Microphase Separation in Low Density Lipoproteins

EVIDENCE FOR A FLUID TRIGLYCERIDE CORE BELOW THE LIPID MELTING TRANSITION*

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Magdalena Preggetter, Ruth Prassl, Bernhard Schuster‡, Manfred Kriechbaum, Fabienne Nigon§, John Chapman¶, and Peter Laggner§

From the Institut für Biophysik und Röntgenstrukturforschung, Österreichische Akademie der Wissenschaften, A-8010 Graz, Austria, §INSERM, Unité 321, Unité de Recherches sur les Lipoproteines et l’Atherogenèse, Hôpital de la Pitié, 75654 Paris Cedex 13, France, and the Center for Ultrastructure Research and Ludwig Boltzmann-Institute for Molecular Nanotechnology, Universität für Bodenkultur Wien, A-1180 Vienna, Austria

The structural organization of the neutral lipid core in human low density lipoproteins (LDL) was investigated in physicochemically defined, distinct human LDL subspecies in the density range of 1.0244–1.0435 g/ml by evaluation of the core lipid transition temperature, chemical composition, and the behavior of spin-labeled core lipids. Calorimetric studies were performed on more than 60 LDL preparations, and the transition temperature, which varied between 19 and 32 °C, was correlated to the chemical composition and revealed a discrepancy at a critical cholesteryl ester to triglyceride ratio of approximately 7:1. For electron spin resonance studies, several LDL preparations were probed with spin-labeled cholesteryl esters and triglycerides, respectively. In LDL with a high triglyceride content, both labels exhibited similar mobility behavior. In contrast, in LDL with only small concentrations of triglycerides, the behavior of labeled cholesteryl esters and labeled triglycerides differed distinctly. The cholesteryl esters were strongly immobilized below the transition temperature, whereas the triglycerides remained fluid throughout the measured temperatures. These results suggest that the critical cholesteryl ester to triglyceride mass ratio of 7:1 corresponds to two concentric compartments with a radial ratio of 2:1, where the liquid triglycerides occupy the core, and the cholesteryl esters form the frozen shell. At higher triglyceride contents, the triglyceride molecules insert into the cholesteryl ester shell and depress the peak transition temperature of the LDL core, whereas at lower triglyceride contents, excess cholesteryl esters are dissolved in the core.

Low density lipoproteins (LDL) are the major carriers of cholesterol in the circulation and are intimately involved in atherogenesis (1 and references therein). These particles represent complex supramolecular assemblies of phospholipids (−20% of the total mass), free (−12%) and esterified (−40%) cholesterol, triglycerides (−5–10%), and a single copy of apolipoprotein B-100, a glycoprotein of 4,536 amino acids (2–4). In addition, LDL transports minor amounts of lipophilic vitamins and drugs (5). The structure of LDL can be described, in general terms, by a quasispherical core-shell model, in which the apolar constituents (cholesteryl esters (CE) and triglycerides (TG)) form a hydrophobic core of about 150 Å diameter, whereas the phospholipids, most of the unesterified cholesterol, and the apoprotein form an outer surface monolayer with a thickness of about 30 Å (6–8).

In this general sense, LDL would appear to fit readily into the structural core-shell scheme of circulating lipoproteins (9). However, LDL exhibits a distinct physical feature that makes it unique among all structural elements of blood: it is the only component to undergo a major structural transition just below physiological body temperature, in the range between 15 and 32 °C (10, 11). The transition temperature varies among individual donors and is correlated to the core lipid composition. This lipid-melting transition directly affects the molecular packing in the core of LDL, i.e. approximately half of the particle mass (12, 13). The local molecular dynamics and polarities in the surface monolayer, including apolipoprotein B, are affected indirectly, such that the transition can be recognized from the particle surface (14, 15). As to the actual transition process, it is generally assumed that it occurs between a low temperature state, in which CE and TG molecules are rigidly packed with their long molecular axes radially arranged in two concentric shells of approximately 36 Å radii each, and a fluid, oily droplet state above the transition (12, 13). A quantitative description of the two states and of the processes involved in the transition is still lacking. However, more detailed structural information is required to understand the biochemical effects of the transition. Several important biological activities of LDL have been shown to be tightly controlled by the physical state of the core lipids: the activity of lecithin-cholesterol acyltransferase (16) and of CE transfer protein (17) and the susceptibility of LDL to copper-induced lipid peroxidation (18).

The present study was performed to obtain detailed information on the relationship between core lipid composition and the physical states involved in the transition, by differential scanning calorimetry (DSC) analyses of a large number (n > 60) of LDL subspecies. Defined LDL particle subspecies have the advantage over the broad continuum of total LDL of possessing a high degree of structural homogeneity with respect to hydrated density, chemical composition, and physicochemical properties (19). The range of chemical compositions of the core
LDL Core Organization

1335

Spin-labeled LDL was concentrated using Centricron 10 concentrators (Amicon, MA) to a final concentration of 7 mg/ml protein.

For preparations of spin labels in pure triolein or olive oil, lipids were dissolved in chloroform and deposited as a dry, thin film on the glass tubing. Triolein or olive oil was added and incubated with moderate mixing for 10 min at room temperature.

For ascorbate quenching, the solutions of ascorbic acid 3 \cdot 10^{-3} M were prepared immediately before use (phosphate-buffered saline with 0.1 g/liter EDTA, pH 7.4) and added to the sample at a ratio of 1:10 (v/v). The ESR signal was measured as quickly as possible after mixing (after approximately 4 min). All ascorbate-quenching measurements were performed at 37 °C.

**DSC**—Calorimetry experiments were performed on the high sensitivity, differential adiabatic scanning microcalorimeter DASM-4 (Bio-biror, Pushchino, Russia) designed by Privalov (26). The scanning rate was 1 °C/min; samples (1–2 mg/ml total LDL cholesterol) were loaded into the calorimeter sample cell at room temperature, and an equal amount of buffer was loaded into the reference cell. The cells were pressurized with nitrogen to 250 kilopascals (2.5 bar). Heat capacity functions were obtained after base-line subtraction and normalization of experimental data, given in μW, to heat capacity units (dlʃ/C° of Ce).

Data were collected on heating runs from 1 °C to 45 °C. Each sample was scanned two or three times and held at 1 °C for approximately 30 min between runs. The transition temperature (Tm) was taken as the peak of the maximum change in heat capacity.

**ESR Measurements**—ESR measurements were performed in glass capillaries (1-mm inner diameter) on an X-band Bruker ECS 106 ESR spectrometer (Bruker, Rheinstetten, Germany). The following settings were used for determination of the rotational correlation times: microwave power, 2 mW; modulation amplitude, 1 G; modulation frequency, 100 KHz; scan width, 100 G. The temperature was controlled to within ±0.1 °C by a Bruker temperature controller B-VT 2000. The initial temperature was 4 °C and was increased stepwise to 40 °C. The preparations were allowed to equilibrate for at least 5 min at each temperature before data acquisition. 10–20 scans were accumulated per temperature point.

The determination of the spectral parameters used in the evaluation of ESR data is shown in Fig. 3A. Correlation times (τ) for the approximately isotropic motion were calculated from line widths W₀ as described by Kivelson (27) where h₀ and h₁ refer to first derivative line heights of the midfield line and the highfield line, respectively.

For the sample of 16-CE in LDL displaying a high CE/TG weight ratio, it was not possible to determine the rotational correlation time because of the more anisotropic motion of this spin label. Equation 1 cannot be used for anisotropic spectra with relatively slow motion. Thus, as a parameter related to the motional characteristics, the ratio h₀/h₁ has to be used (28, 29).

To estimate the polarity of the environment of the spin probes, the isotropic hyperfine coupling a₀ was used:

\[ \tau = (6.5 \cdot 10^{-3})W₀/h₀h₁^{1/3} - 1 \]  

where h₀ and h₁ refer to first derivative line heights of the midfield line and the highfield line, respectively.

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same preparation would make it impossible to discuss differences in thermotropic behavior reliably.

The transition temperatures of the LDL core varied between 15 and 32 °C, although most samples fell into the range of 24–30 °C. The weight ratio of CE to TG was chosen as chemical parameter because CE and TG are the principal LDL core lipids. When free cholesterol was included in the calculations, the results were unaffected. Plotting $T_m$ versus the weight ratio of CE/TG (Fig. 2) led to the observation that a single linear approximation was insufficient to fit the data points. Rather, it appeared that a discontinuity occurred at CE/TG ratios of approximately 7. Such a discontinuity in the concentration dependence of a transition temperature in a mixed lipid system is a clear indicator of a phase separation at this point.

To understand the molecular basis of this nonlinear behavior further and to obtain additional evidence concerning the hypothesis that a microphase separation occurs within the core lipids, spin probes were introduced into the LDL core, and the mobility and polarity of their environment were investigated (Fig. 3).

Temperature series of ESR measurements were performed on various LDL subspecies labeled either with 16-CE or with 16-TG. To characterize the situation above and below the break point in the plot of $T_m$ versus CE/TG ratio (see Fig. 2), some representative measurements are introduced.

At high CE/TG ratios, the 16-CE label incorporated into the LDL core displayed two distinctly different types of spectrum. ESR spectra recorded at $T < T_m$ showed line shapes that are characteristic for anisotropic motion, as is most clearly reflected in the splitting of the low field line (see arrows in Fig. 4A). At $T > T_m$, the motion of the spin-labeled CE became a more isotropic one but still remained restricted as seen by the relatively broad spectral lines. Because of the anisotropic slow motion of the label, the line/height ratio was used to follow the temperature course (Fig. 4C).

In contrast, the recorded spectra for 16-TG-labeled subspecies with a high CE/TG ratio display a rather isotropic behavior (Fig. 4B). The temperature dependence of the rotational correlation time $\tau_c$ showed no change around $T_m$ (Fig. 4D), contrary

FIG. 1. Average chemical composition, mean of 14 donors ± S.D., of LDL subspecies in the density range of 1.0244–1.0435 g/ml. In g/ml: subspecies 5, 1.0244–1.0271; 6, 1.0271–1.0297; 7, 1.0297–1.0327; 8, 1.0327–1.0358; 9, 1.0358–1.0393; 10, 1.0393–1.0435 g/ml. The contents of protein (empty bars), free cholesterol (FC, hatched bars), TG (black bars), and phospholipids (PL, cross-hatched bars) are presented as a percentage of total mass.

FIG. 2. Dependence of $T_m$, determined by DSC, on the CE/TG ratio, by weight, in the core of defined LDL subspecies. ●, subspecies 5; □, 6; ■, 7; ●, 8; ○, 9; △, 10; ▽, unfractionated LDL; ○, LDL labeled with 16-CE; ○, LDL labeled with 16-TG. Densities of the LDL subspecies classes are given in Fig. 1. Broken lines, linear regressions below and above CE/TG = 7. Inset, typical calorimetric scan.
to the temperature profile of 16-CE.

At the other extreme of the CE/TG range, one has to examine the situation at a CE/TG ratio below 7. In this case, the behavior of 16-CE and 16-TG labels in the LDL core are almost identical. One cannot distinguish between LDL subspecies labeled with 16-CE or with 16-TG on the basis of their spectral parameters (see Fig. 5, A and B). Neither the spectra of 16-CE nor of 16-TG reveal the degree of immobilization displayed with 16-CE in LDL with a high CE/TG ratio. Also, at $T > T_m$ a highly mobile situation is reached. Plotting $\tau_c$ versus $T$ results in essentially congruent plots (Fig. 5, C and D).

To compare the behavior of 16-TG in LDL with that in a TG-rich environment, the ESR spectra of 16-TG were measured in olive oil and triolein. The spectra of 16-TG in LDL of CE/TG ratio $> 7$ and those in olive oil or triolein showed the same spectral characteristics but were shifted by about 20 °C (Fig. 6). Results for 16-TG in triolein and in olive oil were identical.

The notion that 16-TG within LDL with a CE/TG ratio $< 7$ is located in a TG-rich domain is supported by comparing $\tau_c$ for 16-TG in olive oil and LDL. In this case, the same temperature-dependent decrease with an almost identical decay constant of the regression fits was observed.

Furthermore, the isotropic hyperfine coupling $a_N$ was calculated to obtain information on the polarity of the 16-TG environment (data not shown). These calculations clearly reveal that 16-TG is located in a low polarity environment. Somewhat surprising is the fact that the polarity of 16-TG in olive oil is higher than the polarity of 16-TG in LDL, despite the presence of groups that increase the polarity of LDL (hydroxyl group of cholesterol, ester groups, etc.). The degree of unsaturation in LDL is similar to that in olive oil. We tend to assume that

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**Fig. 3.** A, typical ESR spectrum of 16-CE or 16-TG in LDL. Spectral parameters, used for the calculation of $\tau_c$, as described under “Experimental Procedures,” are depicted schematically. B, chemical formulas of the spin probes used: 16-CE, 16-TG, (mixed isomers).

**Fig. 4.** Temperature dependence of the ESR spectra and $\tau_c$ of spin labels in LDL with a high CE/TG weight ratio. A, spectra 16-CE; B, spectra 16-TG; C, line/height ratio of 16-CE; and D, $\tau_c$ of 16-TG. The middle spectrum represents the spin probe at the actual transition temperature $T_m$. Arrows in C and D indicate the actual calorimetric midpoint transition temperature.
mobility effects play a role in the determination of polarity through $\alpha'$. In any case, however, these results confirm that 16-TG is located in an extremely apolar environment in LDL.

To assure the validity of the results, it was essential to confirm that the lipid spin labels were intimately associated with and situated in the core of LDL particles. This question was approached by use of ascorbate quenching. Ascorbate is one of the most efficient water-soluble reducing agents for nitroxide radicals (34, 35). To determine the accessibility of each spin-labeled lipid type within LDL to the aqueous phase and to verify the location of the spin labels in the LDL core, the rates of ascorbate-induced reduction of the nitroxyl moiety in the LDL core and in the microemulsions labeled with either 16-CE or 16-TG were examined by monitoring the loss of the ESR signal.

Adding ascorbate solution to LDL preparations containing 16-TG or 16-CE resulted in a very slow loss of ESR signal intensity. The ESR signal remained stable for a period of more than 2 h. On the contrary, the addition of ascorbate solution to the microemulsion containing either large quantities of 16-CE or 16-TG led to an almost immediate (approximately 5 min) loss of paramagnetism. Comparing the rate of reduction with that of 5-DSA and 16-DSA in LDL, it can be stated that 16-TG and 16-CE in LDL were reduced by a lower rate (Fig. 7). These observations confirm the notion that the 16-CE and 16-TG spin probes were localized in the core of the LDL particle (see Fig. 8).

To ensure that the integrity of LDL was not impaired by the incubation and reisolation processes, polyacrylamide gel electrophoresis was performed. The labeled LDL behaved in the same manner as the control preparation.

DISCUSSION

The present results strongly suggest that the apolar lipids TG and CE, in the core of LDL, undergo phase separation below the core lipid transition. This finding is in variance with most of the models proposed in the literature, and it is therefore necessary to recall the evidence that has led to these models.
ratios the CE and TG in the LDL core at two representative CE/TG apolipoprotein B (symbols used for CE, TG, free cholesterol (FC), phospholipids (PL), and apolipoprotein B (apoB) are indicated in the figure.

Before that, however, a more general feature of a quasispherical core-shell model, as is consistent with LDL, has to be considered. The relative weight of the shells varies with the third power of the radius, such that the innermost parts are only a very minor fraction. For instance, in a three-shell structure with radii 1:2:3, the core contributes only 3.7% to the total volume. Therefore, any mass-dependent measurement, as e.g. SAXS, SANS, or NMR, will attain the limits of resolution in terms of the definition of the details of core structure. On the other hand, molecular probe methods such as spin probe ESR, which rely inherently on the exclusive signal from the probe, are not limited by such restraints as long as the probe can be considered nonperturbing. Similarly, DSC signals from a melting transition are sensitive to minor contamination of the melting matrix, which would hardly be detectable by scattering or NMR methods. This feature makes the methods chosen in this study well suited for studies of questions concerning the state of a minority component (TG) in the excess of a second component (CE).

Key evidence for the presently most widely accepted model of the neutral lipid core in LDL has come from the combined x-ray and calorimetric studies by Deckelbaum et al. (10, 11), which was confirmed later by SAXS and SANS data (12, 13, 36). In all of these studies, little attention has been given to the problem of the packing in the innermost core, mainly because of the above mentioned limitations. The original notion, that the strong SAXS peak at spacings of 1/36 Å⁻¹ of LDL at low temperatures has its basis in a layer structure with a 36–Å repeat (10) has been accommodated in later models designed to be consistent with the x-ray results in terms of a concentric spherical shell structure (6, 8, 13). However, a simple model simulation, as shown in Fig. 9, shows that this consistency is not critical in discerning details of the inner core structure of a LDL-type particle. Equally, the results from neutron scattering experiments with selective deuteration of CE (36) would not distinguish between such models as simulated in Fig. 9.

The present notion of a phase separation is primarily derived from the composition dependence of the calorimetric transition temperature (Fig. 2). These data show a break at a CE/TG ratio of 7, by weight. No evidence for such a phenomenon can be found in the literature on isolated, mixed systems of CE and TG (20–22). We are led to assume, therefore, that the particular spatial constraints within the LDL particle promote this behavior. What are these spatial constraints at CE/TG ratios of 7:1? One clue to a possible answer comes from the behavior above and below the critical ratio of 7:1. The almost constant Tm at ratios higher than 7:1 suggests that under these conditions, TG does not act as a perturbant for the presumably smectic CE arrangement (37). Below 7:1, however, TG has a strong effect. It seems, therefore, that above 7:1, TG does not enter the smectic CE compartment whereas below it does, implying the existence of two compartments with an approximate volume ratio of 7:1 (we can neglect here the minor differences in specific volumes of TG and CE, respectively). Adhering to the scheme of spherical shells, one finds that this can be realized easily by a core-shell structure with radial ratio 2:1; there, the volume ratio of shell to core is 7:1.

These facts lead us to the hypothesis that the TG compartment corresponds to the innermost core of radius of approximately 36Å, whereas the CE compartment represents the first shell between 36Å and about 72Å. Only the CE compartment would undergo the calorimetric transition. On the basis of this argument, the composition dependence can be understood readily (see model in Fig. 8). For CE/TG > 7, the CE shell is largely free of contaminating TG, and therefore the Tm remains almost constant. For CE/TG < 7, the amount of CE is insufficient to cover the TG core, and thus the CE shell will be enriched progressively by TG, which leads to a strong depression of Tm. We have, for reasons of simplicity, neglected here the presumed presence of free cholesterol in the apolar lipid core (38). It can be calculated easily that this would have only minor effects on the above geometric argument.

So far, this is merely a geometrical plausibility argument, and alternative explanations for the observed composition dependence of Tm might exist but are difficult to substantiate. We rather prefer to test the validity of the present phase separation concept by independent methods. One such method is spin label ESR, which should, through the suitable choice of labels, detect different microenvironments for CE and TG. Considering two possible cases of LDL core composition there is, on the one hand, LDL with a CE/TG ratio > 7 (Fig. 8B). In this case, our model predicts a practically unperturbed CE shell. Indeed, the 16-CE spin probe exhibits a highly defined change in be-
behavior around $T_m$. It is sensitive to the melting of the CE shell. Even above $T_m$, the 16-CE spectrum remains noticeably restricted, which suggests that some motional anisotropy and hindrance due to the confined space remains effective. The 16-TG spin probe behaves quite differently. No change in behavior around $T_m$ can be observed. Instead, the rotational correlation time of 16-TG decreases monotonously with temperature as expected for the viscosity of a liquid. This finding confirms that the triglyceride compartment remains in the same physical state throughout the measured temperature range, from well below to well above the LDL core phase transition. Furthermore, it was demonstrated that the spectra of 16-TG in LDL are closely similar to those in olive oil, or triolein, at lower temperatures. At the measured temperatures, they are evidently in a liquid state.

At a low CE/TG ratio (<7), on the other hand, our present model predicts that there is too little CE to cover the core of TG by a smectic layer; this would necessitate the formation of a progressively increasing mixed CE/TG microphase. Indeed, our spin label results show that the 16-CE and 16-TG probes display the same types of spectra and mobility, which confirms that both apolar components share the same compartment within LDL, at CE/TG < 7.

The actual location of the labeled apolar lipids in the LDL core is strongly suggested by fluorescence, NMR, and our previous neutron scattering studies (24, 36, 39). Independently, our present ascorbate-quenching results confirm this notion. One might further speculate on the presence of some esterified cholesterol in the outer phospholipid monolayer of LDL. The results presented here allow only for a minor fraction of the CE in the outer monolayer. 16-DSA, a fatty acid spin label carrying the nitroxide in position C-16, which is supposed to reside in the outer monolayer, is quenchable by ascorbic acid (35). 16-CE and 16-TG are quenched much slower, probably only during the short times that the esterified fatty acids protrude dynamically into the phospholipid monolayer. Although the quantification of components is most difficult by ESR, the shape of the spectra recorded for 16-CE or 16-TG remained unaltered during the course of ascorbate reduction, indicating that the signal originates from one lipid environment only, hardly accessible to ascorbic acid. By comparison, 16-CE or 16-TG residing in microemulsions are reduced rapidly by ascorbic acid, in marked contrast to spin labels in LDL, which are hardly reduced at all. Consequently, one can postulate that the LDL particle with its typical lipid composition and its association with one copy of apolipoprotein B100 has an optimal assembly and is remarkably protective of its core components.

What could be the relevance of the model discussed above in relation to the function of LDL in lipid metabolism? Under normal body core temperature, the LDL core is most likely in a fluid state at all times. But, as can be seen by examination of the ESR spectra of 16-CE (Figs. 4A and 5A), the fluidity of the
apolar lipid core differs considerably in LDL with different compositions, even above $T_m$.

The physical state of the LDL core presents a major variable for the activity of CE transfer protein and lecithin-cholesterol acyltransferase; indeed both of these proteins display much higher activity when the core is in a liquid state, probably because of easier access (16, 17). However, it is not only the metabolism of LDL in the blood circulation which is affected. The intracellular degradation of LDL by the lysosomal lipase metabolism of LDL in the blood circulation which is affected. Because most of our donors exhibited a CE/TG ratio around 7, their LDL also varies with the physical state of the core (40). Because of easier access (16, 17). However, it is not only the higher activity when the core is in a liquid state, probably because of easier access (16, 17). However, it is not only the physical state of the LDL core presents a major variable for the activity of CE transfer protein and lecithin-cholesterol acyltransferase; indeed both of these proteins display much higher activity when the core is in a liquid state, probably because of easier access (16, 17). However, it is not only the higher activity when the core is in a liquid state, probably because of easier access (16, 17). However, it is not only the

Considering the fact that lipophilic molecules transported by LDL, e.g., drugs or vitamins, are more highly soluble in a fluid than in a solid phase, the microphase separation could potentially be of mechanistic significance. Thus, the LDL core could act as a microreactor of variable volume. At temperatures above $T_m$, the whole fluid apolar core of LDL with a radius of about 90Å (including the hydrocarbon part of the surface monolayer) would be available for lipophilic solutes, whereas below $T_m$, the reaction space is reduced by about 1 order of magnitude. This effect could lead to concentration of lipophilic molecules to within the local TG core, while CE is frozen out. This would enhance significantly the local reactivities of, for example, neutral antioxidants and could account for the much slower oxidation of LDL lipids below the core $T_m$ (18). Even the solubility of lipophilic molecules in LDL might be controlled by the state of the core, which may be of special relevance when attempts are made to use LDL as a carrier for lipophilic drugs (41, 42).

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