Phospholipid–Protein Interactions in Human Low Density Lipoprotein Detected by ^{31}P Nuclear Magnetic Resonance†

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ABSTRACT: ^{31}P nuclear magnetic resonance (NMR) spectra of human low density lipoprotein (LDL) have been obtained and the major phospholipid components identified. Analysis of the spectra revealed two phospholipid environments: one occupied by 4/5 of the phospholipid with high resolution resonances possessing properties similar to phospholipids in vesicles, and a second occupied by 1/5 of the phospholipid with broad lines indicative of immobilization. Limited trypsin treatment of the particle cleaved all of the B peptide into smaller molecular weight peptides which remained with the particle. Trypsin-treated LDL eluted from a Sepharose CL-6B column similarly to native LDL so that the modified particle remained intact. ^{31}P NMR spectra of trypsin-treated LDL showed little or no immobilized phospholipid. The immobilization in the native LDL particle is attributed to lipid–protein interactions between 1/5 of the phospholipid and the B peptide.

Human plasma low density lipoprotein (LDL),[1] a particle weighing approximately 2.5×10^6 daltons consists of about 20% protein and 80% lipid by weight. Although the lipid compositions of LDL preparations have been extensively characterized (see Morrisett et al., 1975, for recent review), much less is known concerning those interactions between the lipid and protein components which stabilize their association. It has been hypothesized that both hydrophobic interactions between lipid hydrocarbon moieties and nonpolar amino acid side chains, as well as electrostatic interactions between lipid head groups and charged amino acid residues, may play general roles in promoting lipid–protein associations in soluble lipoproteins (Morrisett et al., 1975) as well as in biological membranes. In the case of LDL, there is much less evidence concerning the nature of interactions between lipids and its major protein (B protein) than is known about associations between lipids and the C peptides of very low density lipoprotein (VLDL) or the A proteins of high density lipoprotein (HDL).

We now wish to report the results of examining human LDL by ^{31}P NMR. It has been found that in the native particle approximately 1/5 of the phospholipid ^{31}P resonances are greatly broadened, and the results suggest that this broadening results from lipid–protein interactions.

Materials and Methods

Acrylamide, 97%, was purchased from Aldrich Chemical Co. It was routinely recrystallized from boiling acetone prior to use. *N,N'*-Methylenebisacrylamide was purchased from Eastman; it was routinely recrystallized from acetone. Sodium dodecyl sulfate, purchased from Sigma Chemical Co., was recrystallized from boiling 95% ethanol prior to use. Agarose was purchased from Bio-Rad Laboratories. Sepharose CL-6B was a product of Pharmacia. TRTPCK trypsin, purchased from Worthington Biochemical Corp., was dissolved in cold 10 mM HCl just prior to use. Soybean trypsin inhibitor SI was a Worthington product. Fluorescein isothiocyanate, 10% on Celite, was obtained from Calbiochem.

Goat antiserum to normal human serum (batch 201 FO 42) was supplied by Kallestad Laboratories. Phenylmethanesulfonyl fluoride was obtained from Sigma Chemical Co. and ethylmercurithiosalicylic acid, sodium salt (thimerosal), was from Aldrich Chemical Co. Egg phosphatidylcholine was prepared and purified as described by Huang (1969).

Mixed lipid vesicles were formed by colyophilization and sonication in aqueous 0.1 M NaCl at 2 °C in a Heat Systems W-350 sonicator at 2 °C until a clear dispersion was obtained. Phospholipid concentrations were calculated from their phosphorus content as measured by the Bartlett (1959) method. Unless otherwise specified, protein concentrations were measured by the method of Lowry et al. (1951).

Human plasma low density lipoprotein ($1.019 < d < 1.063$, LDL) was prepared from several donors by adaptations of published methods (Hatch and Lees, 1968; Havel et al., 1955; Mahley and Weisgraber, 1974). Normal human blood was drawn into ACD solution and processed immediately. Cells and plasma were separated by centrifugation at 10 000*g* for 20 min in an International B-20 centrifuge. To each 100 mL of plasma were added 10 mg of thimerosal, 2 mg of phenylmethanesulfonyl fluoride in 0.4 mL of 1-propanol, and 100 mg of Na_2EDTA. The density of the protein-free solvent was measured as follows. Two-milliliter aliquots of plasma were heated in a steam bath for 2 min. After cooling to room temperature, the heated plasma was centrifuged for 3 min at 10 000*g*; 0.1-mL aliquots of protein-free supernatant were withdrawn into calibrated, weighed micropipets, and the weight of the contained liquid was obtained. The density was 1.005 g/mL. Twenty-five-milliliter portions were overlaid with $d = 1.005$ NaCl–KBr solution and centrifuged at 17 500 rpm in a 30 rotor of a Spinco Model L 3-50 ultracentrifuge for 30 min at 20 °C; the turbid chylomicron layer was withdrawn, the protein-free solvent density of the subnatant plasma was adjusted to 1.019 by addition of 4.3 mL of $d = 1.346$ solution per 100 mL of plasma, and the plasma was centrifuged at 29 500

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[1] Abbreviations used: LDL, low density lipoprotein; NMR, nuclear magnetic resonance; NOEE, nuclear Overhauser effect enhancement; PC, phosphatidylcholine; PS, phosphatidylserine; SPM, sphingomyelin.

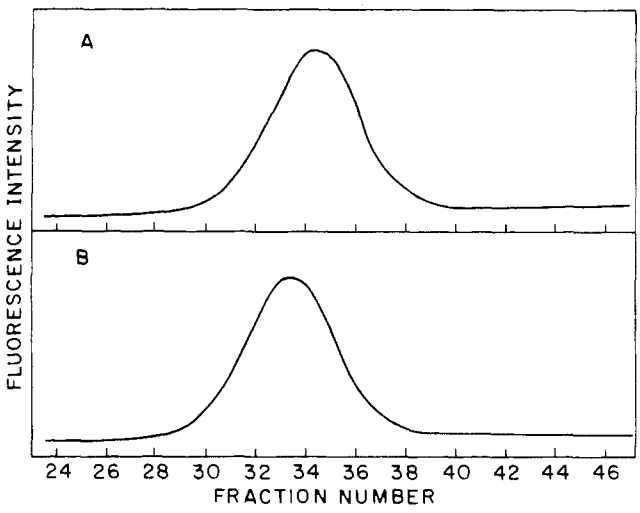

FIGURE 1: Gel chromatography of LDL and trypsin-digested LDL. Both samples were rendered fluorescent by treatment with fluorescein isothiocyanate and were chromatographed on a 1 × 52 cm column of Sepharose CL-6B as described in Materials and Methods. The top panel (A) depicts emergence of native LDL, and bottom panel (B) illustrates the emergence of trypsin-treated LDL from the same column.

rpm in a Spinco 30 rotor for 23 h at 15 °C. The turbid supernatant VLDL layer was withdrawn, and a sample of the clear, colorless, adjacent subnatant layer was utilized to check the protein-free solvent density; it was 1.019. The pelleted proteins were carefully resuspended in the remaining solvent. To each 100 mL of plasma was added 15.6 mL of $d = 1.346$ solution to yield a protein-free solvent density of 1.063, and the plasma was centrifuged at 30 000 rpm for 28 h in a Spinco 30 rotor. The top yellow layer, which was separated by several centimeters of clear, colorless fluid from the sedimented proteins at the bottom of the tube,was withdrawn from each tube and mixed with 10 volumes of $d = 1.059$ solvent. This was recentrifuged at 30 000 rpm for 18 h, and the yellow floating band of LDL was again removed; it was stored at 4 °C in a sealed serum bottle in the dark under nitrogen until used. Low density lipoproteins prepared in this way were used for the NMR studies and, as will be shown, were essentially free of other serum proteins or lipoproteins when examined by gel chromatography, immunoelectrophoresis, or by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the apoproteins. Lipoprotein concentrations were usually determined by dry weight measurements which had previously been found to give more reliable values for lipoprotein apoprotein concentration than either the Lowry method or the ultraviolet absorption (Margolis and Langdon, 1966a).

Tryptic digestion was carried out in the following manner. LDL solutions containing approximately 25 mg of lipoprotein (5 mg of apoprotein) per mL were dialyzed overnight at 4 °C vs. 100 volumes of 150 mM NaCl containing 10 mM $NaHCO_3$; after dialysis, the pH of the LDL solutions was 7.8. To 1.2 mL of a dialyzed lipoprotein solution was added 160 μg of trypsin in 10 μL of 0.01 N HCl. After 2.25 h at 25 °C, 560 μg of soybean trypsin inhibitor was added. The resulting digests were used for NMR studies, for gel chromatography, and for examination of the peptide composition of the trypsin-digested lipoprotein. Both native lipoprotein preparations and tryptic digests were chromatographed on a 1 × 52 cm Sepharose CL-6B column equilibrated with 150 mM NaCl buffered with 5 mM sodium phosphate (pH 7.0). In most cases, the preparation was made fluorescent by allowing it to remain in contact for 5 min with 1 μg of 10% fluorescein isothiocyanate on Celite; following this brief incubation, the Celite was removed by centrifugation. The fluorescent components in the column effluent were detected by an Aminco Fluoromonitor filter fluorimeter equipped with a flow cell and the fluorimeter output was recorded on a strip chart recorder.

Agarose immunoelectrophoresis against goat antiserum to normal human serum was carried out exactly as described by Hatch and Lees (1968). The antiserum gave the expected complex precipitin pattern against whole serum and gave precipitin lines in the expected positions when tested against purified VLDL, LDL, and HDL.

The apoprotein compositions of lipoprotein preparations and of tryptic digests were examined by polyacrylamide gel electrophoresis in 4–24% acrylamide gradients, using equipment resembling that described by Margolis and Kenrick (1968) purchased from the Gradipore Division of Isolab, Akron, Ohio. Gel slabs (100 × 75 × 4 mm) were cast having a concave gradient of 4–24% acrylamide cross-linked with 0.2–1.2% *N,N'*-methylenebisacrylamide in 40 mM Tris–glycine buffer (Arcus, 1970) containing 1% sodium dodecyl sulfate. The reservoir buffer was also 40 mM Tris–glycine buffer containing 0.1% sodium dodecyl sulfate. Lipoprotein samples containing approximately 1 mg of protein per mL were made 2% in sodium dodecyl sulfate by addition of 0.1 volume of 20% sodium dodecyl sulfate and were heated for 1.5 min in a steam bath. The cooled solution was made 2% in mercaptoethanol and 10% in sucrose. Aliquots of 20 μL were placed in each gel slot and electrophoresis was carried out at 30 mA per gel slab until the bromphenol blue tracking dye added to one of the samples had reached the bottom of the slab; this required 2.5–3 h. Occasionally, electrophoresis at 100 V was allowed to proceed for 18 h until the protein samples had reached their "pore limit" (Margolis and Kenrick, 1968). This allowed better resolution of larger proteins but allowed smaller peptides to leave the gel. Following electrophoresis, the gels were fixed and stained with Coomassie blue as described by Fairbanks et al. (1971).

^{31}P NMR spectra were obtained in 10-mm tubes using a JEOL PS 100 P/EC100 Fourier transform spectrometer at 23 °C and 40.48 MHz. Data points (4K) were obtained in the frequency domain with a spectral width of 5 kHz, using a JEOL 5-kHz RF crystal filter. Most spectra were obtained with successive 90° pulses (20 μs) incorporating a delay of 4–5 T_1 between pulses. T_1 measurements were made with the 180°–τ–90° pulse sequence, with continuous proton noise decoupling, using 7–9 data points and analyzed by least-square procedure. Nuclear Overhauser effect enhancements (NOEE) were measured using gated proton decoupling (Yeagle et al., 1975a). All spectra from which intensity measurements were taken were obtained with the proton decoupler gated to remove the nuclear Overhauser effect. Intensities were evaluated using computer integration plus cutting and weighing copies of the spectra. Each LDL spectrum consisted of about 4000 pulses collected in an 8–10-h period.

Results

As shown in Figure 1, over 98% of typical LDL preparation emerged as a single, symmetrical peak well within the included volume of Sepharose CL-6B column ($K_D = 0.33$). Using the fluorescein-labeling procedure, it was possible to detect contamination of less than 1%. Under the conditions with which this column was operated, control experiments showed that VLDL ($K_D = 0.15$) emerged prior to LDL and the two peaks were well separated. HDL emerged with a K_D of 0.65. Mixtures of VLDL, LDL, and HDL were easily separated by the column employed. Figure 1b shows the chromatographic be-

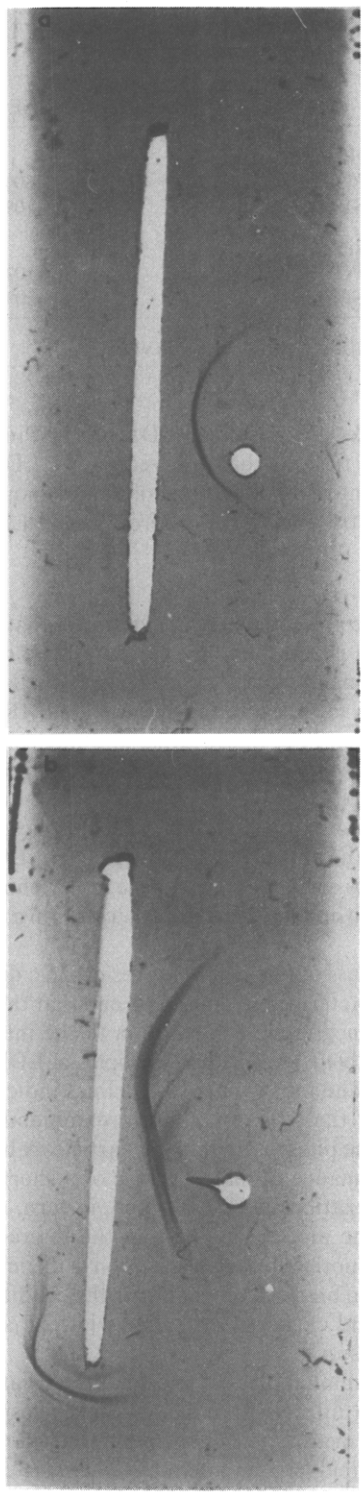

FIGURE 2: Immunoelectrophoresis of purified LDL and crude HDL vs. goat antiserum to human serum. Both pictures were taken after 4 days of diffusion vs. antiserum. The top panel (a) is purified LDL, the bottom panel (b), a d = 1.21 supernatant.

havior of trypsin-treated LDL. It emerged from the Sepharose CL-6B column at the same position as native LDL.

In Figure 2a, agarose immunoelectrophoresis of a typical LDL preparation used in these studies revealed only one precipitin arc when tested against antiserum to whole human serum. For comparison, in Figure 2b, immunoelectrophoresis of an impure HDL preparation against the same antiserum resulted in numerous precipitin arcs. Each LDL preparation

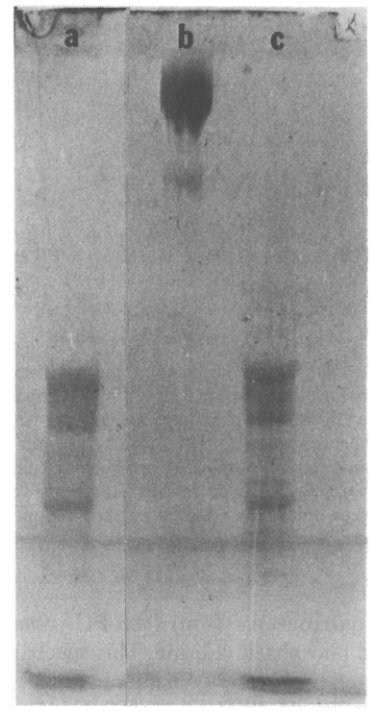

FIGURE 3: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of purified LDL, of trypsin-treated LDL, and of trypsin-treated LDL after gel chromatography. (a) Trypsin-treated LDL; (b) native LDL; (c) trypsin-treated LDL after gel chromatography; this represents material eluting in the major peak of Figure 1B.

used in these experiments was found to give a single precipitin line under the same circumstances.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the apoproteins in an acrylamide gradient, as illustrated in Figure 3 for a typical preparation, uniformly revealed the presence of a major protein component of about 2.5×10^5 daltons and a minor component having a mass of 1×10^5 daltons. These protein components have been regularly observed in many different LDL preparations, and it is thought that they both are intrinsic protein components of the LDL particles because they are reproducibly present in LDL prepared by density flotation, molecular sieve chromatography, polyanion precipitation, or a combination of these methods. These apoproteins have been separated and characterized, as will be reported elsewhere (Kuehl et al., 1977). It is important to note that no C or A peptides, characteristic of VLDL and of HDL, were present in detectible amounts, and that no other protein bands corresponding to major serum proteins were found. It thus seems clear that the protein and lipid components present in those experiments were indeed contributed exclusively by LDL.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of trypsin-treated LDL apoprotein (see Figure 3) demonstrated complete cleavage of the B protein into several peptides of lesser molecular weight. Gels run both before and after Sepharose CL-6B column chromatography indicated that all large resulting peptides remained with the particle, and that only a very small percentage by weight of the protein was removed as small peptides.

The ^{31}P NMR spectrum of normal, whole LDL appears in Figure 4a. The major phospholipids of LDL are phosphatidylcholine (PC) and sphingomyelin (SPM). By comparison with chemical shifts in vesicles (Berden et al., 1974), the upfield peak can be assigned to PC and the downfield peak to SPM,

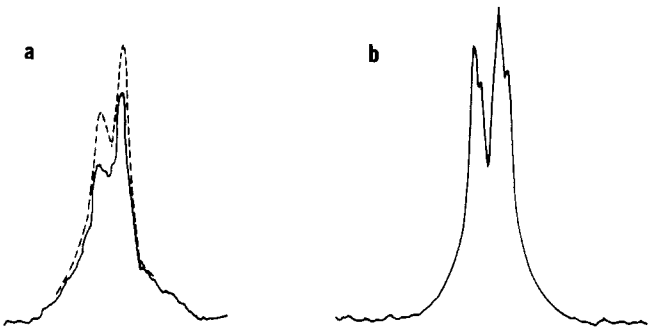

FIGURE 4: (a) ^{31}P NMR spectrum of LDL; (—) native LDL; (- - -) trypsinized LDL. Concentration is about 10 mg/mL protein. Upfield peak is phosphatidylcholine and downfield peak is sphingomyelin. The magnetic field increases to the right. Separation between the two peaks is 0.3 ppm. (b) ^{31}P NMR spectrum of PC/SPM (1.2:1) vesicles. Upfield peak is phosphatidylcholine and downfield peak is sphingomyelin. Structure within each peak is due to phospholipids on the inside of the vesicles possessing a slightly upfield chemical shift relative to phospholipids residing on the exterior of the vesicles.

with small contributions from lyso-PC, phosphatidylethanolamine, and phosphatidylserine. This spectrum is similar to spectra reported previously (Assman et al., 1974; Henderson et al., 1975). Figure 4b shows a ^{31}P NMR spectrum of a PC/SPM (1.2:1) vesicle for comparison. Properties of the lipid head groups in these two systems are similar, as reflected in Table I.

The substantial NOEE observed for LDL necessitates that care be taken to eliminate the NOEE when obtaining intensity measurements, which can be done by suitably gating the proton decoupler (Yeagle et al., 1975a). In these experiments a known amount of HPO_3^-, which has a chemical shift well downfield of the phospholipids, was added as an internal intensity standard. Areas of the HPO_3^- and sample resonances were compared to determine the concentration of phospholipids contributing to the high resolution spectrum of LDL. This value was compared with the phosphate concentration determined chemically (aliquots for phosphate analysis were removed from the sample before the addition of HPO_3^-) which allowed the fraction of phospholipid not seen in the high resolution spectrum of LDL to be calculated. The average of four determinations in samples from three different donors is 21%. Henderson et al. (1975) reported that all the phospholipid in LDL contributed to the high resolution spectrum, but they did not correct for the NOEE. The NOEE for the pyrophosphate standard used in their work has been measured (Yeagle et al., 1975a) and, making appropriate corrections to both the standard and LDL for NOEE, we calculate that about 26% of the phospholipid is not seen, a figure which agrees well with the value reported here. Finer et al. (1975) calibrated ^1H spectra of the *N*-methyl resonance due to PC and SPM in porcine LDL and concluded that 28% of the phospholipid was not contributing to the high resolution spectrum, an amount similar to the results reported here.

The trypsin-treated LDL described above was subjected to the same analysis as native LDL, and values of 1–4% for the phospholipid not contributing to the high resolution spectrum were obtained (see Figure 4). The result is markedly less than the 21% in native LDL.

Comparing linear additions of computer-generated Lorentzian lines with the spectra of LDL before and after trypsinization demonstrated that a change in intensity of both SPM and PC ^{31}P resonances in the LDL spectrum was necessary to explain both spectra. Removing 21% of the intensity from both peaks in trypsinized LDL resulted in a simulated spectrum that fit well the observed native LDL spectrum. Reducing the intensity of only one of the resonances produced simulated spectra quite different from native LDL.

TABLE I: ^{31}P NMR Parameters for Components of LDL and Small Vesicles.

	T_1[a] (s)	NOEE (%)[b]
LDL		
PC	1.6	60%
SPM	1.7	40%
Vesicle[c]		
PC	1.3	40%
SPM	1.9	20%

[a] ±20%. [b] ±10%. [c] Yeagle et al. (1976b).

Discussion

The evidence presented here demonstrates that phospholipid head groups in native LDL are present in two distinctly different environments. One class of phospholipids, comprising about 4/5 of the total, exhibits narrow ^{31}P NMR resonances. Chemical shifts, T_1's, line widths, and nuclear Overhauser effect enhancements of the narrow lines of LDL are similar to PC/SPM vesicles (Table I). Since these parameters are a measure of conformation and motion of the phosphorylcholine head group of the phospholipid (Yeagle et al., 1975b, 1976b), the head groups of the majority of the major phospholipids in LDL exist in an environment similar to that in a phospholipid vesicle.

The other class of phospholipids resides in a different environment, characterized by lines so broad that these phospholipids make a negligible contribution to the intensity of the narrow lines. About 1/5 of the phospholipids of LDL exist in this environment. The great width of the lines indicates that the head groups of these phospholipids are immobilized relative to the rest of the phospholipid in the particle. A broadening of the NMR resonance is characteristic of a shortening of the transverse relaxation time, T_2, which, in turn, reflects a retardation of the motion modulating the interactions determining T_2. A more detailed description is difficult, since the resonance is too broad to detect under the conditions of these experiments and chemical shift, line width, T_1s and NOEE cannot be measured.

This latter, smaller class of phospholipids that gives rise to broad lines is so different from the rest of the phospholipids that it appears to be in a special environment which our data suggest is provided by lipid–protein interactions. First, a change in size of the LDL particle or state of aggregation cannot explain the results since both the native particle and the trypsin-treated particle behaved as unaggregated particles of similar size on a Sepharose CL-6B column. Second, removal of immobilization of 1/5 of the phospholipid by trypsin treatment of the LDL particle, which resulted in cleavage of the protein into smaller peptides, indicates that the original source of the immobilization was the B protein of LDL, and that immobilization requires an intact polypeptide chain.

The LDL protein probably binds strongly both PC and SPM since the intensity of both ^{31}P resonances is sensitive to trypsinization. The experiments were insensitive to the behavior of minor lipid components like phosphatidylethanolamine or phosphatidylserine, so the degree of specificity for

the choline moiety cannot be defined. The tightness of binding of the phospholipid to the protein is reflected in the clear distinction between the two phospholipid environments. The phospholipids appear to be in the slow exchange limit since the narrow resonance component is not perturbed in any way by the immobilized component. If the rate of exchange between the two environments was other than slow on the NMR time scale, the narrow component would have been broadened.

A question still remaining concerns the nature of the lipid–protein interactions causing the observed immobilization. Results from cytochrome *c* binding to negatively charged phospholipid vesicles, which are of similar size to LDL particles, demonstrates that electrostatic interactions between phospholipid head groups and charged amino acid residues on the protein can cause line-broadening effects (Yeagle, manuscript in preparation). However, phospholipid vesicles of dipalmitoylphosphatidylcholine also exhibit line broadening effects when lowered below the phase transition temperature (Uhing, 1975), so that chain immobilization can cause ^{31}P line broadening as well as head-group immobilization. Since ^{31}P T_1 could not be measured for the immobilized phospholipid, no direct evidence is available to distinguish between head group and chain immobilization by the protein.

The means by which the immobilization is disrupted provides a clue to the nature of the lipid–protein interactions. Trypsin treatment cleaves all the B peptide and almost all the fragments remain with the LDL particle. It is therefore unlikely that immobilization of 1/5 of the phospholipid involves portions of the B peptide which are removed by trypsin; rather the protein probably changed its conformation after trypsin treatment in such a way that the phospholipid was no longer immobilized. At the same time, most opportunities for lipid–protein interactions must remain viable since the lipoprotein particle remains intact after tryptic cleavage. One way in which this could be accomplished is to conceive of the B protein as a collection of covalently linked "subunits," which can immobilize small phospholipid pools, or individual phospholipids, in crevasses between protein components, and which when cleaved by trypsin, separate, eliminating immobilization of the phospholipid. Phospholipid constrained in this manner by protein would not be expected to exchange rapidly with bulk phospholipid, which is what is observed experimentally. A lack of preferential binding, as is apparently seen in the immobilized phospholipid component, would also be expected.

Preliminary experiments with human high density lipoprotein show no comparable immobilization of phospholipids. A similar conclusion can be drawn from the data for porcine high density lipoprotein (Finer et al., 1975). Thus the B peptide appears to have a special way of interacting with phospholipids not possessed by the major peptides of high density lipoprotein.

It has recently been suggested that the B protein forms a fundamental structure which persists through the VLDL–LDL life cycle (Havel, 1975), and the model described here may provide a plausible mechanism for that behavior. The B protein and 1/5 of the phospholipid interact strongly to form a phospholipid–protein complex which has the potential of organizing orientation and association of the B protein with larger amounts of lipid, propagated by the amphipathic nature of the bound phospholipid. This complex might form the fundamental structure suggested and play a role in the initial biosynthetic organization of the lipoprotein particle.

Acknowledgments

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