Vol. 105, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 30, 1982 Pages 674-680

FLUORESCENT CHOLESTERYL ESTERS IN THE CORE OF LOW DENSITY LIPOPROTEIN

Larry A. Sklar,* William W. Mantulin, and Henry J. Pownall

Department of Medicine, Baylor College of Medicine, Houston, Texas 77030 and Department of Immunopathology,* Scripps Clinic and Research Foundation, La Jolla, CA 92037

Received February 16, 1982

Fluorescent cholesteryl esters with chromophores placed either on the acyl chain (cholesteryl-cis-parinarate), the sterol ring system (5,7,9-cholesteryl oleate), or the side chain (naphthylcholenamide oleate) have been incorporated into the core of low density lipoprotein. The temperature dependence of several fluorescence parameters has been evaluated. An analysis of the fluorescence lifetime components of cholesteryl-cis-parinarate reveals coexisting environments whose proportion varies and reflects the thermotropic reorganization of the core of the particle. An analysis of the motion by dynamic depolarization suggests that the motions of the acyl chains in the core of the particle are highly restricted.

INTRODUCTION

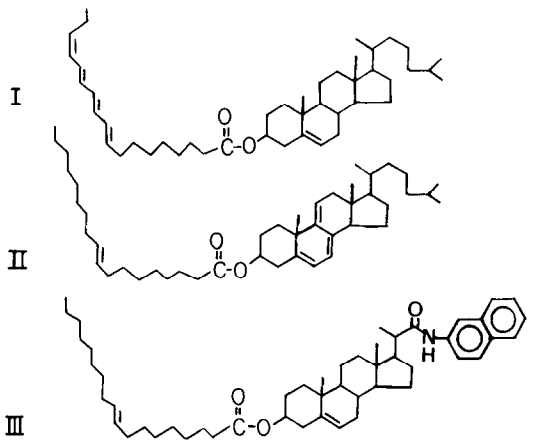
There is considerable interest in the structure of serum low density lipoprotein (LDL). A thermotropic transition, associated with the melting of the cholesteryl esters in the core of the particle, has been characterized by several physical techniques (1-11). The fluorescent probe, diphenylhexatriene, which is sensitive to the motion of membrane lipids proved to be insensitive to the melting of the cholesteryl esters in the particle (2,12). We have now incorporated fluorescent cholesterol esters whose acyl chain, (cholesteryl-cis-parinarate, I), or sterol, (5,7,9-cholestatrienyl-oleate, II) or side chain (naphthycholenamideoleate, III) contained a fluorophore and we have examined several fluorescence parameters to gain further information about the structure of the core of the particle.

METHODS

The fluorescent probes I, II, and III were prepared as described previously (11,13). The starting material, naphthylcholenamide (14), from which III was synthesized, was a generous gift of Drs. Louis Smith and Y.J. Kao. Probes were incorporated into human LDL by the enzymatic method using a lipid transfer factor present in human plasma (11).

The fluorescent sterol esters were chosen on the basis of several criteria. 1) The chromophores represent minimal perturbations to three distinct regions (acyl chain, steroid ring, side chain) of a typical

0006-291X/82/060674-07$01.00/0



cholesteryl ester (e.g. cholesteryl oleate). 2) The emission spectra of these probes overlap the absorption spectrum of the polar, surface lipid analogue, hexadecanoylaminofluorescein. Since all three probes have similar R_0 values (28Å for I or II, 27Å for III at 25°) for resonance energy transfer to this chromophore, their location in the core can be verified (15). 3) Both parinaric acid (16,17) and cholestatrienol (18) probes are sensitive to phospholipid structural reorganization and, in addition, are energy transfer acceptors for the tryptophan emission (19,20) of LDL protein.

The absorbance, induced circular dichroism, and steady-state fluorescence parameters (polarization ratio $I_{//}/I_{\perp}$, and fluorescence quantum yield) of the labeled LDL particles, were measured as described previously (11,15). Dynamic depolarization measurements and fluorescence lifetime analyses were performed on an SLM 4800 phase-shift fluorometer essentially as described by Mantulin and Weber (21). The data were analyzed as described by Weber (22,23) and data analysis applied to membranes followed Lakowicz, et al. (24).

RESULTS

We have shown that thermal reorganization in LDL was detected by a temperature-dependent induced circular dichroism of the incorporated cholesteryl esters I and II (11). The midpoint of the melting of these LDL particles with incorporated chromophoric esters as detected by calorimetry and induced circular dichroism was ~15° (Figure 1A). The fluorescence lifetime of I exhibits short and long lifetime components whose proportion varies strongly over the temperature range of the melting of the cholesteryl esters in LDL (Figure 1B and Table I). We have previously observed a similar behavior of the lifetimes of parinaric acid probes in phospholipid phase transitions. In phospholipids, the short lifetime component was associated with parinaric acid probes in disordered phase and the long component was associated with the probe in ordered phases (16,17).

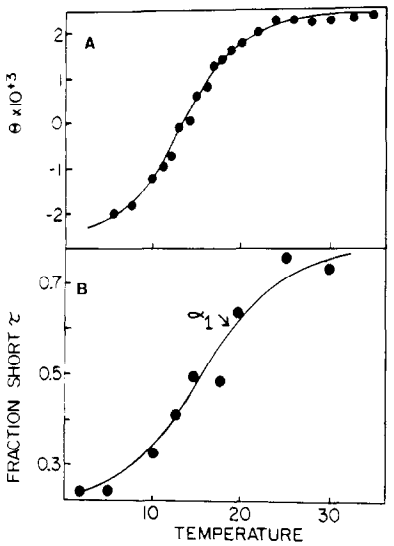


FIGURE 1 - Correspondence between temperature dependence of induced circular dichroism (molar ellipticity in millidegrees, A) and fraction of the short lifetime component B) of I in LDL. The sample contained ~2 mg/ml LDL in which cholesteryl parinarate comprised ~5 percent of the LDL cholesteryl ester. The absorbance of the solution at 325 nm was 1.5 O.D. Circular Dichroism measurements were performed as described previously (ref. 11, Fig. 5B). Lifetimes were measured and analyzed according to the Methods.

We performed an analysis of the dynamic depolarization of fluorescence (Table II). The parameter $\Delta\tau$ (the differential lifetime) is measured as the difference in lifetime determined parallel and perpendicular relative to the exciting light and reflects the dynamics of the acyl chain motion. Using values of $\Delta\tau$, r (the steady-state anisotropy) and the average lifetime $\bar{\tau}$, we calculate the parameters r_∞ (24), which reflects the angular extent of the ester acyl chain motion, the order parameter S (25), and the rotational relaxation rate R (24). These parameters were not strongly influenced by the melting of the core of LDL. Their magnitudes are comparable to those observed for parinaric acid probes (17) or diphenylhexatriene in phospholipid bilayers and membranes where the lipids have been immobilized to a considerable extent (26).

We examined the temperature dependence of several steady-state parameters (Figure 2): 1) the quantum yield of I, II, III, 2) the polarization of I and II, and 3) resonance energy transfer from I, II, and III to the surface acceptor hexadecanoylaminofluorescein or from LDL tryptophan to I and II (not

Table I. Fluorescence Lifetime Analysis of Cholesteryl-cis Parinarate in LDL

Temp	Measured Lifetimes					Calculated Lifetimes		
	30 MHz: A[a]	ϕ[b]	10 MHz: A[a]	ϕ[b]	τ_1	τ_2	α_1[c]	
29.6	6.2	5.5	7.0	6.4	4.7	11.6	.73	
25	7.2	6.5	8.1	7.4	5.8	13.9	.76	
20	8.4	7.3	9.6	8.6	5.9	14.6	.63	
17.5	9.1	7.5	10.6	9.1	5.6	13.9	.48	
14.9	9.9	8.2	11.6	10.0	6.2	15.7	.49	
12.7	10.7	8.9	12.4	10.7	6.3	15.7	.41	
10.1	11.6	9.3	13.5	11.5	6.2	16.2	.33	
5.2	13.3	10.5	15.4	13.1	6.2	17.4	.24	
1.8	14.7	11.4	17.2	14.4	6.8	19.7	.24	

a Lifetime (nsec) measured by amplitude modulation
b Lifetime measured by phase shift
c Fraction of short lifetime component

shown; for example, see ref 15). These parameters were not strongly influenced by LDL melting. The apparent insensitivity of the quantum yields of I and II and the polarization of I contrasts with the response of parinaric acid probes and cholestatrienyl probes to phospholipid melting (see refs. 16-18). The spectroscopic responsiveness to phospholipid melting of

Table II. Dynamic Depolarization Cholesteryl cis-Parinarate in LDL

Temp	τ_{av}[a]	$\Delta\tau$[b]	r[c]	Rx10^{-8} (sec^{-1})[d]	r_∞[e]	S[f]
29.6	6.6	.474	.239	1.09	.204	.724
25	7.7	.491	.246	.99	.215	.743
20	9.1	.478	.254	.99	.229	.767
17.5	9.9	.457	.261	.99	.239	.784
14.9	11.0	.408	.263	1.18	.247	.797
12.7	11.8	.449	.264	1.01	.246	.795
10.1	12.9	.391	.267	1.18	.254	.808

a Average lifetime (nsec) = $\alpha_1\tau_1 + \alpha_2\tau_2$ from Table I.
b The difference in lifetime (nsec) measured parallel and perpendicular to the exciting light at 30MHz
c The measured steady-state anisotropy $(I_{//} - I_\perp)/(I_{//} + 2I_\perp)$
d The calculated rotational relaxation rate (24)
e The calculated limiting anisotropy (±0.01) (24)
f The calculated order parameter (±0.015) (25)

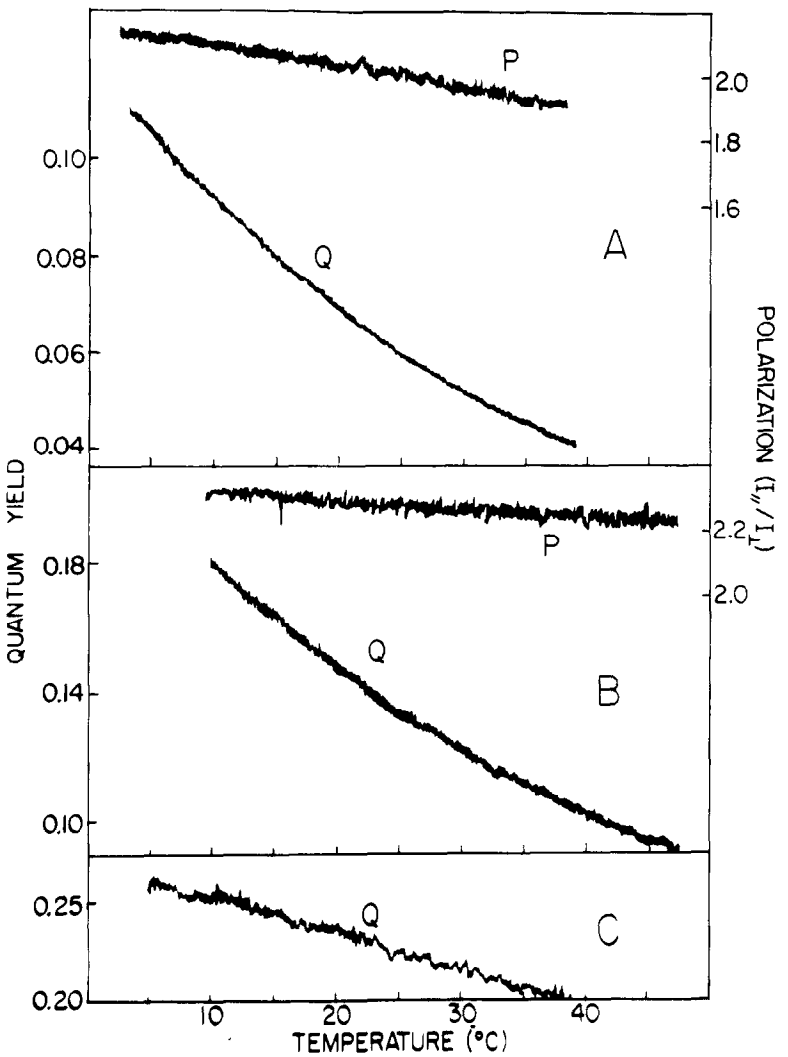


FIGURE 2 - Temperature dependence of the polarization ($I_{//}/I_{\perp}$) and the quantum yield of choromphoric cholesteryl esters in LDL. The sample contained LDL labeled with: A) cholesteryl-cis-parinarate; B) Cholestatrienyl oleate, C) Naphthylcholenamide oleate prepared as in ref 12. The data were recorded on an X-Y recorder, the X-axis driven by the output of a Bailey BAT 8C digital thermocouple thermometer with the sensor placed in the cuvette. For polarization (P) determinations, $I_{//}$ and $I_{\perp}$ were measured simultaneously and the ratio $I_{//}/I_{\perp}$ was displayed on the Y-axis. In these polarization measurements, the background fluorescence of LDL, representing no more than 5 percent of total fluorescence has not been taken into account. The quantum yield (Q) measurements were made by recording the fluorescence intensity as a function of temperature and calibrating the total emission intensity of the sample at a single temperature to standards of known quantum yield (i.e., cis parinaric acid in ETOH, Q=0.020 at 25°). All of these data were reversible with temperature.

esterified probes I and II at 1 mole percent is essentially identical to the unesterified chromophores from which they are derived (L.A. Sklar, unpublished results).

DISCUSSION

This work provides new insight into the environment of cholesteryl esters in the core of LDL. The lifetime components of cholesteryl-cis-parinarate reveal heterogeneity in the core of LDL which is responsive to its melting. The lack of an impact of LDL melting on the motion of this probe is consistent with the results obtained with diphenylhexatriene (2,12) and also with nuclear magnetic resonance (NMR) studies (6-8) which suggest that distal portions of the ester acyl chains are not affected by the melting of the core.

The dynamic depolarization measurements suggest a relatively high degree of immobilization of ester acyl chains, at least on the time scale of fluorescence measurements, while magnetic resonance suggests that LDL ester acyl chains are highly mobile on the NMR time scale (microseconds). This disparity between NMR and fluorescence is reminiscent of the current controversy over the immobilization of lipids by proteins in bilayers. The controversy appears to be resolved in terms of differing time scales on which the lipid motions are analyzed (27,28).

It appears that the fluorescent sterol ester acyl chains are subject to environmental influences in LDL which are different from those of acyl chains in phospholipid bilayers. The environmental factors which give rise to the induced circular dichroism and lifetime heterogeneity in LDL are not yet understood. While the environment is modulated by the melting of the cholesteryl ester acyl chains (hence by lipid-lipid interactions), we cannot rule out the possibility that the environmental heterogeneity arises in part from interactions of the cholesteryl esters with the protein in LDL.

Acknowledgements

This work was supported in part by National Institutes of Health grants HL 19459 and HL 17269, and the American Heart Association to H.J.P. L.A.S. was supported in part by a Helen Hay Whitney postdoctoral fellowship and National Institutes of Health Grant AI 17354. W.W.M. received support in part from the American Heart Association, Texas Affiliate.

References

1. Deckelbaum, R.J., Shipley, G.G., Small, D.M., Lees, R.S., and George, P.K. (1975) Science 190, 392-394.
2. Pownall, H.J., Shepherd, J., Mantulin, W.W., Sklar, L.A., and Gotto, A.M. (1980) Atherosclerosis 36,299-314.

3. Atkinson, D., Deckelbaum, R.J., Small, D.M., and Shipley, G.G. (1977) Proc Nat Acad Sci, U.S.A. 74,1042-46.
4. Laggner, P., Degovics, G., Glatter, O., Kratky, O., Kostner, G., and Holasek, A. (1977) Hoppe-Seyler's Z. Physiol. Chem. 358,771-778.
5. Luzzati, U., Tardieu, A., and Aggerbeck, L.P. (1979) J. Mol. Biol. 134,435-473.
6. Hamilton, J.A., Oppenheimer, N., Cordes, E.H. (1977) J. Biol. Chem. 252,8071-8080.
7. Hamilton, J.A., Cordes, E.H. and Glueck, C.J. (1979) J. Biol. Chem. 254,5435-5441.
8. Kroon, P.A. (1981) J. Biol. Chem. 256.5332-5339.
9. Chen, G.C. and Kane, J.P. (1974) Biochemistry 13,3330-3335.
10. Chen, G.C., Krieger, M., Kane, J.P., Wu, C.C., Brown, M.S., and Goldstein, J.L. (1980) Biochemistry 19,4706-4712.
11. Sklar, L.A., Craig, I.F., and Pownall, H.J. (1981) J. Biol. Chem. 256,4286-4292.
12. Jonas, A. (1977) Biochim. et. Biophys. Acta 486,10-22.
13. Patel, K.M., Sklar, L.A., Currie, R., Pownall, H.J., Morrisett, J.D., and Sparrow, J.T. (1979) Lipid 14,816-818.
14. Kao, Y.J., Soutar, A.K., Kong, K.Y., Pownall, H.J., and Smith, L.C.
15. Sklar, L.A., Doody, M.C., Gotto, A.M., and Pownall, H.J. (1980) Biochemistry 19,1294-1301.
16. Sklar, L.A., Hudson, B., and Simoni, R.D. (1977) Biochemistry 16,819-828.
17. Wolber, P.K., and Hudson, B.S. (1981) Biochemistry 20,2800-2810.
18. Roger, J., Lee, A.G., and Wilton, D.C. (1979) Biochim. Biophys. Acta 552,23-37.
19. Sklar, L.A., Hudson, B., and Simoni, R.J. (1977) Biochemistry 16,5100-5108.
20. Smith, R.J.M., and Green, C. (1974) J. Biochem. 137,413-415.
21. Mantulin, W.W., and Weber, G. (1977) J. Chem. Phys 66,4092-4099.
22. Weber, G. (1978) Acta Phys. Pol. A 54,859-865.
23. Weber, G. (1981) J. Phys. Chem. 85, 949-953
24. Lakowicz, J.R., Prendergast, F.G., and Hogen, D. (1979) Biochemistry 18,508-518.
25. Heyn, M.P. (1979) FEBS Lett 108,359-364.
26. Kawato, S., Kinoshita, K., and Ikegami, A. (1978) Biochemistry 17,5026-5030.
27. Watts, A. (1981) Nature 294.,512-513.
28. Jahnig, F. (1979) Proc. Nat. Acad. Sci. U.S.A. 76,6361-6365.