Heparin Binding to Human Plasma Low-Density Lipoproteins: Dependence on Heparin Sulfation Degree and Chain Length

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ABSTRACT: Binding between low-density lipoproteins (LDL) and fluorescein-labeled heparin was studied quantitatively with a modified form of a published procedure [Cardin, A. D., Randall, C. I., Hirose, N., & Jackson, R. L. (1987) *Biochemistry 26*, 5513-5518], using fluorescence anisotropy titrations. Assumption of binding site equivalence satisfactorily interpreted experimental data. Accordingly, the apparent total capacity, n, and the average dissociation constant, K_d, were estimated as $n \approx 24$ disaccharides per LDL particle and $K_d \approx 4$ μM in 0.05 M HEPES/0.1 M NaCl, pH 7.4, 22 °C. Competition experiments with unlabeled heparins were exploited for the quantitative study of K_d as a function of heparin chain length and sulfation degree (n_s = sulfate groups per disaccharide). The former parameter was investigated with a series of bovine lung heparin fractions with M_w ranging from 1800 to 21 000 and constant sulfation degree ($n_s = 2.8 \pm 0.1$). A series of physically fractionated or chemically modified heparins having $1.2 < n_s < 3.5$ were used to explore the dependence on sulfation degree. LDL affinity was found to increase with increasing both n_s and M_w: an empirical $M_w^{-1.6}$ dependence represented very well the chain length data set; a linear dependence was observed for log K_d as a function of n_s, after appropriate allowance was made for chain length differences among samples. This regularity confirmed that LDL-heparin binding is mainly driven by electrostatic forces. Consistently, the K_d dependence on heparin chain length was analyzed in terms of a linear lattice binding model requiring about 10 disaccharides to fit each independent binding site on apolipoprotein B-100, the protein component of each LDL particle. Furthermore, this model implies an approximately 2-fold increase of LDL binding capacity with respect to the estimate of the equivalent site model, as a consequence of a statistical site exclusion effect. When this correction was applied, each LDL particle was found to contain 4-5 heparin binding sites, each capable of binding about 10 disaccharidic units. Together with the steep dependence of the dissociation constant on salt concentration ($d \log K_d/d[NaCl] = 8.2 \pm 0.3$), this result indicates that about 40-50 positively charged residues in apoB-100 are involved in heparin binding.

Binding of low-density lipoprotein (LDL)[1] particles, the main carriers of cholesterol in the bloodstream, to proteoglycans on the arterial walls is thought to be one of the early events in the formation of atherosclerotic plaque (Berenson, 1984). Heparin, a sulfated glycosaminoglycan of widespread clinical usage for its anticoagulant and antithrombotic activity, effectively competes with LDL uptake by arterial tissue (Srinivasan, 1986) and is also claimed to possess antiatherosclerotic properties (Engelberg, 1990). These observations have motivated several studies of the interaction between LDL and heparin or, more generally, sulfated glycosaminoglycans. By means of various physicochemical methods, the formation of soluble or insoluble (mostly in the presence of divalent metal ions) LDL complexes with glycosaminoglycans has been well documented by these studies, and heparin is reported to be one of the most effective in this regard (Iverius, 1972; Srinivasan et al., 1975; Nakashima et al., 1975; Pan et al., 1978; Bihari-Varga et al., 1983; Cardin et al., 1984, 1987; Hirose et al., 1986; Wegrowski et al., 1990).

In fact, heparin is known to bind several proteins either from plasma, or from cell membranes, or from the extracellular matrix (Lane, 1988). Most of these interactions are supported by electrostatic forces originating from the highly negative charge density of this polyanion. However, interpretation of heparin affinity data for a given protein in structural terms is made difficult by the heterogeneity of native heparin samples, composed of molecules with different chain lengths and variable sulfation patterns. The most common structures of the constituent disaccharidic unit of heparin are represented in Figure 1. For more details, the reader is referred to a recent review (Casu, 1989). In one case, i.e., the formation of a complex between antithrombin III and heparin, a well-defined pentasaccharidic sequence is recognized by the protein with a high level of specificity (Choay et al., 1981; Casu et al., 1981; Thunberg et al., 1982). More generally, low specificity is observed, and clusters of basic amino acid residues constitute the heparin recognition site in the protein. This appears to be the case of LDL, where Lys- and Arg-rich fragments of the constituent protein, apolipoprotein B-100, have been recognized as responsible for heparin affinity (Weisgraber & Rall, 1987; Hirose et al., 1987).

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[1] Abbreviations: LDL, low-density lipoprotein(s); apoB-100, apolipoprotein B-100; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; RH, reference heparin; FRH, fluoresceinated reference heparin; M_w, weight-average molecular weight; M_n, number-average molecular weight; HPLC, high-performance liquid chromatography.

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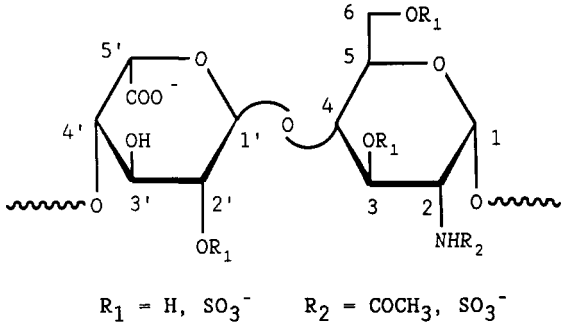

FIGURE 1: Structure of the predominant disaccharidic unit in heparin. This polysaccharide is constituted by a variously sulfated, repeating sequence of an acidic sugar (uronic acid) linked through a 1,4-glycosidic bond to glucosamine. The uronic acid is most frequently the iduronic residue (shown), rarely a glucuronic residue (not shown).

In an attempt to elucidate the structural parameters which are most relevant to the LDL–heparin interaction, recent studies have focused on the isolation of high LDL affinity fractions from native heparin samples (Cardin et al., 1987, 1989; Srinivasan et al., 1991). These investigations have not succeeded at identifying a well-defined, highly specific molecular structure, but have indicated a general trend of increasing affinity with increasing heparin chain length and sulfation degree. With a complementary approach, we decided to prepare heparin samples of known sulfation degree and molecular weight and then quantitatively determine the effect of these two factors on the LDL–heparin dissociation constant.

The fluorescence anisotropy change of a fluoresceinated reference heparin (FRH) was used to monitor the LDL–heparin binding equilibrium. This effect is a consequence of the hydrodynamic mobility difference between free and LDL-bound heparin (Lakowitz, 1983). Previous studies (Cardin et al., 1987) had already demonstrated the feasibility of such measurements, but only after radical elimination of light-scattering artifacts were we able to obtain reproducible and consistent quantitative results. Furthermore, titrations were carried out in the presence of 1 mM EDTA to prevent artifacts due to aggregation induced by contaminant multivalent cations. Under such strictly controlled experimental conditions, it was thus possible to determine dissociation constants between LDL and unlabeled heparins by competition experiments with labeled heparin as a reference. Studies of the molecular weight dependence were carried out with paucidisperse fractions obtained from one batch of beef lung heparin. This material was selected for its highly homogeneous sulfation pattern (Casu et al., 1983; Linhardt et al., 1988; Nader et al., 1990). Samples with various sulfation degrees were obtained either by fractionation of heparins of various biological origin or by chemical modification.

Experimental Procedures

Materials

Three different heparin samples from porcine mucosa were used: PMA was obtained from Fluka (Buchs, Switzerland) as the ammonium salt; PMB from Laboratorio Derivati Organici (Trino Vercellese, Italy) and PMC from Opocrin (Corlo, Italy) were both obtained as sodium salts. Beef lung heparin was purchased from Hepar Industries (Franklin, OH) as the sodium salt. In general, the concentration of heparin samples was determined on the basis of the dry weight and is expressed in terms of the molarity of the constituent disaccharide. The concentration of reference heparin (RH) solutions was determined with the carbazole–borate method (Bitter & Muir, 1962), using pig mucosa heparin (sample PMA) as a reference. Heparinase (heparin lyase, EC 4.2.2.7) was purchased from Seikagaku Kogyo Co. Ltd. (Tokyo, Japan). Its specific activity was 1.5 units/mg (1 unit liberates 1 μmol of hexuron-4-enate/min at 35 °C, pH 7). Human low-density lipoproteins were prepared batchwise from blood bank plasma by sequential ultracentrifugation in density gradients (Havel et al., 1955) and stored at 4 °C in the presence of 0.01% NaN_3, 0.5 mM PMSF, and 1 mM EDTA. The concentration of apolipoprotein B-100 was determined by the Peterson modification of the Lowry assay (Peterson, 1979) with bovine serum albumin (Pierce) as a reference. The molar concentration is computed with the molecular weight (513 K), as derived from the known protein sequence (Chen et al., 1986). Acetylated LDL was prepared by exhaustive treatment with acetic anhydride, followed by ninhydrin determination of the acetylation degree (Frankel-Conrat, 1957). Its concentration was determined from OD_{280} after scattering correction, with the assumption of unchanged molar extinction after acetylation. 5-Fluoresceinamine (isomer I) was purchased from Sigma. Reagent-grade solvents, chemicals, and buffer salts were obtained either from Sigma or from Carlo Erba Reagenti.

Elemental analyses (C, H, N, S) were carried out at the Analytical Service of Farmitalia C. Erba. A Perkin Elmer MPF-66 spectrofluorometer equipped with a standard polarization accessory was used for fluorescence measurements. Absorption spectra were obtained with a Perkin Elmer Lambda 4 spectrophotometer. NMR spectra were obtained with a Bruker AC-300 spectrometer, operating at 300 MHz in the ^1H mode and 75 MHz in the ^{13}C mode, and with D_2O solutions of the polysaccharides. Semipreparative gel filtration chromatography (10–100-mg scale) was carried out with a Waters 625 chromatographer equipped with a 486 UV–vis detector and a Pharmacia FRAC-100 fraction collector. Desalting of polysaccharides was obtained with the same apparatus, by further monitoring conductivity through a microconductimetric flow cell.

Methods

Preparation and Labeling of Reference Heparin. A fraction with improved LDL affinity was obtained from porcine mucosa heparin (PMA) by affinity chromatography through LDL–Sepharose. The column (120 × 16 mm) was prepared according to a standard procedure (Hirose et al., 1987) with about 10 mg of apolipoprotein B-100/g of CNBr-activated Sepharose 4B (Pharmacia). It was loaded with 100 mg of heparin dissolved in 1 mL of buffer (0.01 M HEPES, 0.01 M $CaCl_2$, and 0.01% NaN_3, pH 8), eluted with 200 mL of the same buffer, that removed the majority of the loaded heparin, and finally eluted with buffer containing 0.2 M NaCl. This afforded about 4 mg of heparin with LDL affinity approximately twice as high as unfractionated material. Samples from several chromatographies were pooled, desalted by gel filtration, and lyophilized. A portion (20 mg) of this material was labeled with 5-fluoresceinamine by the procedure of Glabe et al. (1983), as modified by Smith and Knauer (1987). Unreacted fluoresceinamine and salts were removed by gel filtration through Sephadex G-25. The degree of labeling was estimated as 1 chromophoric group per 45 disaccharides on the basis of the absorbance at 490 nm in 50 mM borate buffer, pH 10 (ϵ_{490} = 89 400 M^{-1} cm^{-1}; Ogamo et al., 1982).

Ethanol Precipitation of Heparin. According to Casu et al. (1986), beef lung heparin (3 g) was dissolved in 60 mL of distilled water and cooled at 4 °C, and an equal volume of cold 95% ethanol was added under magnetic stirring. The precipitate, collected by centrifugation (1 h, 2000*g*), redissolved in water, and lyophilized, weighed 1.86 g (sample BL1, 62%

yield). Heparin of porcine mucosa (samples PMB and PMC) was fractionated by a similar procedure (Bianchini et al., 1985) into a precipitate (samples PMB1 and PMC1) and a supernatant (samples PMB2 and PMC2). Electrophoresis on cellulose acetate strips with barium acetate buffer (Oreste et al., 1980) confirmed the predominance of the slow-moving fraction in samples PMB1 and PMC1.

Preparation of Heparins with Altered Sulfation Pattern. The following derivatives were obtained from heparin with chemical modifications involving sulfation, desulfation, and N-acetylation. The sulfation and acetylation pattern of these derivatives was confirmed by ^1H and ^{13}C NMR spectroscopy. The starting material used for the preparation of each derivative is identified by the alphabetic part of its code. Sample PMB3, nonspecifically O-sulfated, was prepared by heparin treatment with sulfur trioxide pyridine complex for 1 h at room temperature (Ogamo 1988). Fully N-acetylated heparins were prepared from porcine mucosa (sample PMB4) and, respectively, bovine lung heparin (sample BL2) according to Inoue et al. (1976). Sample PM5, fully O-desulfated at C_6 of glucosamine and partly O-desulfated at $C_{2'}$ of the iduronic residue, was prepared by N- and C_6-O-desulfation in solvolytic conditions (Inoue et al., 1976): 7 h at 100 °C in dimethyl sulfoxide/methanol (9/1, v/v), followed by resulfation at the amino group with sulfur trioxide trimethylamine complex in basic aqueous medium (Lloyd et al., 1971). 6-O-Desulfation was confirmed in a ^{13}C NMR spectrum by the absence of the characteristic *N*-sulfoglucosamine 6-*O*-sulfate signal at 68.6 ppm and the appearance of the *N*-sulfoglucosamine signal at 62.07 ppm (Gatti et al., 1979). Sample PMB6, selectively O-desulfated at $C_{2'}$ of the iduronic residue, was prepared with alkaline treatment (Jasej et al., 1989). Both the ^{13}C and ^1H NMR spectra of this sample agreed with those described by Jasej et al. (1989). Low molecular weight, supersulfated heparin (sample PMB7) was prepared by reaction with chlorosulfonic/sulfuric acid (Naggi et al., 1987).

Heparin Fractionation by Ion-Exchange Chromatography. Heparin from porcine mucosa (PMB, 4 g), dissolved in 0.25 M NaCl, was loaded on a DEAE-Sephacel column (6 × 20 cm). Stepwise elution of the column with 1-L portions of aqueous NaCl solutions of increasing concentration (0.25, 0.58, 0.60, 0.65, 1 M) led to heparin resolution into fractions with different charge density. Sample PMB8 was obtained by pooling the fractions which eluted at 0.65 M NaCl, followed by desalting and lyophilization.

Enzymatic Depolymerization of Heparin. This was accomplished with a modification of a published procedure (Rice & Linhardt, 1989), as detailed below. Ethanol-precipitated bovine lung heparin (sample BL1, 150 mg) was dissolved in 6 mL of 5 mM phosphate buffer/0.2 M NaCl, pH 7 at 30 °C. This solution was supplemented with 0.15 unit of heparin lyase, and the reaction was monitored by periodically withdrawing 100-μL aliquots which were examined for molecular weight distribution by gel-filtration HPLC. After 24 h, the reaction was stopped with 1-min exposure to 100 °C and the solution concentrated in a centrifugal evaporator and then filtered through Chelex 100 cartridges (Bio-Rad).

Preparation of Heparin Fractions with Well-Defined Molecular Weight. Fractions with higher molecular weight (1–6 in Table I) were obtained by gel filtration through Sephadex G-75 of ethanol-precipitated bovine lung heparin (sample BL1). Fractions with shorter chain length (7–11 in Table I) were prepared from enzymatically depolymerized heparin by gel filtration through a Sephadex G-50 column. In both cases, elution was accomplished with phosphate buffer (0.01 M)/NaCl (0.1 M), pH 7.4 at 1 mL/min flow, and heparin was detected by monitoring the absorbance at 210 nm. Heparin-containing tubes of each chromatography were pooled into 5–6 fractions with approximately equal heparin content and progressively increasing elution volume. These were desalted on Sephadex G-10 and lyophilized.

Table I: Physicochemical Characterization and LDL Affinity of Bovine Lung Heparin Fractions with Well-Defined Molecular Weight

fraction	n_s[a]	M_n	M_w	K_d (μM)
1	2.79	19800	21300	3.2 ± 0.5
2	2.84	14900	16200	4.3 ± 0.7
3	2.81	13400	14700	6.9 ± 1.0
4	2.79	11800	13100	6.1 ± 0.8
5	2.84	9700	10900	8.5 ± 1.4
6	2.83	7800	8950	13.8 ± 2.0
7	2.65	7700	8900	13.2 ± 2.4
8	2.56	7350	8350	17.1 ± 2.2
9	2.66	3400	3900	63 ± 8
10	2.84	1500	1850	130 ± 16
11	ND[b]	500	630	350 ± 85

[a] Average n_s (fractions 1–10) is 2.76 with 3.6% relative standard deviation. For sample BL1, which was used for the preparation of these fractions, n_s = 2.83. [b] Not determined.

Determination of Heparin M_w and M_n. Two serially connected TSK gel permeation columns (SW2000-XL and SW3000-XL) were used in combination with a TSK (SW-XL) precolumn in a Hewlett-Packard 1090 M chromatographer. Phosphate-buffered (0.01 M, pH 7.4) Na_2SO_4 (0.1 M) was used as the eluent at 0.5 mL/min flow. The columns were thermostated at 40 °C, and the heparin signal was monitored at 210 and 232 nm with diode array detection. Data analysis was accomplished according to De Vries (1989). The system was calibrated either with paucidisperse heparin samples, whose molecular weight had been determined viscosimetrically (Johnson, 1982), or with secondary standards based on these. This calibration was verified in the low molecular weight range by analysis of the heparinase depolymerization pattern (Barrowcliffe et al., 1989): peaks assigned to disaccharide, tetrasaccharide, etc. up to dodecasaccharide could be resolved in the chromatogram and appeared with retention times corresponding to the expected M_w values.

Determination of Sulfation Degree. The sulfation degree, n_s, the number of sulfate groups per disaccharide unit, was determined from elemental analysis as the ratio between sulfur and nitrogen atoms. As a complementary technique, conductimetric titrations were also used (Casu & Gennaro, 1975).

Fluorescence Anisotropy Titrations. Exciting light coming from the monochromator set at 495 nm (5-nm bandwidth) was passed through an interferential filter (Schott-Pal 500 nm, 21-nm half-bandwidth) before reaching samples. A cutoff filter with less than 1% light transmittance at 510 nm was placed before the emission monochromator set at 530 nm (7-nm bandwidth). With this optical setup, the effect of fully polarized scattered light was investigated by separately measuring the signals of solutions containing either LDL or FRH. Results indicate that artifacts due to scattered light never exceed 3% of the reported data in our experiments. Anisotropy data are averages of two determinations, and error bars indicate the difference of the two values.

Samples were prepared in 0.05 M HEPES, 0.1 M NaCl, 0.01% NaN_3, and 1 mM EDTA, pH 7.4, and thermostated at 22 °C. Two solutions were prepared for binding studies: both contained the same concentration of fluoresceinated reference heparin, but LDL was present in one of the solutions. Progressively increasing LDL concentration at constant reference heparin concentration was generated by microliter

additions of the second solution to the first in a quartz cuvette. Similarly, in competition experiments, a solution of fluoresceinated reference heparin and LDL in a cuvette was mixed with increasing amounts of a second solution having the same components and the polysaccharide to be tested. Salt-promoted dissociation of the LDL–FRH complex was investigated with the same procedure, by supplementing the second solution with a high salt concentration (0.5 M NaCl).

Analysis of Binding and Competition Data. The association of LDL and heparin was treated with a two-state (bound, free) approximation. Accordingly, the experimental anisotropy, r, could be expressed as the sum of two contributions:

$$r = r_{free}X_{free} + r_{bound}(1 - X_{free}) \quad (1)$$

with $X_{free} = [H]_{free}/c_H$, the molar fraction of free heparin, and r_{free} and r_{bound} are the anisotropy values of reference heparin in the two states. Binding data were analyzed with a model assuming equivalence of all binding sites on LDL. If n is the total binding capacity of apolipoprotein B-100 (measured as disaccharide units) and K_d is the dissociation constant per site (one site binds one disaccharide in this model), then a Langmuir-type binding isotherm may be written:

$$[H]_{bound}/c_{LDL} = \frac{n[H]_{free}}{K_d + [H]_{free}} \quad (2)$$

where c_{LDL} is the total concentration of LDL. In combination with mass conservation for heparin, this leads to

$$X_{free} = \frac{c_H - K_d - c_{LDL} + [(nc_{LDL} + K_d - c_H)^2 + 4K_dc_H]^{1/2}}{2c_H} \quad (3)$$

Equations 1 and 3 have been used for the determination of best-fitting n, K_d, and r_{bound} values in a nonlinear regression procedure carried out with the program MINSQ (Micro-Math Scientific Software, Salt Lake City, UT). The value of r_{free} = 0.080, as determined experimentally for FRH solutions, was used throughout. Competition data were interpreted with eq 4 that was derived according to the formalism introduced by Feldman (1972) for the case of two ligands, H and I, competing for the same sites on LDL where C_I is the total concentration of I and $K_{d,I}$ is the corresponding dissociation constant for the LDL complex. This equation was solved numerically in the $0 < X_{free} < 1$ interval by a procedure included in the MINSQ package.

$$C_I = \left[\frac{K_{d,I}}{K_d}X_{free}\left(\frac{nc_{LDL}}{1 - X_{free}} - c_H\right) - K_{d,I}\right]\left(1 + \frac{K_{d,I}}{K_d}\frac{1 - X_{free}}{X_{free}}\right) \quad (4)$$

Analysis of Salt Titration Data. The dependence of the dissociation constant on salt concentration was modeled by the empirical equation:

$$\ln K_d = \ln K_0 + m \ln [(C_0 + C_s)/C_0] \quad (5)$$

where K_0 is the dissociation constant at a reference salt concentration (C_0) and C_s is the added salt concentration. This is an integrated form of eq 6 discussed under Results and is derived from thermodynamic analysis of polyelectrolyte binding (Record et al., 1978). No correction was found necessary for fluorescence anisotropy change as a consequence of increasing solution viscosity within the experimental salt concentration range.

Results

Structural Properties of Reference Heparin. When the physical properties of reference heparin (RH) are compared with those of the source material (PMA) in Table II, an increase of both M_w and n_s can be observed. ^{13}C NMR spectroscopy confirms increased sulfation in RH with respect to PMA: the intensity of 6-O-sulfated and N-sulfated glucosamine signals indicates an increase from 81% to 94% and from 77% to 84%, respectively. No relevant change is detected for the intensity of the 2′-O-sulfated iduronyl residue (≈60% of total iduronic acid content). These results are consistent with the general trend reported in former studies on high LDL affinity fractions of heparin (Cardin et al., 1989; Srinivasan et al., 1991).

Table II: Physicochemical Characterization and LDL Affinity of Heparin Samples with Different n_s Values

sample	comments	n_s	M_w ($\times 10^{-3}$)	K_d (μM)
RH	high LDL affinity	2.37	23.3	4.4 ± 0.8
PMA	commercial sample	2.25	15.8	8.0 ± 0.8
PMB	commercial sample	2.25	14.5	13.4 ± 0.8
PMC	commercial sample	1.83	12	22.8 ± 1.5
PMB1	slow-moving fraction	2.38	16.1	13.1 ± 1.2
PMB2	fast-moving fraction	2.1	12.2	15.0 ± 1.2
PMB3	O-sulfated	2.8	12.5	2.8 ± 0.4
PMB4	N-acetylated	1.68	9.6	230 ± 12
PMB5	6-O-desulfated	1.2	23.9	390 ± 50
PMB6	2′-O-desulfated	1.5	9.3	110 ± 12
PMC1	slow-moving fraction	2.05	18.6	12.8 ± 0.9
PMC2	fast-moving fraction	1.80	11.5	29.6 ± 1.7
PMB7	supersulfated	3.5	4.8	4.5 ± 0.4
PMB8	high charge density	2.27	16.6	7.8 ± 0.8
BL1	slow-moving fraction	2.83	15.7	4.5 ± 0.7
BL2	N-acetylated	1.68	13.9	370 ± 30

Binding of Reference Heparin to LDL. By increasing the LDL concentration in a solution having constant FRH concentration, the fluorescence anisotropy changed from 0.080 (r_{free}) to about 0.160 (r_{bound}). These values reasonably agree with fluorescence polarization values reported by Cardin et al. (1987) for fluoresceinated heparin alone and in the presence of an excess of LDL. The use of anisotropy is preferred here, for this parameter is linearly related to the molar fraction of free heparin (eq 1), whereas polarization is not (Lakowitz, 1983). In contrast with the above study, no correction for viscosity was found necessary up to the highest LDL concentration investigated in our experiments (6 μM). One example of binding data and corresponding analysis is shown in Figure 2A; it also reports data of fluorescence anisotropy measured in the presence of increasing concentration of acetylated LDL. The constancy of the fluorescence anisotropy in this latter case is strong evidence that the observed effect is entirely due to binding. When scattering artifacts are not entirely eliminated, an apparent dependence of anisotropy on Ac-LDL concentration develops. This is due to increasing contributions of fully polarized scattered light, as is easily verified by a dependence of the effect on FRH concentration (data not shown).

The simple model assuming equivalent sites explains very well all data reported in Figure 2A: three binding curves, corresponding to different FRH concentrations. More complex models were unsuccessful at improving the quality of fit. A second experiment with a different LDL batch produced very similar data, with slightly different values of the best-fitting parameters (n, K_d, r_{bound}). Averaged values for the two data sets are n = 23.8 ± 1.0, K_d = 4.0 ± 1.0 μM, and r_{bound} = 0.160 ± 0.002. Figure 2 also reports on a competition experiment where FRH binding to LDL is challenged by increasing concentrations of unlabeled RH (panel B). These data are very well interpreted with eq 4, as expected for a fully reversible association. The two dissociation constants, K_d = 4.0 ± 1.0 μM and $K_{d,I}$ = 5.3 ± 0.3 μM, of the labeled and un-

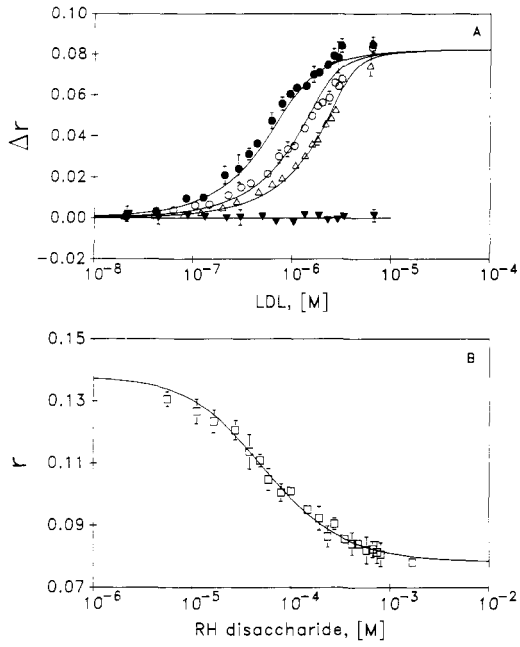

FIGURE 2: Analysis of three FRH–LDL binding curves and one FRH–RH competition curve. Experiments were carried out at 22 °C in 50 mM HEPES, 0.1 M NaCl, 0.01% NaN_3, and 1 mM EDTA, pH 7.4. (A) Binding curves were measured at constant FRH concentration [respectively 20 (●), 46 (○), and 80 (△) μM] and variable LDL concentration. On the ordinate axis, the difference between the anisotropy of the titrated solution and that of a reference solution containing only FRH (r_{free}) is reported. In this case, the best-fitting binding parameters are K_d = 3.8 ● 0.9 μM, n = 22.9 ± 0.9, and $r_{bound} - r_{free}$ = 0.082 ± 0.002. Values reported in the text are averages obtained with two different LDL batches. Results of a titration experiment with acetylated LDL are also reported for comparison (▼). (B) The competition experiment was carried out at [FRH] = 17.3 μM and [LDL] = 1 μM and analyzed with the same binding parameters as in (A); the best-fitting value of the dissociation constant for the RH–LDL complex was K_i = 5.2 ± 0.3 μM.

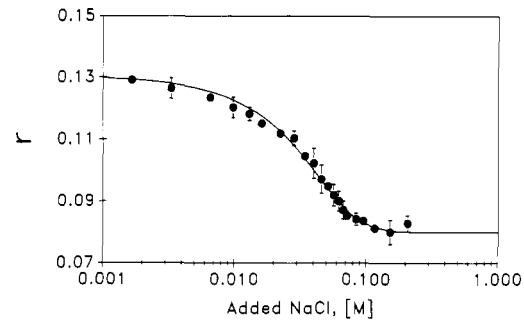

FIGURE 3: Salt-induced displacement of FRH from its complex with LDL. The starting buffer contained 50 mM HEPES, 0.1 M NaCl, 0.01% NaN_3, and 1 mM EDTA, pH 7.4. Temperature, 22 °C; the concentrations of FRH (17.3 μM) and of LDL (0.75 μM) were kept constant throughout the experiment. Binding parameters: K_d = 4.0 μM; n = 23.8. The best-fitting curve was obtained with a model incorporating the salt dependence of K_d, as described by eq 5 with m = 8.2.

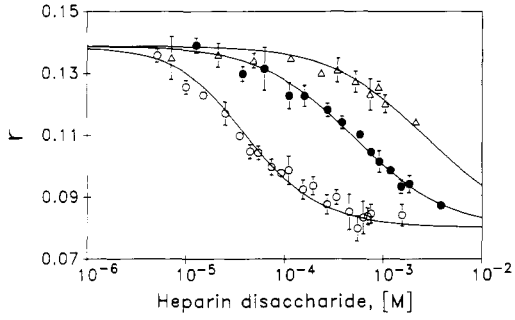

FIGURE 4: Competition curves of heparin fractions 1, 9, and 11 (Table I) with FRH for the binding to LDL. Heparin concentration is referred to the disaccharide unit. Buffer, temperature, and FRH and LDL concentrations are the same as in Figure 1B. Data were analyzed keeping the following parameters constant: K_d = 4.0 μM; n = 23.8; r_{free} = 0.080; r_{bound} = 0.160. The three curves correspond to the following K_d values: 3.2, 63, and 350 μM.

labeled species do not exactly coincide, though their difference marginally exceeds the relative errors. As the total heparin concentration in competition experiments is much higher than that used in binding, the difference between K_d and $K_{d,I}$ may be related to the presence of weaker binding sites not detectable in binding experiments. As the experimentally observed difference is marginal, so is the importance of such sites, if any, and no effort was done to further characterize them.

Salt Titration of the LDL–Heparin Complex. The association of LDL and heparin is highly sensitive to salt concentration (Iverius, 1972) as a consequence of its electrostatic nature. This property is also exploited in the purification of heparin fractions with high LDL affinity (Cardin et al., 1989; Srinivasan et al., 1991). When the salt concentration is progressively increased in a solution with constant LDL and FRH concentrations, the fluorescence anisotropy gradually decreases (Figure 3) as a result of the dissociation of bound heparin. The experimental data are very well interpreted by a simple salt dependence of the dissociation constant, that derives from thermodynamic analysis of binding to polyelectrolytes (Record et al., 1978):

$$\frac{\text{d log } K_d}{\text{d log [salt]}} = m \approx Z\Psi \qquad (6)$$

where Z is the number of positively charged groups in one LDL binding site and Ψ is the counterion association parameter of heparin. $\Psi \approx 0.84$ is roughly estimated from physical constants and structural data: negative charges per disaccharide ($1 + n_s = 3.3$) and chain length per disaccharide (8.4 Å) from solid-state data (Atkins & Nieduszynski, 1976). In combination with the experimental value of m = 8.2 ± 0.3, this leads to $Z \approx 10$, with an accuracy that largely depends on the crude estimate of Ψ.

Chain Length Dependence of the Dissociation Constant. Table I collects number- and weight-average molecular weights, M_n and M_w, respectively, of beef lung heparin fractions prepared by gel filtration. Sulfation degrees, n_s, as measured by elemental analysis, and the corresponding dissociation constants of LDL complexes are also reported in Table I. These latter were obtained by competition experiments as detailed in the examples reported in Figure 4, and are averages of two measurements with different LDL batches. Properties of the unfractionated beef lung heparin sample are also reported for comparison. Fraction 11 contains a mixture of disaccharides, and was produced in insufficient quantity for a full analytical characterization. LDL affinity increases (K_d decreases) with increasing heparin chain length. This dependence is very well described by the empirical equation:

$$K_d = K_0 M_w^a \qquad (7)$$

Best-fit values of the parameters are obtained from a log K_d vs log M_w plot (r^2 = 0.9998): log K_0 = 1.4 ± 0.3 and a = −1.59 ± 0.08.

Dissociation Constant as a Function of n_s. Sixteen heparin samples with various degrees of sulfation ($1.2 < n_s < 3.5$) were obtained either by exploiting the natural variability, or by further discriminating this parameter through ethanol precipitation or ion-exchange chromatography, or by selective chemical modification. These are listed in Table II together

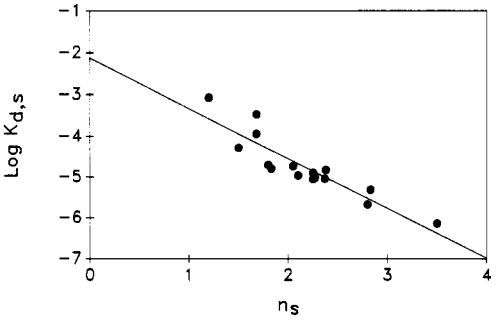

FIGURE 5: Dependence of log $K_{d,s}$ on the degree of sulfation. The LDL affinity of 16 heparins is plotted against the corresponding number of sulfate groups per disaccharide (n_s), after correction for chain length dependence with eq 8. Products with the highest affinity are at the lower right side.

with the corresponding n_s and M_w values. Dissociation constants of the LDL complex were determined through competition experiments with FRH, and are also reported in Table II. Unfortunately, the preparation of heparins with various n_s values and constant molecular weight proved to be an extremely laborious task, and thus a considerable spreading of M_w values is exhibited by these samples. However, by extending the aforementioned empirical relationship between K_d and M_w to samples of any degree of sulfation, a simple correction is found, which permits the extrapolation of standard dissociation constants $K_{d,s}$, as expected for heparins with a common molecular weight $M_{w,s}$, from experimental K_d values:

$$K_{d,s} = K_d(M_{w,s}/M_w)^a \quad (8)$$

For convenience, the average value of M_w among samples in Table II (15 000) was used as $M_{w,s}$; the value $a = -1.6$ was assumed on the basis of the previous analysis. These standardized dissociation constants are plotted (as log $K_{d,s}$) versus n_s values in Figure 5. Correlation between the two parameters is significant ($r^2 = 0.9957$), though not as good as for the M_w dependence of K_d. Data spreading increases at low n_s values, perhaps as a result of some specificity in the interaction between LDL and poorly sulfated heparins. Nevertheless, the general data trend is most simply interpreted in terms of electrostatic forces dominating the energetics of LDL–heparin binding.

Discussion

One of the first possible questions about the nature of the LDL interaction with heparin concerns the existence of a particular heparin substructure that may be recognized with high specificity by apolipoprotein B-100. Our data cannot rule out such a hypothesis, but strongly disfavor it. In agreement with our findings, former observations (Fransson & Havsmark, 1981; Cardin et al., 1989; Srinivasan et al., 1991) reported that high LDL affinity fractions of heparin have a higher sulfation degree and moleclar weight than the starting material. More significantly, our systematic study with well-characterized heparin fractions and derivatives indicates a regular dependence of LDL affinity on the heparin molecular weight and on the degree of sulfation. The present data restrict the value of the empirically found relationships (see Figure 5 and eq 8) to sulfated polysaccharides with the heparin backbone. There is no reason, however, to exclude a similar behavior for other sulfated polysaccharidic structures. In fact, LDL binding to several other sulfated polysaccharides is well-known, and comparative studies have been presented (Iverius, 1972; Fransson & Havsmark, 1982; Wegrowski et al., 1986; Srinivasan et al., 1988; Christner & Baker, 1990). In view of our findings, however, a careful evaluation with a series of well-characterized samples is required for an appropriate ranking of preferential LDL binding with any of these structures. Such studies are currently in progress at our laboratories and will be the subject of a future report.

As all experimental evidence is in favor of, though not proving, a nonspecific interaction model, we decided to adopt such a model as a working hypothesis for data analysis. As we shall see, this choice leads to a picture of LDL–heparin interaction in fairly good agreement with results of biochemical studies. One of the consequences of nonspecific interaction between a large ligand (one LDL site) and a linear polymer (heparin) is the site exclusion effect (McGhee & von Hippel, 1974) that is expected to markedly affect binding isotherms. This has been recently recognized in the interaction between heparin and thrombin (Olson et al., 1991). However, a characteristic signature of this effect, bending of Scatchard plots (Scatchard, 1949) at high polymer saturation level, is very difficult to highlight in the case of LDL–heparin binding, as light absorption by LDL irreparably corrupts fluorescence measurements at a high LDL/heparin ratio. Scatchard representation of data or, better, the construction of a binding isotherm (Klotz, 1982) indicates that significant measurements cannot exceed more than about 60% coverage of the available binding sites in the equivalent site model. With this restriction, site exclusion is not quantitable through fluorescence anisotropy measurements, but it will nevertheless produce two important consequences of the physical meaning of the two parameters, K_d and n, as obtained with the application of a Langmuir-type isotherm (McGhee & von Hippel, 1974). The dissociation constant K_d, though referred to repeating units of the polymer (disaccharides), is a measure of the interaction between one full site on the protein and the matching heparin segment (N_0 disaccharides long); the LDL capacity, n, as deduced by the simple model, underestimates the true binding capacity by as much as a factor of 2, the limit for large N_0. With this correction, the true LDL binding capacity increases up to $n \approx 40$–50 disaccharide units.

Our binding data do not discriminate between the two interpretive models (with or without site exclusion); however, a bent Scatchard plot has been reported for the heparin–LDL interaction (Cardin et al., 1987) in a filter binding assay with radioactively labeled heparin. The site exclusion effect is an alternative to the proposed interpretation (multiple sites with different affinity on LDL). Furthermore, a linear lattice binding model with site exclusion offers an interesting interpretation to LDL–heparin affinity as a function of polysaccharide chain length. In this model, chain termination in polymers of finite length is taken into account with a correction which depends on polymer length (McGhee & von Hippel, 1974). In the limit of low saturation (high heparin to LDL ratio), that is consistent with our displacement experiments, this correction leads to the following apparent dependence of K_d on N, the number of repeating units (disaccharides) in the polymer:

$$K_d(N) = K_d(\infty)\frac{N}{N - N_0 + 1} \quad (9)$$

where N_0, as previously defined, is the number of disaccharides that are bound at one site on LDL and $K_d(\infty)$ is the dissociation constant for an infinitely long chain. Because this dependence is more easily recognized in reciprocal form, a plot of $1/K_d$ versus N is reported in Figure 6. The curve is a best-fitting with eq 9, upon excluding the first two data points ($N < 10$), and corresponds to $N_0 = 10.9 \pm 1.5$ and $K_d(\infty) = 2.5 \pm 0.5$ μM. As is evident from the figure, data with longer chains ($N > 30$) would be required to validate this interpre-

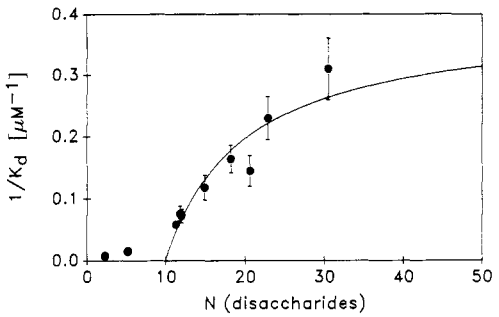

FIGURE 6: Dependence of the reciprocal of the K_d on the average chain length (N = number of disaccharide units) of heparin fractions. N is computed from M_n (Table I) as $N = M_n/650$. The curve is a result of best fit with eq 9 on data with $N > 10$, and corresponds to $m = 10.9 \pm 1.5$ and $K_d(\infty) = 2.5 \pm 0.5$ μM.

tation, but these data are difficult to obtain in view of the natural limits of heparin chain length. Incidentally, the length of the polysaccharidic chain that is bound at one site on LDL is approximately half that of an average heparin chain ($M_w \approx 15000$).

A structural model of the heparin–LDL interaction is finally derived from a combination of the binding capacity of the LDL particle after correction for site exclusion ($n \approx 40$–50 disaccharide units), the length of the bound heparin segment per LDL site ($N_0 \approx 11$ disaccharide units), and the number of positively charged groups in one binding site ($Z \approx 10$). Apolipoprotein B-100 contains 4–5 heparin binding sites, with average dissociation constant $K_d = 4$ μM; each of these sites is capable of binding about 10–12 disaccharides and contains approximately 10 positively charged residues. The presence of several heparin binding sites is consistent with studies on peptide fragments of apoB-100 (Weisgraber & Rall, 1987; Hirose et al., 1987): from five to seven heparin binding portions were identified in the protein sequence with the help of affinity chromatography and peptide sequencing. Furthermore, the total number of basic amino acid residues contained in these sequences (35–40) approximately agrees with our estimate (40–50) based on a combination of binding capacity and salt dependence study.

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Redox Titrations of Carbon Monoxide Dehydrogenase from *Clostridium thermoaceticum*†

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ABSTRACT: Redox titrations of carbon monoxide dehydrogenase (CODH) from *Clostridium thermoaceticum* were performed using the reductant CO and the oxidant thionin. Titrations were followed at 420 nm, a wavelength sensitive to redox changes of the iron–sulfur clusters in the enzyme. When CODH was oxidized by just enough thionin to maximize A_{420}, two molecules of CO per mole of CODH dimer (4 equiv/mol) reduced the enzyme fully. Likewise, 4 equiv/mol of thionin oxidized the fully-reduced enzyme to the point where A_{420} maximized. The four $n = 1$ redox sites which titrated in this region were designated *group I* sites. They include at least two iron–sulfur clusters, $[Fe/S]_A$ and $[Fe/S]_B$, and two other sites, A′ and B′. The $[Fe_4S_4]^{2+/1+}$ cluster in CODH is included in this group. $[Fe/S]_B$ and B′ have reduction potentials (at pH 8) below −480 mV vs NHE; $[Fe/S]_A$ and A′ have reduction potentials above that value. The reduction potential of either $[Fe/S]_B$ or B′ is near to the CO/CO_2 couple at pH 8 (−622 mV). When CODH was oxidized by more than enough thionin to maximize A_{420}, some of the excess thionin oxidized the so-called *group II* redox sites. These sites have reduction potentials more positive than group I and do not exhibit changes at 420 nm when titrated. Titration of group II sites required 1–2 equiv/mol. EPR of reduced group II sites exhibited the $g_{av} = 1.82$ signal. When these sites were oxidized, the only signal present had g values at 2.075, 2.036, and 1.983. The $g_{av} = 1.82$ species either is not an iron–sulfur cluster or is one with unusual optical and redox properties. Thionin in large molar amounts slowly inactivated the enzyme by an oxidation process. Exposure of 100 equiv/mol of thionin to CODH for 1 week completely inactivated the enzyme. These so-called *group III* oxidations are not involved in the catalytic mechanism of the enzyme.

Carbon monoxide dehydrogenase (CODH) is the central enzyme in the acetyl coenzyme A (acetyl-CoA)[1] or Wood pathway [see Ragsdale et al. (1988) for a review], an autotrophic pathway used by acetogenic bacteria to obtain cellular carbon from CO_2. CODH catalyzes the synthesis of acetyl-CoA from CO, coenzyme A, and a methyl group, as well as the reversible oxidation of CO to CO_2 (Ragsdale & Wood, 1985; Lu et al., 1990). The enzyme has an $(\alpha\beta)_3$ quaternary structure and a molecular weight of 440 000 (Ragsdale et al., 1983).

CODH is one of only four enzymes known to contain nickel (Lancaster, 1988). Each $\alpha\beta$ dimer is believed to contain 2 nickels, 11–13 irons, and ~14 acid-labile sulfides (Ragsdale

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[1] Abbreviations: CoA, coenzyme A; NHE, normal hydrogen electrode; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure spectroscopy; ENDOR, electron nuclear double magnetic resonance; THF, tetrahydrofuran; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; FCI, ferrous component I.

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