FLUORESCENCE AND CIRCULAR DICHROISM STUDIES ON HUMAN SERUM LOW DENSITY LIPOPROTEIN PARTICLES AND LIPID-DEPLETED DERIVATIVES

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The low density liporpotein from human serum, and derivitives prepared free of neutral lipids and total lipids, have been studied by fluorescence and circular dichorism methods. Removal of the neutral lipids had little effect on the tryptophan fluorescence at neutral pH. However, by the criteria of circular dichroism, over the range of 200 nm to 250 nm, there was a reduction in secondary structure of over 75%. Removal of the remaining phospholipids resulted in a qualitatively different structure by both fluorescence and circular dichroism criteria.

Neutral lipids were removed from LDL in a step-wise fashion in order to determine the exact amount of neutral lipid required for the native circular dichroism spectrum. The circular dichroism band intensity was constant until approximately 10% of the total cholesterol (as cholesterol ester) remained. The intensity then abruptly dropped as more cholesterol was removed.

We concluded that the two spectroscopic methods report on two distinct aspects of LDL structure. The tryptophan fluorescence appears to be sensitive to the presence of phospholipids. The circular dichroism, however, appears to be sensitive to the binding of a small amount of neutral lipid. These findings suggest that a functional and geometric separation of binding sites may exist for these two classes of lipids. Such a distinction is predicted by the icosohedral model of the quaternary structure of LDL. In this model, the phospholipids are located on the surface of the particle, in the holes of an icosohedrally symmetric surface network of protein subunits; the neutral lipids are located in the particle core.

Finally, we suggest that functional significance may be attached to our finding that relatively few cholesterol ester molecules are needed to maintain the native secondary structure of LDL. This provides a mechanism whereby the amount of bound neutral lipid could be raised or lowered (for transport and transfer to cells) without affecting the protein in any structurally significant manner.

INTRODUCTION

Studies on the structure of the low density lipoprotein particle (LDL, $1.019<d<1.063$) from human serum have been widely pursued. It is the functional basis of lipid transport in most mammals and has been implicated in the etiology of atheroscleorosis (1, 2). The particle also has been viewed as a natural model membrane (3). Recent electron microscopic (4, 5) and X-Ray diffraction (6) studies on LDL have led to a consistent structural

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model for the particle. Protein subunits are arranged as an icosohedrally symmetric surface network, with phospholipids in the holes of the net. The neutral lipid component is assumed to be located in the particle core. Studies on the thermal dependence of denaturation of LDL have shown that the quaternary structure (4) and the structural stability of the protein component (7) are dependent on the presence of all constituent lipids. However, the nature of the contributions of the neutral lipid core and of the surface phospholipids to the total organization are not understood.

We have approached this problem by studying the structural consequences of the stepwise removal of phospholipid and neutral lipid from LDL particles. The structural changes were monitored using two independent spectroscopic methods: fluorescence of the tryptophan chromophore and circular dichroism. As predicted by the model for quaternary structure, the removal of the two different classes of lipid had markedly different structural consequences. A preliminary account of these studies has been recently published (8).

MATERIALS AND METHODS

Purification of LDL

LDL was prepared on equilibrium sucrose gradients in the presence of 0.001 M EDTA and 0.01M borate, pH 9.1. Solutions of serum or purified fractions of LDL were adjusted to density 1.08 with solid sucrose. Density was measured by refractometry. Centrifuge tubes were filled one-half full of density 1.006 sucrose in buffer. Dense solutions of LDL were then injected by syringe under the less dense layer. Tubes were spun for 18 hours at 16°C in a type 30 or 42 rotor on a Beckman L2-65B ultracentrifuge. LDL was recovered by puncturing the bottom of the tube and collecting serial fractions. The process was repeated until the LDL was pure by immunological and electrophoretic criteria. The average density of the LDL was 1.040.

Extraction of Lipids

Neutral lipids were selectively extracted from LDL as previously described (4). Briefly, 1 ml samples of LDL, containing 0.5–1.0 mg/ml protein, were mixed with 50 ml of peroxide-free diethyl ether and agitated gently at 4°C on a rotating wheel. Extraction was performed in a glass-stoppered test tube, and air bubbles were excluded. Complete extraction was achieved by continuing the extraction for 24 hours. Partially delipidated species of LDL were obtained by stopping the extraction at different times. The neutral-lipid-free LDL was termed NLF-LDL.

Lipid-free LDL (apo-LDL) was prepared by extraction of lipids with chloroform-methanol (2:1, v/v) and the protein was solubilized from the ether powder at pH 11.5. The exact procedure was previously described (7).

Fluorescence and Circular Dichroism

Fluorescence measurements were performed on a modified Aminco-Bowman spectrofluorometer that was linear over the range of 300 to 400 nm (9). Spectra were corrected for self-absorption for both the exciting and emitted rays. Curvettes were 0.3 cm in length. Cicular dichroism spectra were obtained using the Cary 60 spectropolarimeter. Path length was 0.49 mm. For most studies, the circular dichroism and fluorescence data were obtained from the same sample.

Protein solutions were adjusted to specific pH values by addition of known volumes of NaOH using a radiometer pH meter equipped with a B-type single electrode (Thomas). Over the pH range studied no corrections for Na+ ion activity were necessary. An interval of at least 30 minutes was allowed to elapse between adjustment of pH and recording of spectra. This was done in order to minimize kinetic effects.

Chemical Analysis

Protein was determined by the method of Lowry (10) using crystalline bovine serum albumin as standard. Total phosphorus was determined by the method of Fiske and Subba-Row (11). Total cholesterol was determined by the method of Zak (12). Semiquantitative estimates of specific lipids were obtained by thin layer chromatography. Briefly, samples were delipidated with chloroform: methanol (2:1, v/v) and spotted on 250 micron thick layers of silica gel. Phospholipids were developed with a solvent consisting of chloroform: methanol: acetic acid: water (65:25:8:2, v/v). Neutral lipids were developed with a solvent of petroleum ether: diethyl ether (95:5, v/v). Spots were visualized with iodine vapor.

RESULTS

Fluorescence Studies

The intrinsic fluorescence of proteins, due mainly to tryptophan residues, has widely been used as an indicator of protein structural features (13). The fluorescence emission spectra for LDL, neutral-lipid-free LDL (NLF–LDL) and lipid-free LDL (apo-LDL) are shown in Fig. 1. The areas under the curves (except for the scatter peaks) are proportional to the relative quantum yields. The emission maximum for LDL was 330 nm compared with 333 nm for NLF–LDL, but the quantum yields for the two species were nearly the same. However, further removal of the phospholipids to give apo-LDL results in an emission maximum of 334 nm and a reduction in quantum yield of about 30%. The removal of lipids thus is accompanied by a shift in fluorescence to longer wavelengths, suggesting that the tryptophans are now in a more polar environment. However, even an emission maximum of 334 nm is characteristic of a significantly more hydrophobic environment than available to a fully exposed tryptophan residue which would emit maximally at 340–345 nm (14, 15).

The scatter peaks shown in Fig. 1 are roughly proportional to the molecular size of the individual species, as would be expected from light scattering theory (16).

Although the quantum yield of apo-LDL is significantly less than that for LDL, the fluorometric titration data (Fig. 2) suggest that they are both rather stable to changes in pH. There are no sharp breaks in the titration curves; this argues against any major conformational transitions over a wide range of pH. The quenching at extremes of pH can be attributed to quenching by – COOH groups and by ionized tyrosyl residues (13). The differences in the curves noted in the alkaline region may reflect differences in the accessibility of tyrosyl groups; however, variations in stability towards alkali cannot be ruled out. No data for NLF–LDL in the acid region were obtained due to precipitation of the protein.

Circular Dichroism Studies

The circular dichroism spectrum of lipoproteins, over the range of 250 to 180 nm,

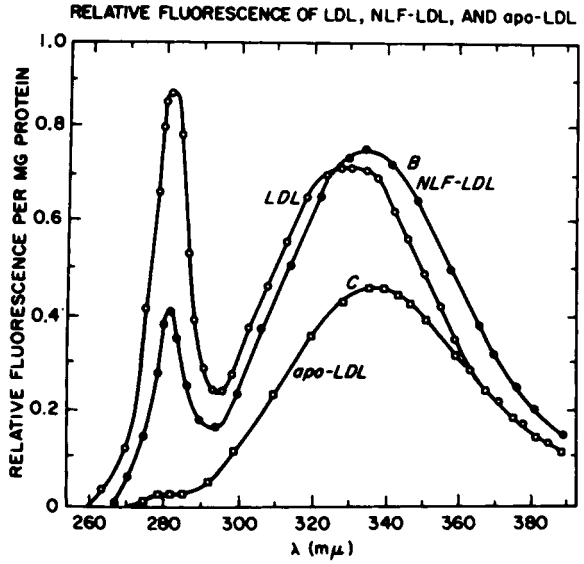

Fig. 1. Fluorescence emission spectra of LDL and delipidated derivatives. Excitation was at 278 nm. Samples were dialyzed vs. 0.01 M borate buffer pH 9.1 containing 0.001 M EDTA for 24 hours and clarified by centrifugation.

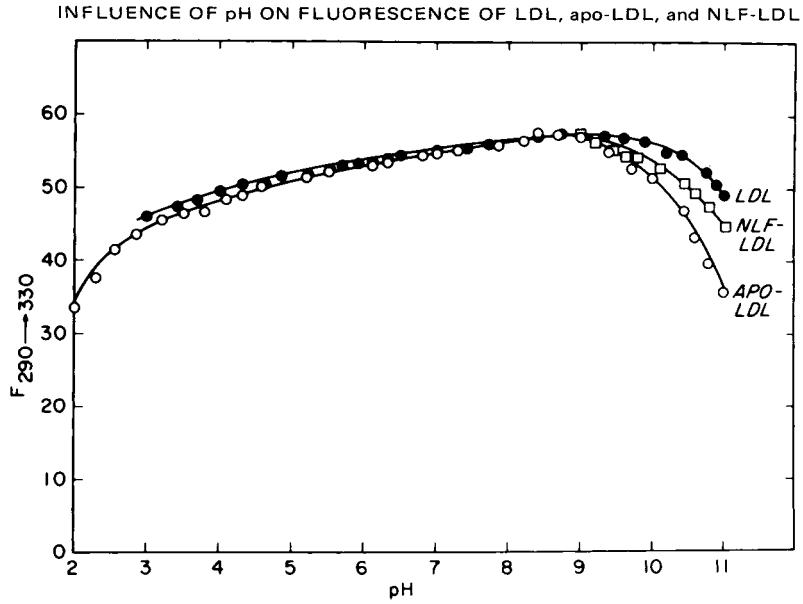

Fig. 2. Influence of pH on the fluorescence of LDL and derivatives. The solutions initially contained borate buffer, pH 9.1, as in Fig. 1. pH was vaied by addition of 1 M HCl or KOH. Excitation was at 290 nm and the emission of each species was monitored at its emission peak. Protein concentration, 0.8 mg/ml. Volume corrections were small. Temp., 27°C.

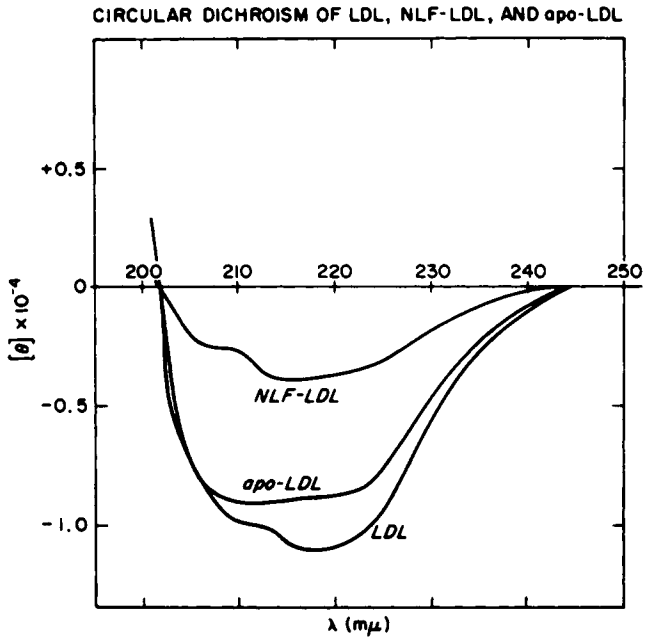

Fig. 3. Circular dichroism of LDL, NLF–LDL and apo-LDL.

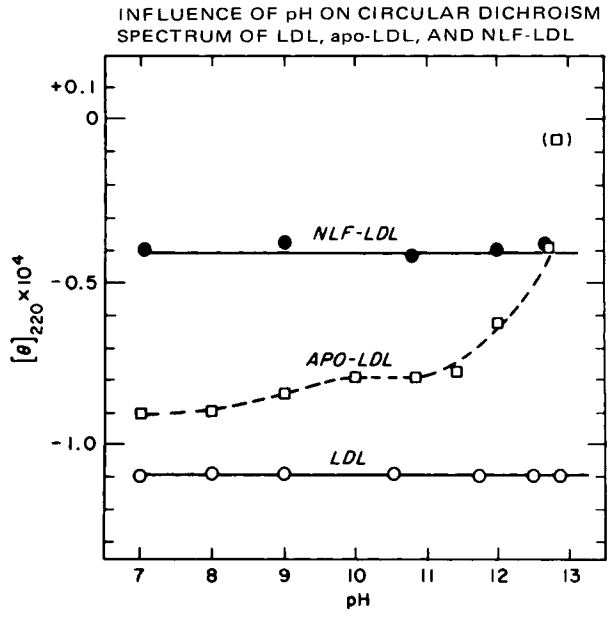

Fig. 4. Influence of pH on circular dichroism spectrum of LDL, NLF–LDL, and apo-LDL.

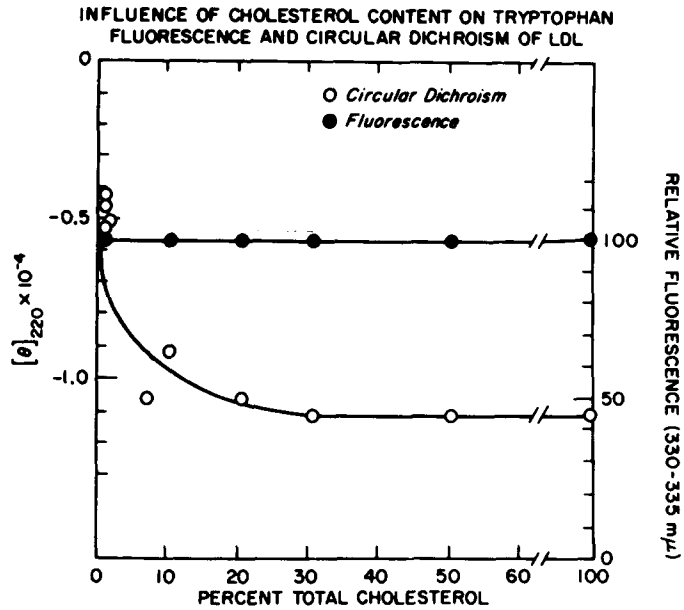

Fig. 5. Influence of cholesterol content on tryptophan fluorescence and circular dichroism of LDL.

has been used as a measure of protein secondary structure (7). The circular dichroism spectra for LDL, NLF–LDL, and apo-LDL at pH 9.0 are shown in Fig. 3. The striking finding here is that apo-LDL is apparently much more ordered than the NLF–LDL, but slightly less ordered than the native LDL. Absorption flattening as a possible cause for the differences in intensity could be ruled out since the optical density of these species in solution was negligible at 570 nm and 720 nm.

In order to assess the stability of the secondary structure, circular dichroism spectra were obtained over the pH range of 7–13. A graph of ellipticity at 220–222 nm over this range of pH is shown in Fig. 4. The secondary structures of both LDL and NLF–LDL are stable to elevation of pH as far as pH 13, though the spectra are qualitatively and quantitatively different. By contrast, the apo-LDL is quite susceptible to denaturation by elevated pH. The denaturation of apo-LDL was found to be reversible.

Influence of Neutral Lipid Content on Structure

The marked dependence of the circular dichroism spectrum on the presence of neutral lipids led us to examine LDL from which various amounts of neutral lipids had been extracted. This is shown in Fig. 5, where as a control the fluorescence of each species was also measured. The total cholesterol was taken to represent the neutral lipid component. The intensity of the circular dichroism band at 220 nm was found to remain constant until approximately 10% of the total cholesterol remained. Thin layer chromatography revealed that the residual cholesterol was solely esterified, and all triglyceride was removed. In each case, the percentage of cholesterol was related to the amount of protein measured in each sample. A small amount of phospholipid was lost by extraction procedure (5%); however, this amount was lost by the time that 50% of the total cholesterol had been removed. Therefore, phospholipid loss could not be implicated in the loss in secondary structure that occurred when the last 10% of the cholesterol ester was removed from LDL.

DISCUSSION

These data suggest that phospholipids and neutral lipids apparently influence different aspects of LDL structure. Structural modifications in LDL protein induced by removal of phospholipids appear to be specifically monitored by the fluorescence of the tryptophan chromophore. However, the influence of neutral lipids on LDL protein structure appear to be monitored exclusively by the circular dichroism spectrum from 200 nm to 250 nm. These findings suggest that the two lipid classes play different structural roles in LDL. They may be bound at physically distinct sites in the LDL particle, as, in fact, is predicted by the now widely accepted icosohedral model for LDL (3).

The icosohedral model for LDL is derived from electron microscopic and X-Ray studies showing that the essential structure of LDL is based on a protein network of globular subunits arranged on the surface of a sphere with icosohedral symmetry. Phospholipids are located on the surface of the particle, in the holes of the protein network. By contrast, neutral lipids, mainly cholesterol esters, are located in the core of the particle. Interpreted in terms of this model, the fluorescence data report on that portion of the protein near the surface of the particle, proximal to the phospholipids. The circular dichroism data report on protein structure towards the center of the particle, proximal to the neutral lipids. We may also infer that the protein region surrounding the phospholipid reporter chromophore is less ordered than the region influenced in secondary structure by neutral lipids.

Only a few neutral lipid molecules are needed to maintain the circular dichroism spectrum of LDL. This finding may be of great potential importance for the understanding of LDL function. The data in Fig. 5 show that only 10% (or 20 molecules) of cholesterol ester per particle interact with, or are responsible for the maintainance of, the ordered structure defined by the circular dichroism spectrum. The rest of the neutral lipid molecules in the native LDL may simply interact with other neutral lipid molecules in the particle interior. This means that a large variation in the neutral lipid content of LDL can be accommodated without concommitant changes in protein structure. Such a mechanism would allow LDL to bind and transfer neutral lipid for eventual release without large energetic demands such as those involved in protein conformational changes.

These chemical studies do not, of course, constitute independent evidence for the icosohedral model of LDL. They are merely consistent with such a model. However, these studies do show that the different lipid classes intereact with different geometric aspects of the protein moeity of LDL. Alternate models for LDL, that may be developed in the future, must accomodate this finding.

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