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Distribution of Lipid-Binding Regions in Human Apolipoprotein B-100†

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ABSTRACT: The distribution of lipid-binding regions of human apolipoprotein B-100 has been investigated by recombining proteolytic fragments of B-100 with lipids and characterizing the lipid-bound fragments by peptide mapping, amino acid sequencing, and immunoblotting. Fragments of B-100 were generated by digestion of low-density lipoproteins (LDL) in the presence of sodium decyl sulfate with either *Staphylococcus aureus* V8 protease, pancreatic elastase, or chymotrypsin. Particles with electron microscopic appearance of native lipoproteins formed spontaneously when detergent was removed by dialysis from enzyme digests containing fragments of B-100 and endogenous lipids, or from incubation mixtures of delipidated B-100 fragments mixed with microemulsions of exogenous lipids (cholesteryl oleate and egg phosphatidylcholine). Fractionation of the recombinant particles by isopycnic or density gradient ultracentrifugation yielded complexes similar to native LDL with respect to shape, diameter, electrophoretic mobility, and surface and core compositions. Circular dichroic spectra of these particles showed helicity similar to LDL but a somewhat decreased content of β-structure. Most of the fragments of B-100 were capable of binding to lipids; 12 were identified by direct sequence analysis and 14 by reaction with antisera against specific sequences within B-100. Our results indicate that lipid-binding regions of B-100 are widely distributed within the protein molecule and that proteolytic fragments derived from B-100 can reassociate in vitro with lipids to form LDL-like particles.

Low-density lipoproteins (LDL),[1] the major carriers of cholesterol and cholesteryl esters in human plasma, are spherical particles (18–25 nm in diameter) that consist of a neutral lipid core (mainly composed of cholesteryl esters) surrounded by a polar surface shell (phospholipids, unesterified cholesterol, and protein) (Deckelbaum et al., 1977; Gotto et al., 1986). Apolipoprotein B-100 is the sole protein component of LDL and is the ligand responsible for the binding of LDL to the LDL receptor (Brown & Goldstein, 1986; Mahley et al., 1977, 1984). Moreover, B-100 is one of the largest single polypeptides known, consisting of 4536 amino acid residues, as deduced from cDNA clones (Knott et al., 1986; Law et al., 1986; Yang et al., 1986), and appears to possess a very high affinity for lipids, exemplified by its inability to transfer among lipoprotein particles and by its insolubility in aqueous media after delipidation. This high affinity for lipids suggests a structural role for B-100 in the formation and maintenance of lipoprotein particles (Kane, 1983).

Various model LDL systems have been employed to study the detailed structural organization of LDL particles and the nature of the interaction of B-100 with lipids (Atkinson & Small, 1986). These systems involve the reassembly of intact B-100 with surface phospholipids alone (Watt & Reynolds, 1981; Walsh & Atkinson, 1983; Dhawan & Reynolds, 1983) or with surface and core lipids (Krieger et al., 1978; Ginsburg et al., 1984; Lundberg & Suominen, 1984). Such systems have clearly demonstrated the ability of B-100 to bind lipids and to form model LDL complexes that exhibit some physicochemical and biological properties similar to those of native LDL. In addition, small tryptic peptides (M_r <5000) (Cardin & Jackson, 1986) and large thrombolytic fragments (Corsini et al., 1987) of B-100 also possess high enough affinity for lipids to form stable complexes when interacting with dimyristoylphosphatidylcholine liposomes or cholesterol-induced canine high-density lipoproteins, respectively. However, few details are known about the distribution of lipid-binding regions

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[1] Abbreviations: LDL, low-density lipoprotein(s); VLDL, very low density lipoprotein(s); apo B, apolipoprotein B; SP, *Staphylococcus aureus* V8 protease; EL, pancreatic elastase; CH, chymotrypsin; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride); K_D, equilibrium dissociation constant; CD, circular dichroism.

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of B-100, although data from amino acid sequence analysis predict that lipid-binding structures in the form of amphipathic β-sheets, hydrophobic regions, and some amphipathic helices are distributed throughout the length of the molecule (Knott et al., 1986).

To study further the distribution of lipid-binding regions within the B-100 protein, we sought to determine which polypeptide fragments derived from B-100 would bind to lipids. For this purpose, B-100 fragments were first generated by proteolytic digestion of LDL in the presence of sodium decyl sulfate with three different enzymes specific for different types of cleavage sites. Then the detergent was removed either from the enzyme digests or from incubation mixtures of delipidated B-100 fragments mixed with lipid microemulsions to determine whether the B-100 fragments would reasssociate with the endogenous lipids of LDL or with exogenous lipid microemulsions, respectively. After isolation by ultracentrifugation, the techniques of peptide mapping, amino acid sequencing, and immunoblotting were used to characterize the lipid-bound fragments. Our findings showed that most of the proteolytic B-100 fragments could bind to lipids of either endogenous or exogenous origin, that fractionation of the recombinant particles yielded complexes similar to native LDL in shape, diameter, and chemical composition, and that the lipid-binding regions were widely distributed within the B-100 molecule.

Materials and Methods

Materials. Methylene blue and sodium decyl sulfate were purchased from Eastman Kodak Co. (Rochester, NY). Sodium decyl sulfate was recrystallized from ethanol to remove contaminants. *Staphylococcus aureus* V8 protease (SP) was purchased from Pierce Chemical Co. (Rockford, IL), porcine pancreatic elastase (EL) from Boehringer Mannheim Biochemicals (Indianapolis, IN), and α-chymotrypsin (CH) from Worthington Diagnostic Systems (Freehold, NJ). Sodium [^{125}I]iodide (13–17 mCi/μg of I) and ^{125}I-labeled staphylococcal protein A were purchased from Amersham Corp. (Arlington Heights, IL). Poly(vinylidene difluoride) (PVDF) membrane (Immobilon-P Transfer Membrane) was purchased from Millipore Co. (Bedford, MA). Deuterium oxide (99.8 atom %) and KBr were purchased from Sigma Chemical Co. (St. Louis, MO).

Isolation of LDL. LDL were isolated from the serum of normal fasting (14 h) subjects within the density interval 1.025–1.055 g/mL by sequential ultracentrifugation (Havel et al., 1955). Solvent densities were adjusted with KBr, and all ultracentrifugal solutions contained 0.05% NaN_3, 0.04% EDTA, pH 7.5, and 0.05 mg/mL gentamicin. After isolation, LDL were dialyzed extensively at 4 °C against 0.9% NaCl containing 0.05% NaN_3 and 0.04% EDTA, pH 7.5. LDL were iodinated with Na^{125}I by the method of McFarlane (1958), as modified for lipoproteins by Bilheimer et al. (1972). The specific activity of the iodinated LDL was generally between 200 and 300 cpm/ng of protein.

Proteolytic Digestion of LDL. To generate B-100 fragments, limited proteolysis of LDL was carried out in the presence of sodium decyl sulfate (Cleveland et al., 1977) because it dissociates lipids from B-100 (Gotto et al., 1969), rendering more cleavage sites available to enzymes, and because it has a high critical micellar concentration (Mukerjee & Mysels, 1971), facilitating its removal by dialysis (see below). The proteolytic digestion was carried out after adding 0.5% sodium decyl sulfate to 3 mg/mL of LDL (protein) and heating the solution to 95 °C for 3 min. After cooling, 0.1 M Tris-HCl buffer, pH 8.4, was added (10 mM $CaCl_2$ was also added for the CH digestion). One of the following three enzymes was added at a mass ratio of enzyme to B-100 of 1 to 125: (1) SP, dissolved in 0.9% NaCl at 1 mg/mL; (2) EL, suspended in H_2O at 10 mg/mL; and (3) CH, dissolved in 10 mM $CaCl_2$ plus 1 mM HCl at 1 mg/mL. The mixtures were then incubated at 37 °C for 90 min, and the digestions were stopped by adding sodium decyl sulfate to 1.5% and heating the mixtures to 95 °C for 3 min.

Delipidation of Enzyme Digests. The enzyme digests of LDL were delipidated with 20 volumes of ethanol/diethyl ether (3:1) (Brown et al., 1969) and then solubilized in 1% sodium decyl sulfate plus 0.1 mM Tris-HCl buffer, pH 8.4. Residual phospholipids in the delipidated B-100 fragments ranged from 2 to 3 μg/mg of protein.

Recombination of B-100 Fragments with Lipids. Two different methods were used. Method I (dialyzed digests): For recombination of B-100 fragments with the endogenous lipids of LDL, the enzyme digests of LDL were dialyzed against 2 L of 0.15 N NaCl plus 0.5 mM Tris-HCl buffer, pH 7.5, at 22 °C (one to three changes of dialysate, 16–20 h each) to remove sodium decyl sulfate. Method II (incubation mixtures): For recombination of B-100 fragments with exogenous lipids, the enzyme digests were delipidated and solubilized in sodium decyl sulfate, as described above, and then incubated with lipid microemulsions (cholesteryl oleate and egg phosphatidylcholine) prepared by an injection method (Chen et al., 1984) at a mass ratio of 1:1 or 1:4 at 37 °C for 1 h. This mixture was then dialyzed for 2–3 h against the same buffer as before.

Following dialysis, the lipid-bound B-100 fragments were isolated by one of two procedures.

(1) Stepwise ultracentrifugation: The density of the dialyzed enzyme digests or incubation mixture was adjusted to 1.06 g/mL with deuterium oxide and ultracentrifuged at 100000g at 12 °C for 16 h. The 1.06 g/mL top layer containing lipid–protein complexes was collected, its density was adjusted to 1.02 g/mL, and it was ultracentrifuged as before. The 1.02 g/mL infranatant layer was collected, which contained the recombinant LDL-like particles.

(2) Ultracentrifugation through a density gradient containing deuterium oxide or KBr: Two milliliters of 0.9% NaCl (d = 1.006 g/mL) was added to the bottom of a 6.1-mL nitrocellulose tube, and 2 mL of a solution containing 0.6 mL of deuterium oxide and 1.4 mL of 0.9% NaCl (d = 1.037 g/mL) or 2 mL of a solution containing KBr and NaCl (d = 1.037 g/mL) was layered beneath. Then 2.1 mL of a solution containing 1.19 mL of deuterium oxide and 0.91 mL of dialyzed enzyme digest (d = 1.063 g/mL) or 2.1 mL of a solution containing 0.8 mL of KBr and NaCl at d = 1.155 g/mL and 1.3 mL of dialyzed enzyme digest was layered beneath the previous layer. The samples were centrifuged in a Beckman SW 41 rotor at 39000 rpm at 12 °C for 18 h. The bands with yellowish colors, indicative of carotenoid content, were collected. Densities were determined with a pycnometer. Fractions isolated with KBr were dialyzed against 0.9% NaCl and 0.5 mM Tris-HCl buffer, pH 7.5. The recovery of protein in these particles was about 15–25%. The extent of detergent removal, based on the methylene blue assay (Hayashi, 1975), was 98% at 1 day, 99.2% at 2 days, and 99.8% at 3 days. The residual detergent in all the isolated recombinant particles was 0.015–0.03 mg/mg of B-100 protein.

Electron Microscopy. After negative staining with 2% potassium phosphotungstate, samples were examined and photographed with a Siemens 101 electron microscope (Hamilton et al., 1980). Particle diameters were measured on the photographs with a Hipad Digitizer (Chen et al., 1984).

Gel Electrophoresis. To assess the electrophoretic integrity and mobility of the recombinant LDL-like particles, agarose gel electrophoresis of native LDL and of the recombinant LDL-like particles was performed by the method of Noble (1968). To display the polypeptide array of the enzyme digests and the recombinant particles, analytical SDS–PAGE was performed using a 4.5–18% linear gradient slab gel and a discontinuous buffer system (Laemmli, 1970). Prior to SDS–PAGE, these samples were combined with sodium dodecyl sulfate, glycerol, sodium mercaptoacetate, and bromophenol blue to final concentrations of 1%, 10%, 1%, and 0.003%, respectively. The gels were stained with Coomassie Blue R-250 or with silver.

Equilibrium Binding Studies. Human fibroblasts derived from normal newborn foreskin were grown as described previously and used after 48 h of incubation in 10% lipoprotein-deficient serum (Mendel & Kunitake, 1988). The abilities of ^{125}I-LDL and the recombinant LDL-like particles to bind to the LDL receptor of human fibroblasts were assessed in direct and competitive binding studies, respectively. These binding studies were performed at 4 °C essentially by the method of Goldstein and Brown (1974). Equilibrium dissociation constants (K_D), numbers of saturable sites, and relatively unsaturable ("nonspecific") components of the binding were estimated by nonlinear least-squares curve fitting of the total binding data, as described previously (Mendel et al., 1986).

Circular Dichroism. To assess the secondary structure of the B-100 fragments in the recombinant LDL-like particles, CD spectra of the recombinant particles were measured and compared with those of native LDL. CD was measured on a Jasco J-500 A spectropolarimeter. The CD data were expressed in terms of mean residue ellipticity ($[\theta]$ in degrees centimeter squared per decimole of protein residue) using a mean residue weight of 113. All spectra presented are the average of two or more measurements on each of two to four different samples. The concentration of protein ranged from 0.06 to 0.15 mg/mL, and that of sodium decyl sulfate was estimated to be less than 0.2 mM.

Immunoblotting. Polypeptides were transferred to nitrocellulose filters following SDS–PAGE (Towbin et al., 1979). Unoccupied binding sites were blocked by using 5% nonfat dry milk at 22 °C. The filters were incubated at 22 °C with various region-specific peptide antisera raised in rabbits (Protter et al., 1986; Innerarity et al., 1987; Marcel et al., 1987), washed, and incubated with ^{125}I-labeled staphylococcal protein A at 0.1 μCi/mL, rewashed, and exposed to Kodak X-Omat film at −70 °C.

Purification and Sequence Analysis of B-100 Fragments. The purification of polypeptide fragments was carried out by two different approaches. One was by preparative SDS–PAGE (Hardman & Kane, 1980): Delipidated B-100 fragments of the recombinant particles were separated on a 5% resolving gel. After isolation, the purity of each fragment was tested by analytical SDS–PAGE with silver staining. Each purified fragment was concentrated and transferred to 0.01% SDS–0.1 M ammonium bicarbonate by repeated ultrafiltration with Amicon membranes (PM 10 or PM 30).

Another approach was by electroblotting onto a PVDF membrane (Matsudaira, 1987): Fragments of the recombinant particles were first separated by analytical SDS–PAGE using a 10–18% linear gradient slab gel (14 × 16 cm, 1.5 mm thick) and a discontinuous buffer system (Laemmli, 1970). After SDS–PAGE, the polypeptides were electroblotted onto PVDF membranes for 45–90 min at a constant current of 0.5 A/gel in a blotting apparatus. The PVDF membranes were then stained with Coomassie Blue R-250, and the bands were cut out with a clean razor blade.

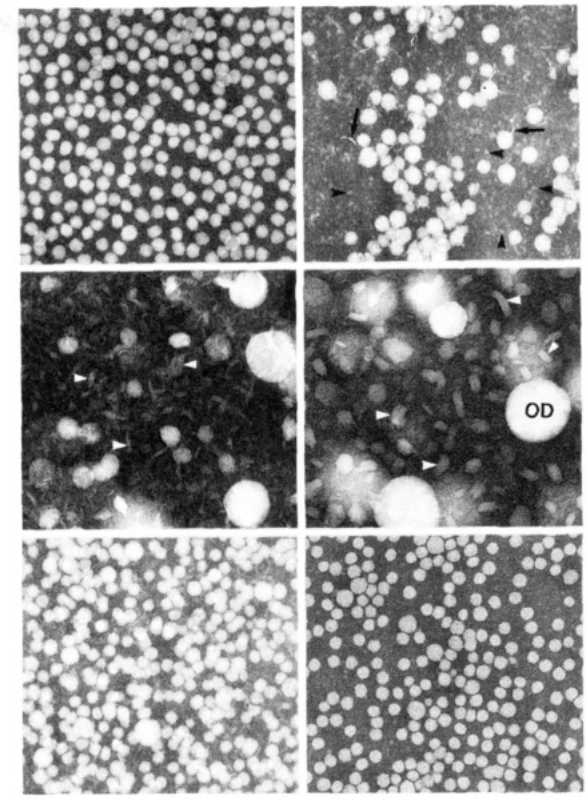

FIGURE 1: Electron micrographs (81000×) of negatively stained native LDL and recombinat LDL-like particles. Top left, untreated native LDL; top right, LDL treated with 0.5% sodium decyl sulfate and heated to 95 °C for 3 min (arrowheads show very small particles, and arrows point to disklike structures); middle, enzyme digests of detergent-treated LDL [arrowheads show irregularly shaped structures among oil droplets (OD)]; bottom left, recombinant spherical LDL- and VLDL-like particles formed after the removal of detergent from enzyme digests by dialysis; bottom right, centrifugally isolated recombinant LDL-like particles of B-100 fragments and endogenous lipids of LDL.

Amino-terminal amino acid sequence analysis of the purified fragments was performed on an Applied Biosystems Model 470 A protein microsequencer (Hunkapiller & Hood, 1983).

Analytical Methods. The concentration of protein was determined (Lowry et al., 1951) with hydrated bovine serum albumin as standard. The contents of cholesterol and cholesteryl ester were determined by an enzymatic procedure (Huang et al., 1975), those of phospholipids as lipid phosphorus (Stewart & Hendry, 1935), and those of triglyceride by the Sigma kit, modeled after an enzymatic procedure (Bucolo & David, 1973).

Results

Electron Microscopy. After 0.5% sodium decyl sulfate was added to native LDL and the solution was heated to 95 °C for 3 min, negatively stained samples showed that some LDL-like particles remained, together with some larger spherical particles, some smaller particles, and a few disk-like structures (Figure 1, top right panel). At higher concentrations of detergent (1%, for example), electron microscopy showed that only a few irregular particles remained (data not shown). When the treated LDL were digested with proteolytic enzymes (SP, EL, or CH) in the presence of 0.5% sodium decyl sulfate, the negatively stained digests contained scattered chylomicron-sized oil droplets together with pleomorphic, elongate

Table I: Chemical Composition of Native LDL and Recombinant LDL-like Particles

	protein	phospholipid	cholesteryl ester (% mass)	free cholesterol	triglyceride
native LDL	23	21	40	10	7
LDL-like particles[a]					
method I					
A	18	22	43	9	8
B	20	23	41	10	6
method II	20	22	58	0	0

[a] Recombinant particles prepared by each of two methods as described under Materials and Methods. Particles prepared by method I were B-100 fragments, generated by SP, EL, or CH digestion, bound to endogenous lipids and isolated either by density gradient (A) or by stepwise (B) ultracentrifugation. Particles prepared by method II were delipidated B-100 fragments (SP or EL digestion) bound to exogenous lipid microemulsions and were isolated by stepwise ultracentrifugation.

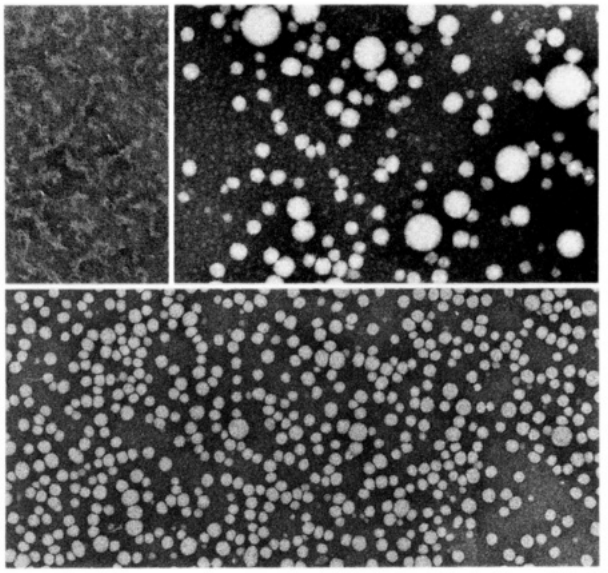

FIGURE 2: Electron micrographs (81000×) of delipidated B-100 fragments and recombinant particles. Top left, delipidated B-100 fragments in 1% sodium decyl sulfate; top right, recombinant particles from delipidated B-100 fragments and exogenous lipid microemulsions of egg phosphatidylcholine and cholesteryl oleate isolated in 1.06 g/mL top fraction; bottom, same experiment except recombinant particles of 1.06 g/mL top were reisolated in 1.02 g/mL bottom fraction.

structures with apparently flattened surfaces (Figure 1, middle panels). When detergent was removed from the three different enzyme digests by dialysis (method I), apparently spherical particles reappeared with diameters predominantly similar to those of native LDL and some VLDL-sized particles (Figure 1, bottom left panel). After fractionation by stepwise or density gradient ultracentrifugation, the isolated recombinant particles appeared to be mainly similar to native LDL in shape and diameter (Figure 1, bottom right and top left panels). In contrast, the delipidated B-100 fragments of the enzyme digests had a fibrillar appearance (Figure 2, top left panel) similar to that reported for delipidated intact B-100 solubilized in sodium decyl sulfate (Gotto et al., 1968a). The recombinant particles prepared by incubating the delipidated B-100 fragments (from SP or EL digests) with preformed exogenous lipid microemulsions (method II) also appeared spherical. However, these particles spanned VLDL and LDL in size in the 1.06 g/mL top layer (Figure 2, top right panel) but were homogeneous and more similar in size to native LDL in the 1.02 g/mL bottom layer (Figure 2, bottom panel). The diameters of all these recombinant particles prepared by both methods were between 15 and 25 nm (19 ± 4 nm, mean ± SD).

Characterization of Recombinant LDL-like Particles. All the recombinant LDL-like particles prepared from the three different enzyme digests, like native LDL, exhibited β-electrophoretic mobility on agarose gel electrophoresis (not shown) and ranged in density from 1.02 to 1.035 g/mL (Figure 3).

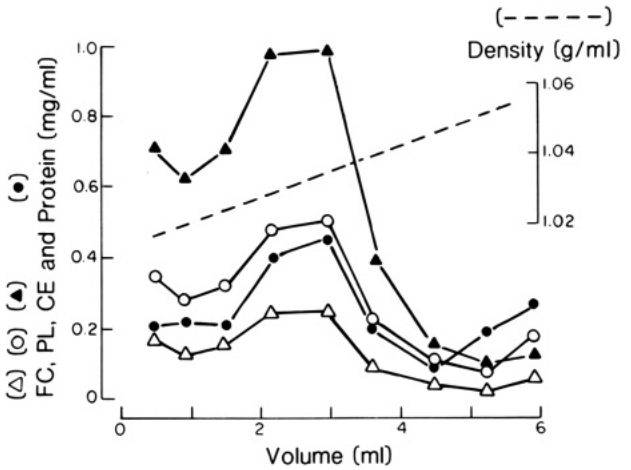

FIGURE 3: Separation by density gradient ultracentrifugation of recombinant LDL-like particles formed after the removal of detergent from enzyme digests. The top of the tube is to the left. Free cholesterol (FC) (△), phospholipid (PL) (○), cholesteryl esters (CE) (▲), and protein (●) concentrations were measured in the fractions.

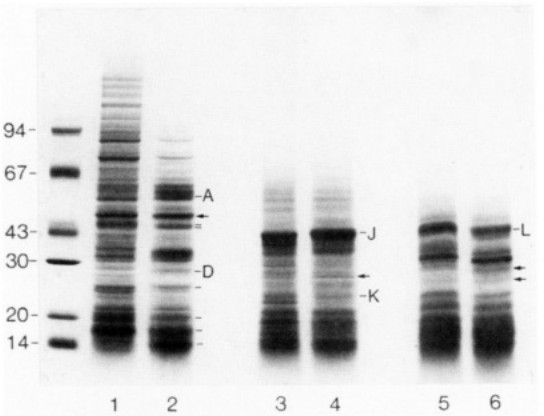

FIGURE 4: Analytical SDS–PAGE patterns. Lanes 1, 3, and 5, polypeptide fragments of enzyme digests of LDL by SP, EL, and CH, respectively. Lanes 2, 4, and 6, fragments of centrifugally isolated recombinant particles prepared by method I from SP, EL, and CH digests, respectively. The horizontal marks indicate fragments of the recombinant particles that have been isolated, sequenced, and positioned in the B-100 molecule as shown in Figure 7. From top to bottom: A, B, C, D, E, F, G, H, I (SP), J, K, (EL), and L (CH). The arrow in lane 2 indicates a multiple-component band, and the arrows in lanes 4 and 6 indicate three fragments recognized by antiserum 2404–2425. On the left, standard proteins for molecular mass estimation (in kilodaltons) are shown.

Moreover, chemical analysis showed that these recombinant particles had surface and core compositions similar to those of native LDL (Table I). It is noteworthy that the yellow color, due to β-carotene and characteristic of LDL in solution, remained in the recombinant complexes of endogenous lipids and B-100 fragments, indicating tight association of β-carotene with the core lipids.

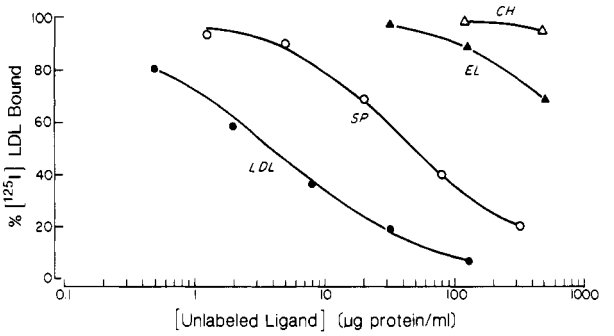

FIGURE 5: Displacement of ^{125}I-LDL bound to the LDL receptor of cultured human fibroblasts by unlabeled LDL and by recombinant particles prepared from SP, EL, and CH digests. Total binding data are shown. Each point shown represents the mean of duplicate determinations. Each curve shown is representative of studies performed with at least three different preparations of recombinant particles of LDL. The free ^{125}I-LDL concentration was 1.0 μg of protein/mL in each assay.

Analytical SDS-PAGE patterns of the enzyme digests showed that no intact B-100 remained after proteolytic digestion of LDL by SP, EL, or CH (Figure 4). Depending on the enzyme used, B-100 was digested into about 15–30 major components with mass from approximately 14 to 150 kDa (Figure 4, lanes 1, 3, and 5). The gel patterns also showed that most of the B-100 fragments, except the few largest ones from SP digests, could bind to lipids to form recombinant LDL-like particles (Figure 4, lanes 2, 4, and 6).

In equilibrium binding studies, ^{125}I-LDL bound to the LDL receptor on human fibroblasts with a K_D of 3.0 μg of protein/mL; the capacity of these receptors was 200 ng of LDL protein/mg of cell protein (data not shown). ^{125}I-LDL were displaced from these receptors by unlabeled LDL with an apparent K_D of 3.5 μg of protein/mL and by the recombinant LDL-like particles prepared from SP, EL, and CH digests with apparent K_D's of 35, >300, and >3000 μg of protein/mL, respectively (Figure 5). The observed differences in K_D's among the different recombinant particles would be at least qualitatively retained if concentrations had been expressed in units of molarity, since all these particles (including native LDL) had similar protein to lipid ratios and similar diameters.

The CD spectra of the recombinant particles showed a wide negative trough around 220 nm, which was similar to that of native LDL, and a deep negative trough around 208 nm, which was not present in native LDL (Figure 6). The general features of the CD spectra of particles prepared from the three different enzyme digests were essentially the same, except for slight variations in magnitude around 220 nm and somewhat larger variations in magnitude around 208 nm. Of the three enzymes used, the CD spectrum of particles prepared from CH digests had the largest negative 208-nm trough. The ratio of the magnitudes of the troughs at 208 to 220 nm was found to be 0.82 ± 0.02 (three different preparations) for native LDL, whereas it was 1.12 ± 0.06, 1.17 ± 0.06, and 1.23 ± 0.11 (five different preparations each) for particles prepared from SP, EL, and CH digests, respectively.

Amino Acid Sequencing and Immunoblotting. To determine the structure of the lipid-bound fragments in the recombinant particles, we have isolated 12 fragments, as indicated by horizontal marks in Figure 4, and subjected them to amino-terminal sequence analysis. Two fragments, designated fragments J and L from EL and CH preparations, respectively, were isolated by preparative SDS–PAGE and analyzed. Nine fragments (A–I) from SP and two fragments (J and K) from EL preparations were isolated by electroblotting onto PVDF membrane following SDS–PAGE and analyzed. Fragment

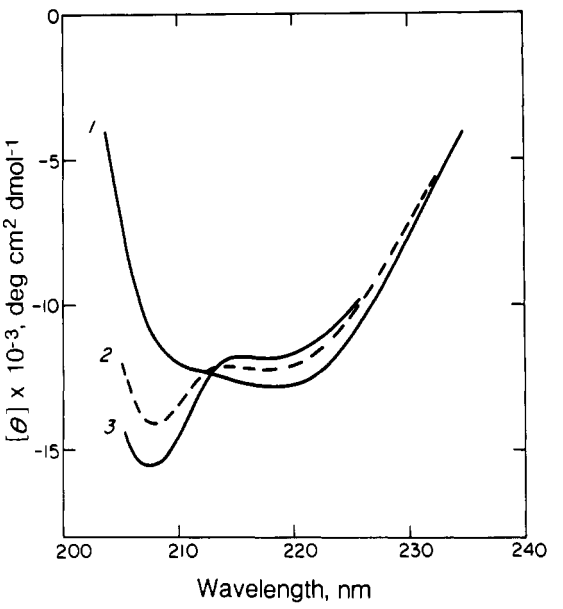

FIGURE 6: CD spectra of native and recombinant LDL-like particles in 50 mM NaCl/20 mM phosphate buffer, pH 7.5, at 25 °C. Curve 1, native LDL; curves 2 and 3, recombinant particles prepared from SP and CH digests, respectively.

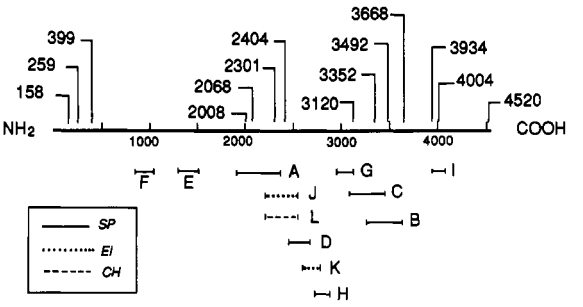

FIGURE 7: Location of the region-specific peptide antisera and the isolated lipid-bound B-100 fragments. The long horizontal line represents the B-100 molecule. The number above the long horizontal line designates the amino-terminal residue of the synthetic peptide. The isolated fragments (A–L) corresponding to the bands in SDS–PAGE pattern, denoted by horizontal marks (Figure 4), were analyzed by amino-terminal sequence analysis. The carboxyl-terminal boundary of the fragments was estimated from the molecular weight of the fragments, as determined by SDS–PAGE. Residues 3147–3157, 3359–3367 (Knott et al., 1986), and 3345–3381 (Yang et al., 1986) are the putative receptor-binding domains of B-100.

J from the EL preparation was thus determined by both approaches. The results of amino-terminal sequence analysis in combination with estimation of the molecular weight allowed positioning these fragments in the B-100 molecule (Figure 7). The molecular weights of the 12 fragments were estimated from their mobilities on SDS–PAGE. A mean residue molecular weight of 112 was used for the two amino-terminal fragments (E and F) and 120 was used for the other fragments, to calculate the length of the isolated fragments, because the amino-terminal region of B-100 was not as highly glycosylated as the rest of the molecule (Knott et al., 1986; Yang et al., 1986). The estimated molecular masses and boundaries are as follows: fragment A, 52 kDa (residues 1910–2344); fragment B, 45 kDa (residues 3240–3616); fragment C, 45 kDa (residues 3108–3484); fragment D, 29 kDa (residues 2419–2662); fragment E, 25 kDa (residues 1288–1512); fragment F, 22 kDa (residues 846–1043); fragment G, 20 kDa (residues 2961–3129); fragment H, 17 kDa (residues 2714–2857); fragment I, 15 kDa (residues 3945–4071); fragment J, 43 kDa (residues 2220–2579); fragment K, 24 kDa (residues 2584–2785); and fragment L, 43 kDa (residues 2222–2581).

In characterizing the isolated bands from SDS–PAGE patterns, often more than one polypeptide was contained within a seemingly pure band in the gel patterns. For example, fragments B and C (approximately 45 kDa) were found in a given electroblotted band on the PVDF membrane, and at least three components appeared in the band just above it, as indicated by an arrow (Figure 4, lane 2). To identify further the lipid-binding regions of B-100, we have employed the technique of immunoblotting, using 14 antisera raised against region-specific synthetic peptides of 17–40 amino acids in length. The synthetic peptide sequences are 158–186, 259–280, and 399–415 (Hardman et al., 1987) and 2008–2024, 2068–2091, 2301–2325, 2404–2425, 3120–3159, 3352–3371, 3492–3511, 3668–3687, 3934–3956, 4004–4021, and 4520–4536 (Innerarity et al., 1987). The results of immunoblotting showed that the amino-terminal 259–280 antiserum reacted with many fragments of SP, EL, and CH preparations but 158–186 and 399–415 antisera only reacted weakly with a few small fragments (<20 kDa) of SP, EL, and CH preparations. The central 2008–2024, 2068–2091, 2301–2325, and 2404–2425 antisera as well as the 3352–3371 antiserum reacted strongly with many fragments of different sizes (15–90 kDa) in all SP, EL, and CH preparations. The carboxyl-terminal 4520–4536 antiserum reacted only with fragments of approximately 20, 24, and 60 kDa from SP preparations and did not react with those of EL and CH preparations. On the other hand, antisera 3120–3159, 3492–3511, and 4004–4021 reacted quite strongly with many fragments of various sizes of SP and EL preparations, but they did not react at all with those of CH preparations. In addition, antisera 3668–3687 and 3934–3956 reacted strongly with many bands of EL preparations, but not with those of both SP and CH preparations. Seven representative patterns of immunoblotting with antisera 259–280, 2008–2024, 2301–2325, 2404–2425, 3120–3159, 3352–3371, and 3492–3511 are shown in Figure 8.

On the basis of the patterns of immunoblotting (Figure 8) and peptide mapping (Figure 4) in combination with sequence analysis (Figure 7), it is apparent that antisera 2301–2325 and 2404–2425 recognized fragments J (residues 2220–2579) and L (residues 2222–2581) but not fragment D (residues 2419–2662). Also, some bands on the SDS–PAGE pattern could be recognized by the immunoblotting study, resulting in the identification of their approximate positions within the B-100 molecule. For example, antiserum 2404–2425 appeared to react strongly with a band right above fragment K (Figure 4, lane 4) and weakly with two bands below fragment L (Figure 4, lane 6), as indicated by arrows, whereas antiserum 2301–2325 did not interact with the band right above fragment K but did interact strongly with the upper one of the two bands below fragment L, suggesting that the sequence of the upper band spanned these two antisera sequences within B-100 and that the amino termini of the other two bands recognized by antiserum 2404–2425 did not extend to 2301–2325. On the other hand, antiserum 2008–2024 recognized many fragments of various sizes of SP and EL preparations, including peptides the size of fragment A (residues 1910–2344), which was isolated from a broad band of SDS–PAGE pattern (Figure 4, lane 2). It is most likely that fragments of different sizes recognized by one region-specific antiserum represent overlapping peptides reflecting a variable extent of digestion of the B-100 molecule.

Discussion

We have shown that proteolytic fragments of B-100, with mass from approximately 14 to 100 kDa generated by three

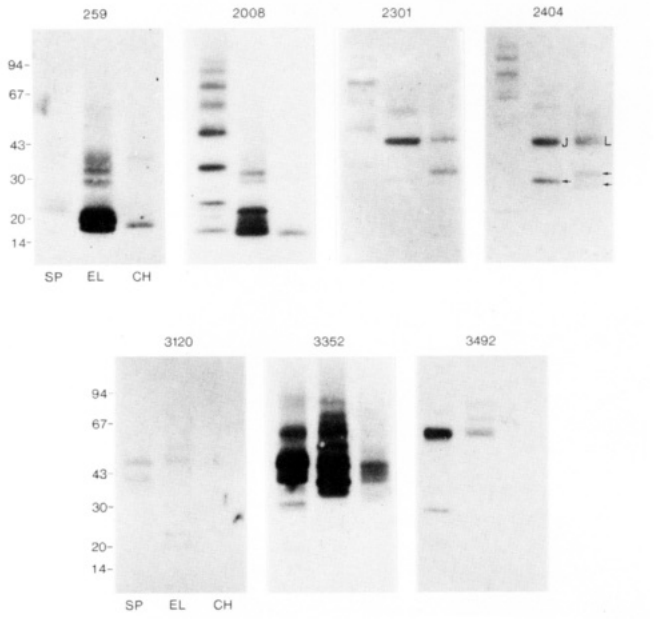

FIGURE 8: Immunoblots of B-100 fragments of the recombinant particles, separated by SDS–PAGE, using region-specific peptide antisera. The number above the lines designates the amino-terminal residue of the synthetic peptide. The bands denoted by arrows correspond to bands denoted by arrows in Figure 4. Approximate molecular masses (in kilodaltons) are indicated.

different proteases, can bind either to endogenous lipids of LDL or to exogenous lipid microemulsions of egg phosphatidylcholine and cholesteryl oleate. The resulting B-100 fragment–lipid complexes have shapes, diameters, electrophoretic mobilities, and chemical compositions similar to those of native LDL. Our results indicate that lipid-binding regions are widely distributed within the B-100 molecules.

The pleomorphic structures observed in the negatively stained enzyme digests (Figure 1, middle panels) indicate the great complexity of micellar formation with detergent in a multiple-component system. For example, in the presence of detergent, the proteolytic fragments of B-100 may expose more hydrophobic surface than the intact B-100 does, and, depending on the protease used, each enzyme digest contained a different array of B-100 fragments, which may be expected to interact somewhat differently with the several lipid constituents of native LDL, resulting in various mixed micelles of lipids, detergent, and polypeptides. Dialysis lowered the concentration of detergent and allowed reorganization of the mixed micelles to form particles. Remarkably, spherical particles similar to native LDL and VLDL appeared to be the major products formed (Figure 1, bottom left panel) with all three dialyzed enzyme digests, despite their varied initial morphologies. Furthermore, similar LDL-like particles could be formed from the interaction of delipidated B-100 fragments with preformed model lipid microemulsions of egg phosphatidylcholine and cholesteryl oleate (Figure 2, bottom panel). When the preformed model microemulsions were treated with 1–1.5% sodium decyl sulfate prior to interacting with the B-100 fragments, followed by heating the mixtures, heterogeneous particles of rounded and other shapes together with some fibrillar structures were formed after removal of the detergent (data not shown). Taken together, these observations suggest that the preformed model microemulsions or the mixed micelles of endogenous lipid constituents of LDL are the preferred environments for the B-100 fragments in the formation of spherical, recombinant LDL-like particles, consistent with the results of experiments in which naturally occurring lipoprotein particles (cholesterol-induced canine high-density lipoproteins)

were used as a lipid source to recombine with thrombolytic fragments of B-100 (Corsini et al., 1987). These observations are also consistent with previous reports showing that a variety of grossly different morphological structures can be formed by the interactions of B-100 and various amphiphilic ligands, depending on the chemical and physical states of the ligands (Atkinson & Small, 1986).

B-100 fragments from all three enzyme digests accompanied both the surface and core lipids during flotation at $d = 1.06$ g/mL and during sedimentation at $d = 1.02$ g/mL, indicating the formation of stable complexes. These complexes had core and surface compositions resembling those of native LDL. Since each enzyme has its own specific cleavage sites, different patterns of B-100 fragments were generated. The recombinant particles contained arrays of polypeptides reflecting the patterns of various enzyme digests (Figure 4). Although the gel pattern of the recombinant particles was enriched in several bands, most of the B-100 fragments could bind to lipids to form LDL-like particles.

In characterizing the lipid-bound fragments, we have isolated and sequenced 12 fragments with several overlapping sequences mostly in the central part of the B-100 molecule (Figure 7). These fragments represented approximately 50% of the total sequence of B-100 but apparently less than 50% of the lipid-binding bands observed in the SDS–PAGE pattern (Figure 4). The results of immunoblotting with antisera against specific regions of B-100 demonstrated that both amino-terminal and carboxyl-terminal fragments also bound to lipids. The observation that fragments B, C, D, E, H, and K are in the vicinities of proline-rich clusters with high amphipathic β-sheet potential (Knott et al., 1986) suggests that this structure is indeed involved in lipid binding. Taken together, the results of proteolytic fragment analysis and immunoactivity indicated that lipid-binding regions are distributed widely within the B-100 molecule.

The CD spectra of recombinant LDL-like particles showed helicity similar to native LDL based on the ellipticity around 220 nm and a markedly deep trough around 208 nm not present in that of native LDL (Figure 6). The appearance of a deep negative trough around 208 nm has been reported for intact LDL or delipidated LDL in the presence of 20 mM or higher sodium decyl sulfate (Gotto et al., 1968b, 1973), or sodium dodecyl sulfate (Scanu & Hirz, 1968), and for other proteins solubilized in ionic detergents (C.-S. C. Wu, personal communication). Thus, it seems possible that the observed spectral difference between native LDL and the recombinant particles was due to the presence of the small amount of detergent in the recombinant particles. However, it has also been shown that low levels of detergent, for example, 0.5 mM (Gotto et al., 1973) to 4 mM (unpublished observations) sodium decyl sulfate or 2 mM sodium dodecyl sulfate (Scanu et al., 1969), did not significantly alter the CD spectra of intact and delipidated LDL. Therefore, the fact that the concentration of detergent present in the recombinant particles used for CD measurements was estimated to be less than 0.2 mM suggests that there may be other explanations for the spectral alteration. Nevertheless, it should be noted that the interaction of B-100 fragments with lipids to form recombinant LDL-like particles occurred after exposure of the B-100 fragments to high concentrations (40–60 mM) of sodium decyl sulfate. During that process, the B-100 fragments might have assumed a different conformation, possibly less β-structure and more disordered form (Gotto et al., 1973), as indicated by much higher ratio of $[\theta]_{208}$ to $[\theta]_{222}$ (1.5 for enzyme digests before dialysis and 1.3 for the delipidated B-100 fragments dissolved in 40 mM sodium decyl sulfate), as compared with those of native LDL or recombinant particles.

It is of interest that the recombinant LDL-like particles prepared from the SP digest, but not from the CH or EL digests, appeared to have some LDL receptor-binding activity, on the order of 10% of that of native LDL. This may have been due to the presence of particular B-100 fragments in the recombinant particles prepared from the SP digest that were not present in the particles prepared from the CH and EL digests, or it may have been due simply to the fact that larger fragments were present in the particles prepared from the SP digest (Figure 4). As regards the former possibility, it should be noted that the recombinant particles prepared from the SP digest contained two identified fragments (B, residues 3240–3616, and C, residues 3108–3484; Figures 4 and 7) that are in the vicinity of the putative receptor-binding domains of B-100 (Knott et al., 1986; Yang et al., 1986). In assessing the binding data as a whole, it is important to realize that although the recombinant particles were obviously heterogeneous, equilibrium binding studies performed with heterogeneous ligands can still yield biologically relevant information. Under usual experimental conditions, the observed K_D in such a setting represents a weighted average of the K_D's of the different particles being studied (Mendel et al., 1985).

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Calitoxin, a Neurotoxic Peptide from the Sea Anemone *Calliactis parasitica*: Amino Acid Sequence and Electrophysiological Properties†

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ABSTRACT: We have isolated a new toxin, calitoxin (CLX), from the sea anemone *Calliactis parasitica* whose amino acid sequence differs greatly from that of other sea anemone toxins. The polypeptide chain contains 46 amino acid residues, with a molecular mass of 4886 Da and an isoelectric point at pH 5.4. The amino acid sequence determined by Edman degradation of the reduced, S-carboxymethylated polypeptide chain and tryptic and chymotryptic peptides is Ile-Glu-Cys-Lys-Cys-Glu-Gly-Asp-Ala-Pro-Asp-Leu-Ser-His-Met-Thr-Gly-Thr-Val-Tyr-Phe-Ser-Cys-Lys-Gly-Gly-Asp-Gly-Ser-Trp-Ser-Lys-Cys-Asn-Thr-Tyr-Thr-Ala-Val-Ala-Asp-Cys-Cys-His-Glu-Ala. No cysteine residues were present in the peptide. Similarly to other sea anemone toxins, calitoxin interacts, in crustacean nerve muscle preparations, with axonal and not with muscle membranes, inducing a massive release of neurotransmitter that causes a strong muscle contraction. The low homology of CLX with RP II and ATX II toxins has implications regarding the role played by particular amino acid residues.

The sea anemone *Calliactis parasitica* or *Calliactis tricolor* is a coelenterate normally present along the European coasts of the Atlantic Ocean. It grows on shells of the gastropod mollusc *Buccinum undatum* that are occupied by hermit crabs (Paguridae). In the Mediterranean Sea, *Calliactis parasitica* lives in symbiosis with the crab *Pagurus bernhardus* or *Pagurus policaris*.

The advantages postulated for anemone-bearing pagurids are camouflage, assistance in capturing prey, protection from

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