels of remaining activity were lower than those obtained with octa- or decasaccharides. In equimolar solutions of LPL and tetra- or hexasaccharides, the activity of the enzyme decreased only moderately slower than in the absence of heparin fragments. Fragment concentrations corresponding to a severalfold molar excess over LPL enhanced the stability. This stabilization was saturable similarly to what was obtained for the longer fragments. This effect might be nonspecific since inositol hexasulfate at a high molar excess was able to stabilize the enzyme to a similar degree (Figure 1F).

Determination of Stoichiometry of the LPL/Decasaccharide Complex by Density Gradient Ultracentrifugation. Direct binding studies were performed by sedimentation in sucrose density gradients. LPL was mixed with radiolabeled heparin fragments, and the mixture was subjected to ultracentrifugation to separate the complexes from free fragments (Figure 2). The LPL/decasaccharide complex could be sufficiently well separated from unbound decasaccharide. After centrifugation of mixtures of decasaccharides and LPL at initial molar ratios of 10:1 and 160:1, respectively, molar ratios of 1:0.81 and 1:0.95 of decasaccharide to LPL were found for the sedimented complexes. These results directly demonstrated that complexes of one decasaccharide molecule per LPL dimer existed in the mixture and that these complexes were sufficiently stable to allow isolation. During the centrifugation, some LPL protein was lost, probably due to formation of inactive aggregates which sedimented to the bottom of the tubes. However, the specific activity of the enzyme at the position of dimeric LPL was as high as for

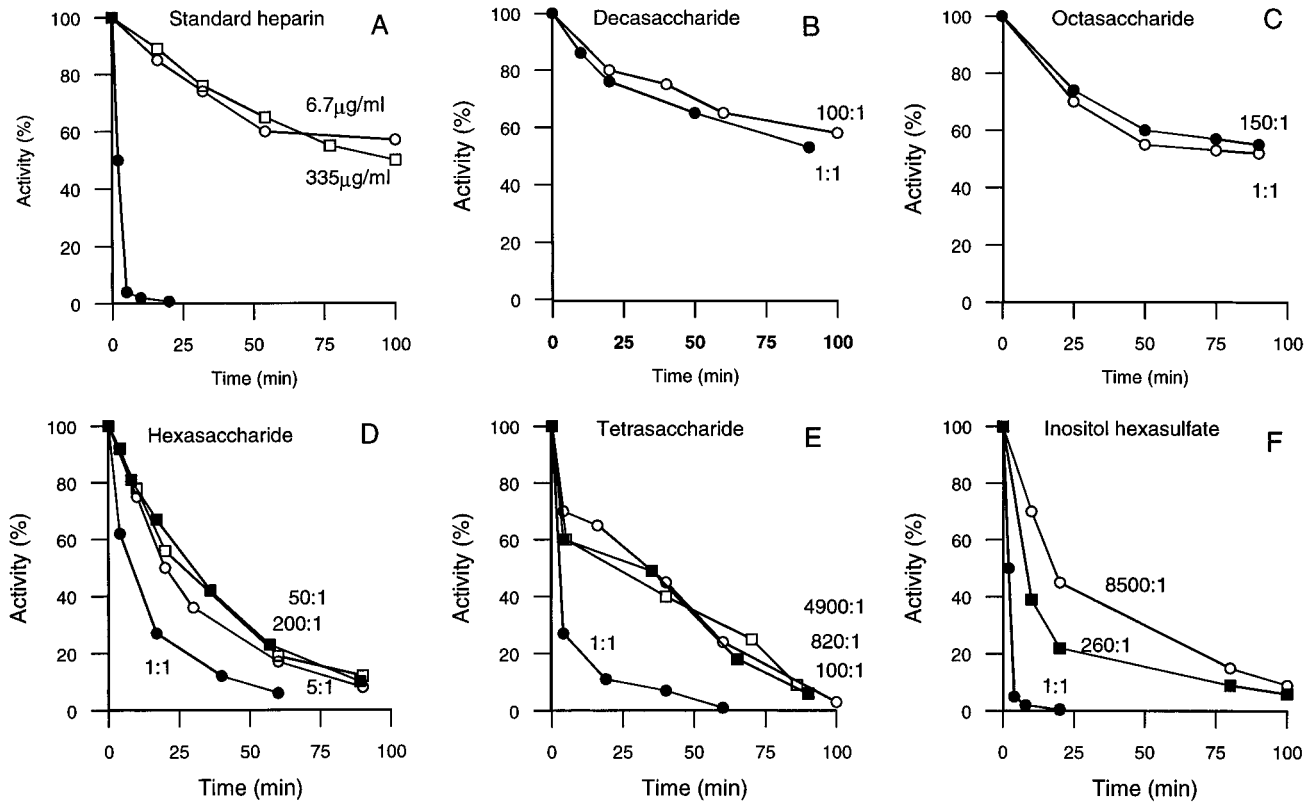

FIGURE 1: Time-dependent inactivation of LPL: effect of heparin oligosaccharides. LPL (0.1 μM) was incubated in 20 mM Tris, 0.15 M NaCl, pH 7.4 at 37 °C. At the times indicated, aliquots of the reaction mixtures were withdrawn for activity measurements. The LPL activities are expressed as percent remaining of the initial activity at time 0. (A) Inactivation of LPL alone (●) and in the presence of standard heparin, 6.7 μg/mL (○) and 335 μg/mL (□). (B) Effect of a 1:1 (●) and of a 100:1 (○) molar excess of decasaccharides over LPL. (C) Effect of a 1:1 (●) and of a 150:1 (○) molar excess of octasaccharides over LPL. (D) Effect of a 1:1 (●), a 5:1 (○), a 50:1 (■), and a 200:1 (□) molar excess of hexasaccharides. (E) Effect of a 1:1 (●), 100:1 (○), a 820:1 (■), and a 4900:1 (□) molar excess of tetrasaccharides. (F) Effect of a 1:1 (●), a 260:1 (■), and of a 8500:1 (○) molar excess of inositol hexasulfate over LPL.

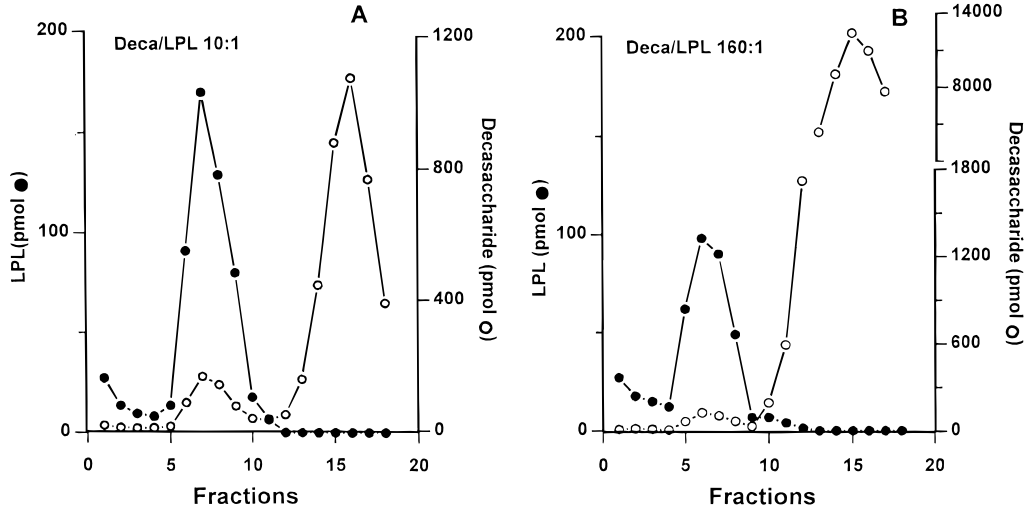

FIGURE 2: Separation of LPL−decasaccharide complexes from free decasaccharide by ultracentrifugation in sucrose density gradients. Gradients of 5−10% sucrose (by mass) were prepared as described under Materials and Methods. Samples of 0.1 mL containing LPL and radiolabeled decasaccharides were loaded on top of the gradients, and the tubes were then subjected to ultracentrifugation for 18 h at 45 000 rpm at 10 °C. (A) 1.2 nmol of LPL was mixed with 12 nmol of decasaccharides. (B) 1.2 nmol LPL was mixed with 192 nmol of decasaccharides. After the centrifugation, fractions (0.25 mL) were collected from the bottom of the tubes, and LPL was determined by assays of activity and protein. The data presented in the figure are calculated for 0.1 mL. The concentration of decasaccharides was determined by radioactivity measurements. The values presented in the figure are mean values of determinations performed in triplicate. The LPL: decasaccharide ratios in the sedimented complexes were 0.81 in (A) and 0.95 in (B).

fully active LPL. In contrast to the case with decasaccharide, LPL lost most of its specific activity during sedimentation experiments with tetra-, hexa-, or octasaccharides. Therefore, stoichiometries for these complexes could not be determined.

Determination of Equilibrium Constants. We used surface plasmon resonance for quantitative characterization of the interaction between LPL and heparin. In our experiments, biotinylated heparan sulfate was attached to streptavidin

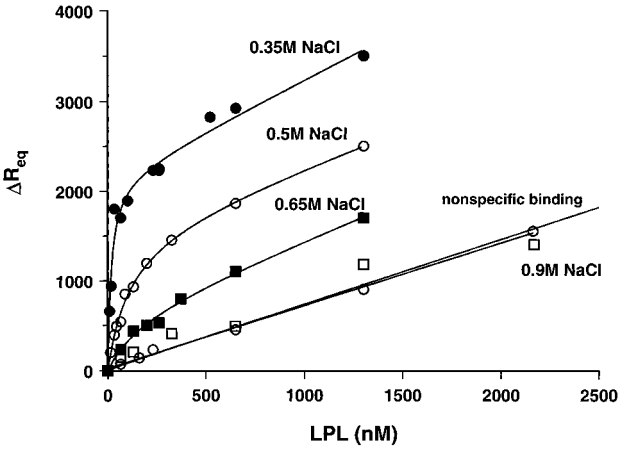

FIGURE 3: Binding of LPL to heparan sulfate at different salt concentrations as studied by surface plasmon resonance. Heparan sulfate was covalently immobilized to the dextran matrix of a sensorchip as described under Materials and Methods. LPL was injected over the heparan sulfate in 10 mM Hepes buffer, pH 7.4, containing 3.4 mM EDTA and NaCl at the concentrations shown in the figure. ΔR_{eq} is response in response units (R) at steady-state. The curves were fitted using eq 6.

which was covalently bound to the dextran matrix on sensorchips. Solutions containing LPL were injected over the heparan sulfate layer, and binding of LPL was registered as an increase in response units (ΔR). The observed change in ΔR is directly proportional to the amount of protein on the surface (Stenberg et al., 1991). The steady-state values of ΔR were taken as equilibrium values, ΔR_{eq}. Figure 3 shows plots of ΔR_{eq} values versus concentration of injected LPL at different salt concentrations. For a simple binding reaction, this dependency should follow a saturable hyperbole. In our case, the ΔR_{eq} changed much at low concentrations of LPL, but less and almost linearly at higher concentrations. Control experiments showed that LPL bound nonspecifically to the streptavidin-coated layer in the absence of heparan sulfate. This binding increased proportionally to the LPL concentration, and it was not dependent on salt concentration (Figure 3). Taking the nonspecific binding into account, the data were fitted using the equation:

$$\Delta R_{eq} = \frac{\Delta R_{max} L}{K_d + L} + bL \qquad (6)$$

where L is the concentration of LPL in the injected solution, K_d is the equilibrium dissociation constant for the specific binding, b is a proportionality constant representing the nonspecific binding, and ΔR_{max} is the maximal capacity for specific binding expressed in response units. The slope calculated for nonspecific binding, $b = 0.70$, was close to the slopes for the linear parts (0.77−1.0) of the binding curves for heparan sulfate-covered layers. The calculated K_d increased with increasing concentrations of NaCl (Table 1). The decrease in the binding affinity was paralleled by a corresponding decrease in ΔR_{max} from 2150 at 0.35 M NaCl to 650 at 0.65 M NaCl. At 0.9 M NaCl, binding to the heparan sulfate-covered layer was not significantly different from the nonspecific binding. When heparin was immobilized to the sensorchip instead of heparan sulfate, binding of LPL was similar, but the absolute values for the K_d values were about 50-fold lower than with heparan sulfate-covered layers (data not shown).

Table 1: Equilibrium and Rate Constants for the LPL/Heparan Sulfate Interaction at Different Salt Concentrations

[NaCl] (M)	K_d[a] (nM)	k_{diss}[b] (s^{-1})	k_{ass}[c] $\times 10^{-8}$ (M^{-1} s^{-1})
0.15	0.30 ± 0.05	0.05 ± 0.01	1.70 ± 0.45
0.22	−	0.07 ± 0.01	−
0.35	17.0 ± 4.0	0.20 ± 0.03	1.25 ± 0.34
0.40	−	0.43 ± 0.06	−
0.50	121 ± 17	0.57 ± 0.23	0.60 ± 0.09
0.65	229 ± 98	>0.5	−

[a] K_d is the equilibrium constant calculated according to eq 6. [b] k_{diss} is the dissociation rate constant determined in the presence of 1 μM LMW heparin. [c] k_{ass} is the association rate constant calculated from the equation $k_{ass} = k_{diss}/K_d$.

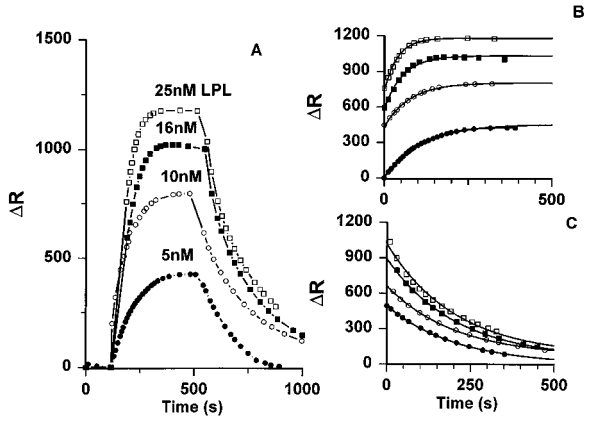

FIGURE 4: Illustration of how binding data were used for calculation of rate constants for the interaction between LPL and immobilized heparan sulfate. Panel A shows raw data obtained from binding experiments with different concentrations of LPL as indicated. Panels B and C show fitted curves for association and dissociation phases, respectively. The association and dissociation curves were simultaneously fitted using eqs 1 and 2 described under Materials and Methods. The experiments were performed in 10 mM Hepes, pH 7.4, with 3.4 mM EDTA and 0.35 M NaCl at 25 °C.

Determination of Association and Dissociation Rate Constants. An advantage with the surface plasmon technique is the possibility to analyze separately on- and off-phases of the interaction. The on-rate (association rate) was determined as rate of increase of ΔR during injection of LPL solution over the layer. The off-rate (dissociation rate) was determined during wash out of bound enzyme by a flow of buffer without LPL. The apparent association rate constants and the apparent dissociation rate constants were calculated by simultaneous fitting of dissociation and association phases using eqs 1 and 2 as described under Materials and Methods. An example of the curve fitting is shown in Figure 4. The plots of $\Delta R/\Delta t$ versus R for the association phases were nearly linear at low concentrations of LPL. The interaction was apparently characterized by a fast association rate constant, 10^6 M^{-1} s^{-1}, which was not dependent on salt concentration. The dissociation rate increased by increasing the concentration of NaCl. At concentrations higher than 0.3 M, it followed a single-exponential decay. At concentrations lower than 0.3 M, the dissociation was more accurately described by a two-exponential decay. This could be explained by a possible heterogeneity of the heparan sulfate chains or by rebinding after the dissociation (Karlsson et al., 1991). To test the latter hypothesis, free heparin fragments were injected into the system during the dissociation phase. This tremendously increased the dissociation rate and led to close to complete dissociation of the bound enzyme (Figure

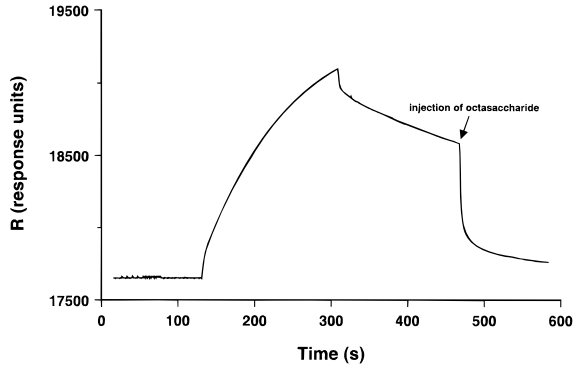

FIGURE 5: Effect of injection of free octasaccharides on the dissociation of LPL from immobilized heparan sulfate shown as a sensogram. First, 30 μL of a solution of LPL (5 nM) in 10 mM Hepes, 0.15 M NaCl, pH 7.4, was injected over heparan sulfate immobilized to a sensorchip. The flow rate was 5 μL/min at 25 °C. At 300 s, the bulk-flow was changed to buffer without LPL, and the dissociation of LPL from the chip was followed. In the dissociation phase, a solution of 1.6 μM octasaccharides in the same buffer was injected. The starting point is indicated by the arrow. Note the marked increase in dissociation rate caused by the free ligand.

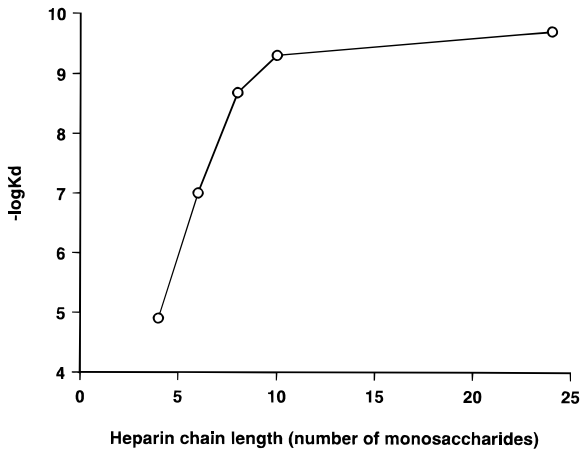

FIGURE 6: Dependency of the dissociation constants (K_d) for the LPL−oligosaccharide interaction on heparin chain lengths. Dissociation constants were determined by competition with oligosaccharides of different chain lengths for binding of LPL to immobilized heparan sulfate in the BIAcore using eqs 4 and 5 described under Materials and Methods. The experiments were performed in 10 mM Hepes, 3.4 mM EDTA, pH 7.4 at 25 °C, in the presence of 0.15 M NaCl. The heparin chain lengths are expressed in numbers of monosaccharide units.

5). Thus, the true dissociation rate is much faster than the apparent rate due to significant rebinding of LPL in systems with a bulk-flow of buffer only. An increase of the flow rate from 5 μL/min to 500 μL/min did not influence the dissociation rate. The true dissociation rate constants (k_{diss}) for the interaction between LPL and heparan sulfate were calculated from data obtained in the presence of a high concentration (1 μM) of low molecular weight heparin (LMW heparin) (Table 1). Control experiments showed that a further increase of the concentration of LMW heparin did not cause any appreciable increase in the dissociation rate constants. The dissociation rate constants were enhanced by factor of 100 in the presence of LMW heparin. Size-fractionated heparin fragments (tetra-, hexa-, octa-, and decasaccharides) also increased the LPL dissociation rate to a similar level, but higher concentrations were needed of the fragments than of LMW heparin (see Figure 5 for the effect of octasaccharide). The true association rate constants, k_{ass}, which characterize direct interaction between LPL and heparan sulfate chains, were calculated from the equation $k_{ass} = k_{diss}/K_d$, where the k_{diss} values were determined in the presence of competing LMW heparin and the K_d values were determined from the equilibrium data as described above. The calculated k_{ass} values at different salt concentrations are shown in Table 1. Monomeric, catalytically inactive, LPL had an association rate constant about 1000 times lower than dimeric LPL for binding to heparan sulfate (data not shown).

The diffusion coefficient for LPL was determined at a very high heparan sulfate surface concentration ($\Delta R_{max} = 4900$) where the rate of binding of LPL was linear with respect to time for at least 20 s. The experimental values for the diffusion coefficients, 1.6×10^{-11} to 2.0×10^{-11} m^2/s, were about 3 times lower than those estimated from the molecular mass of the enzyme.

Determination of K_d for the Size-Fractionated Heparins. Specific binding of LPL to heparan sulfate-coated surfaces was blocked if the enzyme was preincubated with heparin fragments. In contrast, nonspecific binding was not influenced by the presence of free heparin fragments. For determination of equilibrium dissociation constants (K_d), the concentration of LPL was kept constant, while the concentrations of heparin fragments were varied. The steady-state level of bound LPL, expressed in R_{eq}, decreased gradually by increasing the fragment concentration. At 0.15 M NaCl, octa- and decasaccharides blocked LPL binding already at concentrations equal to the enzyme concentration (2 nM). Much higher concentrations of hexa- and tetrasaccharides were needed for complete blocking. Assuming that R_{eq} was proportional to the concentration of free LPL in the injection mixture, we determined the K_d values for each heparin fragment. The calculations were performed according to eq 4, when the concentration of heparin fragment was comparable to the LPL concentration, and according to eq 5, when the fragment concentration was much higher than the LPL concentration. There was a tremendous change in the K_d values from tetrasaccharide to decasaccharide, while decasaccharide had only 4.5-fold higher K_d than LMW heparin (a mean of 24 monomers) (Figure 6).

Table 2: Equilibrium Dissociation Constants for Dimeric and Monomeric LPL with Isolated Decasaccharide Fragments

	Dec 1[a]	Dec 2[a]	Dec 45[a]
LPL dimer	0.80 ± 0.05 nM	0.62 ± 0.06 nM	7.60 ± 0.80 nM
LPL monomer	−	3.61 ± 0.30 μM	−

[a] Dec 1, Dec 2, and Dec 45 were isolated as previously described by ionic exchange chromatography (Larnkjær et al., 1995). The K_d values were calculated from competition studies with immobilized heparan sulfate at 0.15 M NaCl.

The highly sulfated heparin fragments Dec 1 and Dec 2 had about 10-fold lower K_d than the less sulfated Dec 45 (Table 2). Monomeric LPL had 6000-fold lower affinity for Dec 2 than the dimer had (Table 2).

Effect of Salts on the Interaction. According to the protein−polyelectrolyte theory (Manning, 1978), the binding of LPL to heparin should be an ion-exchange-type process involving release of bound sodium ions from the heparin polyelectrolyte chain. Mathematically, the effect of the concentration of a monovalent cation on the protein−

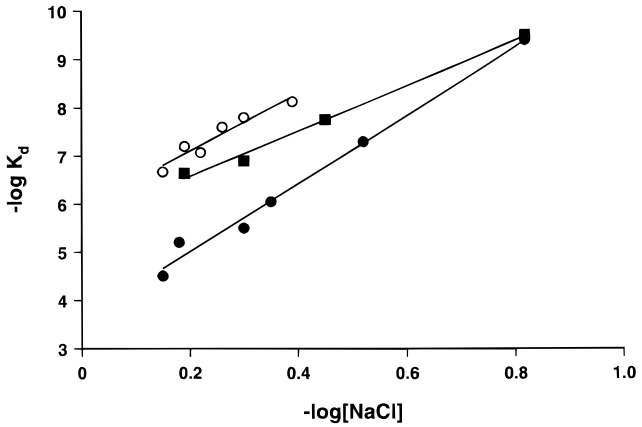

FIGURE 7: Dependency on the salt concentration of the K_d values for the interaction of LPL with Dec 2, standard heparin, and heparan sulfate. The K_d values for the LPL−Dec 2 interaction (●) were calculated from competition studies with immobilized heparan sulfate. The K_d values for binding of LPL to unfractionated heparin (○) and to heparan sulfate (■) were from direct binding studies of LPL to immobilized unfractionated heparin or heparan sulfate, respectively.

polyelectrolyte dissociation constant K_d should follow the equation:

$$\log K_d = \log K_{(0)} - Z\psi \log [M^+] \qquad (7)$$

where $[M^+]$ is the concentration of monovalent ion, $K_{(0)}$ reflects the contribution of nonelectrostatic interaction to the binding, $\log K_d = \log K_{(0)}$ when $[M^+] = 1$ M, Z represents the number of ionic interactions, and ψ is the axial charge density of heparin [for heparin, $\psi = 0.7$ (Olson et al., 1991)]. Linear dependencies of $\log K_d$ on $\log [Na^+]$ for the interaction of LPL with Dec 2, with unfractionated heparin, and with heparan sulfate were obtained (Figure 7) which had slopes equal to 7.1, 6.0, and 4.7, respectively. The almost equal values for Dec 1 and for unfractionated heparin indicated that a similar number of ionic interactions were involved in their binding to LPL (9−10). The lower value for heparan sulfate is most likely caused by a lower axial charge density. Extrapolation of the lines to [NaCl] = 1 M gives the values for $K_{(0)}$, which should represent the nonelectrostatic component of the interactions. The free energy change for the nonelectrostatic interaction can be expressed as $\Delta G_{(0)} = RT \ln K_{(0)}$. The contribution of electrostatics to the binding at 0.15 M NaCl was defined as $(\Delta G - \Delta G_{(0)})/\Delta G$, where ΔG is the change of free energy at 0.15 M NaCl. The electrostatic contribution was calculated to 60% for binding of LPL to Dec 2, 49% for binding of LPL to unfractionated heparin, and 44% for binding of LPL to heparan sulfate.

Effect of Heparin Fragments on the Circular Dichroism and the Fluorescence Spectra of LPL. To study possible effects of heparin on the conformation of LPL dimers, circular dichroism and fluorescence measurements were performed. No significant differences in the circular dichroism spectra of LPL were detected by addition of up to a 1000-fold molar excess of octasaccharides over LPL. The intrinsic tryptophan fluorescence emission spectrum of LPL was also not affected by the addition of heparin fragments under the same conditions as those used for studies of circular dichroism (data not shown).

DISCUSSION

In the present study, we find that heparin fragments with $M_w = 2400-3000$ bind to LPL dimers in a 1:1 ratio. This is not consistent with the results published by Clarke et al. (1983), who reported that heparin fragments with $M_w = 6000-8000$ bind with a stoichiometry of 2:1 to LPL and that only longer heparin fragments ($M_w = 10\,000-18\,400$) bind in a 1:1 ratio.

The discrepancy between the two studies might be due to differences in the experimental conditions. The salt concentration in the previous study was 0.05 M, which is lower than what we used. At low salt concentrations, LPL tends to dissociate into inactive monomers, leading finally to formation of irreversible aggregates (Osborne et al., 1985). In the study by Clarke et al. (1983), there are no data on LPL activity. It is possible that mostly inactive forms of the enzyme were studied.

For analysis of the stabilization of LPL by heparin fragments, we use the two-step inactivation mechanism presented by Osborne et al. (1985). According to this mechanism, the LPL dimers are in a rapid and reversible equilibrium with monomers, which are prone to irreversible changes in conformation. Sufficiently long heparin chains might bind simultaneously to both monomers in the dimer and prevent its dissociation. Since a molar ratio of 1:1 of octa- and decasaccharides to LPL dimers was sufficient for maximal stabilization, these fragments must be long enough to bind both monomers at the same time. A high molar excess of tetra- and hexasaccharides was needed for stabilization, and the maximal stabilization was lower than for the longer fragments. A similar stabilization was obtained by inositol hexasulfate, demonstrating that this effect was rather nonspecific. The results suggest that tetra- and hexasaccharides are not long enough to cover the heparin binding regions in dimeric LPL.

The heparin chain presents itself as a linear lattice which contains overlaping binding sites for LPL. According to the linear lattice model (McGhee et al., 1974), the dissociation constant, K_d, is determined by the ratio of the intrinsic site dissociation constant, $K_{d(0)}$, to the total number of LPL binding sites per heparin chain, $N - N_0 + 1$:

$$K_d = \frac{K_{d(0)}}{N - N_0 + 1} \qquad (8)$$

where N is the number of repeating disaccharide units on a heparin chain and N_0 represents the size of the LPL binding site expressed in number of disaccharide units. By this model, the possibility that the LPL binding site contains less than 10 monosaccharides ($N_0 = 5$) is excluded, because in cases of smaller binding sites the difference between the K_d values cannot be so large as was experimentally determined. If we assume that $N_0 = 5$, then the theoretical value of K_d for LMW heparin (12 disaccharide units) can be calculated by dividing the $K_d = 0.9$ nM for decasaccharide by 6 [$12 - (5 + 1) = 6$]. The result is 0.15 nM, and this is comparable with the experimentally determined K_d (0.2 nM). A similar dependency of the K_d values on heparin chain length was demonstrated by binding experiments on microtiter plates (Larnkjær et al., 1995).

Our results show that LPL has very high affinity for heparan sulfate and for heparin. The K_d values were

remarkably lower than those previously presented (Clarke et al., 1983; Larnkjær et al., 1995). For LMW heparin, a K_d = 0.2 nM was determined here, compared to the previously reported values of 13 nM (Larnkjær et al., 1995) and 43 nM (Clarke et al., 1983). These differences might be due to differences in binding of heparin to immobilized LPL (Larnkjær et al., 1995) or to possible inactivation of LPL in the previous studies. We found here that the K_d for inactive LPL monomers was 6000-fold higher than for active dimers.

The surface plasmon resonance-based technique offered a possibility to study the interaction of LPL with a surface covered with heparan sulfate. This is a functionally important interaction, since this is presumably how the enzyme is bound to the endothelium of the blood vessels. Our data suggest that binding of LPL to a heparan sulfate-covered surface is diffusion-controlled. The experimentally determined diffusion coefficients were, however, about 3 times lower than the one calculated from the molecular weight of LPL dimers (5.2×10^{-11} m^2/s) and than that determined by sedimentation equilibrium centrifugation (4.9×10^{-11} m^2/s) (Iverius & Östlund-Lindqvist, 1976). Since the high concentrations of negative charges on heparan sulfate have strong effects on the structure and viscosity of the surrounding water, it is likely that the environment close to the surface has different properties than the bulk-flow phase (Nieduszynski, 1989). The decreased diffusion coefficient of LPL could be due to hindered penetration of the enzyme to the surface phase.

The direct interaction between LPL and heparan sulfate chains is characterized by very high values for association rate constants, 10^7-10^8 M^{-1} s^{-1}. High values for association rate constants are typical when electrostatics contribute to the interaction (Northrup & Erickson, 1992). As electrostatic forces have long-range effects, they can maneuver the interacting molecules into orientations favorable for binding, a phenomenon known as electrostatic steering (Kozack et al., 1995). The dependency of K_d on the concentration of NaCl (Manning, 1978) was used to calculate the contribution of electrostatics. We found that 44% of the binding energy for the interaction between LPL and heparan sulfate is determined by electrostatics. For the highly sulfated decasaccharide Dec 2, the contribution of electrostatics was 60%. This expected and essential contribution of electrostatics to the interaction may explain the high association rate.

The interaction was also characterized by a fast dissociation rate of LPL from the heparan sulfate chains (0.05 s^{-1} at 0.15 M NaCl) in the presence of free heparin. Fast association and fast dissociation suggest that there is a rapid exchange of LPL molecules between binding sites in the layer without release to the bulk phase (Figure 8). When the number of heparan sulfate chains on the surface is high enough, it is more likely for the dissociated enzyme to bind to another heparan sulfate chain than to leave the surface for the bulk-flow phase. Fast rebinding leads to condensation of the enzyme to the surface. According to Glaser (1995), rebinding is characterized by an increase of dissociation at a higher flow rate and by an increase of dissociation by injection of the free ligand during the dissociation phase. In our case, only the second requirement was fulfilled. The reason why the dissociation was not influenced by the flow rate needs further investigations.

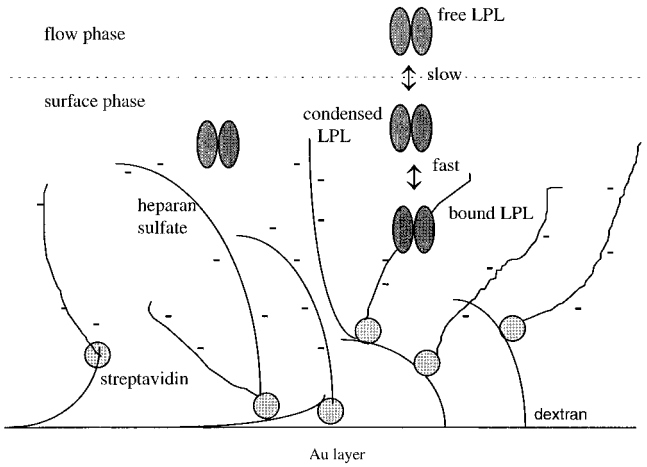

FIGURE 8: Schematic model for interaction of LPL with immobilized heparan sulfate. The negatively charged biotinylated heparan sulfate chains are bound to streptavidin, which is covalently attached to the dextran layer of the sensorchip. The bulk-flow of buffer is from left to right. The accumulation of negative charges on the surface organizes the water close to the surface to a "surface phase" with special properties. The distribution of LPL dimers (dark ovals) between the bulk-flow phase and the "surface phase" is rate-limiting. This is indicated by the double arrow marked "slow". The enzyme is condensed into the surface phase due to avid rebinding. The condenced LPL is in rapid equilibrium with binding sites on individual heparan sulfate chains on the surface (indicated by the double arrow marked "rapid"). Thus, there exist three pools of LPL in the system: free LPL, condensed LPL, and bound LPL.

The model can be applied to LPL on the vascular endothelium *in vivo*. The concentration of LPL in the circulating blood is very low. This is caused partly by a rapid clearance of the enzyme by the liver (Wallinder et al., 1984), and also by an avid reassociation of free enzyme to the endothelium (Chajek-Shaul et al., 1988). The several hundredfold increase in the level of free enzyme after intravenous injection of heparin demonstrates that there must be a large pool of LPL ready to leave the vascular endothelium and that the apparent dissociation of LPL under normal conditions, without free heparin, is a slow process. The rapid increase of the dissociation after injection of heparin fragments in the BIAcore experiments is in good accordance with the experiences *in vivo*.

Analysis of the dependency of the LPL−Dec 2 interaction on the NaCl concentration revealed that there were about 10 ionic interactions between the dimeric LPL and the decasaccharide. This means five ionic interactions per LPL monomer. Four different clusters of positively charged amino acid residues have been proposed to contribute to the heparin binding of LPL (van Tilbeurgh et al., 1994). The total number of positively charged amino acids in those clusters is 15, which is higher than the number of ionic interactions calculated from the salt dependency. It is likely that not all of these are involved in the interaction with heparin. The fourth cluster is situated in the outermost carboxy-terminal end. This part can be cleaved off by chymotrypsin, forming a truncated form of the lipase (Lookene & Bengtsson-Olivecrona, 1993). This form has the same affinity for heparin as intact LPL dimer, as judged from its interaction with heparin−Sepharose (Lookene & Bengtsson-Olivecrona, 1993). Thus, this cluster may not be directly involved in the binding. These and other positively charged residues in excess might, however, be involved in creation of the total electrostatic potential, which should be

important for the initial attraction of LPL to negative surfaces. There are 20 negatively charged groups in the heparin fragment Dec 2 (Larnkjær et al., 1995). Thus, only half of them appear to be directly involved in the interaction.

Circular dichroism and fluorescence measurements (data not shown) revealed that no major conformational changes occurred in LPL on binding to heparin. This was expected, since heparin has no effects on the catalytic function of the enzyme (Olivecrona & Bengtsson-Olivecrona, 1989). In fact, binding to heparin-like ligands should be fully compatible with all other functions of the enzyme, since this is the way LPL is attached on the vessel walls.

In conclusion, our data strongly support the view that heparin fragments longer than octasaccharides can span binding sites on both subunits in the LPL dimer. The interaction of LPL with surface-bound heparan sulfate is a rapid exchange process which leads to accumulation of the enzyme close to the surface. The much higher affinity of the LPL dimer compared with the LPL monomer for heparan sulfate explains why mainly LPL dimers are found on the vascular endothelium.

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