THE REQUIREMENT FOR LIPID FLUIDITY IN THE FORMATION AND STRUCTURE OF LIPOPROTEINS: THERMOTROPIC ANALYSIS OF APOLIPOPROTEIN-ALANINE BINDING TO DIMYRISTOYL PHOSPHATIDYLCHOLINE*

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SUMMARY

By circular dichroic, fluorescence and ultracentrifugal methods, it is shown that apoLP-alanine binds to dimyristoyl phosphatidylcholine in its liquid crystalline state more efficiently than its gel state.

Several studies based on spectroscopic, hydrodynamic, kinetic and chromatographic methods have demonstrated the in vitro binding of serum apolipoproteins to a variety of lipids (1-6). Most of these and other investigations have been directed toward assessing the magnitude of the lipid-protein interaction and have been less concerned with the influence of the physical state of the lipid in regulating the formation of a lipid:protein complex. In a comprehensive study of the binding of apolipoprotein-alanine (apoLP-Ala) to a variety of synthetic and naturally occurring lipids, we have observed some interesting and potentially important effects of temperature and the physical state of phospholipids on the binding efficiency of apoLP-Ala. This communication presents the preliminary results of a representitative portion of that study which strongly suggests that a liquid crystalline state of the phospholipid is a requirement for efficient binding to apolipoproteins. This conclusion is based upon our lipid-binding studies on the system,

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apoLP-Ala:dimyristoyl phosphatidylcholine (DMPC). The gel → liquid crystalline transition temperature (T_c) of DMPC appears at 23° (7) which is within a convenient temperature range for thermal studies of native proteins.

EXPERIMENTAL PROCEDURE

General Methods and Materials

DMPC (Sigma), Trizma base (Sigma) and all other buffer salts (Fisher) were used as received. Optical densities were measured on a Beckman Acta V Spectrophotometer. Centrifugation was accomplished on a Beckman L2-65 ultracentrifuge. Phospholipid dispersions were prepared at 35° by the method of Batzri and Korn (8). The apoLP-Ala was obtained as described by Brown et al. (9) from the very low density lipoproteins of fasting subjects with type IV or V hyperlipoproteinemia.

Physical Studies on the Temperature Dependence of the Formation of Lipid:Protein Complexes

Circular Dichroic Spectra - The circular dichroism (CD) of the lipid:protein samples were recorded on a calibrated Cary 61 spectropolarimeter. The circular dichroic spectra of DMPC liposomes (0.98 mg/ml) and apoLP-Ala (0.241 mg/ml), mixed and maintained at 5°, were measured as a function of increasing temperature in a 1.0 mm path length thermostatted cell. For each spectrum, a blank was measured in which the protein was omitted. The mean residue ellipticity, $[\theta]_\lambda$, was calculated according to the equation,

$$[\theta] = \frac{\theta^\circ_\lambda \text{ MRW}}{10\ 1c}$$

Where MRW represents the mean residue weight of apoLP-Ala (MRW = 111), θ°_λ is the measured ellipticity at wavelength λ, c is the protein concentration in grams/ml and 1 is the cell path length in cm. The α-helical content of the lipid:protein system determined by the procedure of Greenfield and Fasman (10) as modified by Morrisett et al. (1), is

$$\% \text{ α-helix} = \frac{[\theta]_{222} + 3000}{36{,}000 + 3000} \times 100$$

Fluorescence Spectra - Fluorescence spectra were recorded on an Aminco-Bowman spectrofluorimeter equipped with an RCA-1P28 phototube employing excitation and analyzing slits of 0.5 and 1.0 mm, respectively, and an excitation wavelength of 288 nm. Wavelengths have been determined to an accuracy of ±0.75 nm of the given value.

The fluorescence spectra of apoLP-Ala (33 μg/ml) mixed with liposomes of DMPC at ∿5° C were recorded as a function of increasing temperature. A duplicated sample without DMPC was recorded over the same temperature range. A 50:1 DMPC:apoLP-Ala molar ratio was chosen based on the observed stoichiometry of Morrisett et al. (1).

Density Gradient Centrifugation of ApoLP-Ala:DMPC Complexes

Linear density gradients (ranging from d = 1.063 to d = 1.210) of salt were prepared in 5 ml polyallomer tubes using a Buchler Auto DensiFlo gradient maker. All samples contained 10 mM Tris-HCl, pH 7.4 and 1 mM EDTA. Sodium chloride and potassium bromide were contained in the two gradient maker compartments to provide the proper density; these were 0.483 M NaCl and 0.54 M KBr (d = 1.063) or 1.58 M NaCl and 1.80 M KBr (d = 1.210). DMPC liposomes (4 mg) and the apoprotein (0.65 mg) were mixed in a 5° cold room with the low density half of the gradient. The gradient was prepared in the cold room and transferred in an ice bath to a centrifuge which had been pre-cooled to 5° C. A similar lipid:protein mixture was prepared at ∿30° C and transferred to the same centrifuge. Both samples were spun in a Beckman SW 50.1 rotor at 50,000 rpm (234,000 g) for 72 hrs. The contents of each tube were fractionated and analyzed for protein by measuring the fluorescence of an aliquot of each fraction and comparing these values with a standard curve. Total phosphorous was measured by the method of Bartlett (11) and the density of each fraction was obtained from its respective refractive index measured on a Bausch & Lomb Refractometer (13).

RESULTS

The spectral and ultracentrifugal properties of apoLP-Ala and DMPC mix-

tures above T_c are very different from those observed if the lipid and protein are mixed and measured below T_c. The following sections illustrate the important effect which temperature has upon the binding of apoLP-Ala to DMPC.

Circular Dichroic Spectra - Above the T_c of the phospholipid, the α-helical content of apoLP-Ala is much higher than that observed below T_c. In Figure 1, this effect is illustrated by a plot of % α-helix vs temperature in

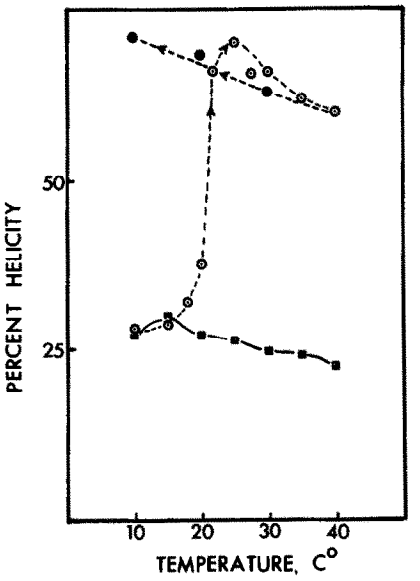

Figure 1: Change in percent helicity of apoLP-Ala with and without DMPC as a function of temperature. ■ apoLP-Ala alone (heating and cooling curves are superimposable); ⊙ DMPC + apoLP-Ala, 50:1. Empty and filled circles represent increasing and decreasing temperatures, respectively.

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a 50:1 DMPC:apoLP-Ala mixture which was prepared at 5°. Very little change is observed below 20° but above 20° a rapid increase in % α-helix is produced. This is maximal at 25° (70% helix) above which a gradual decrease in helicity is observed. Cooling below the T_c (23° C) of DMPC does not reverse the helical development. Similar behavior is not observed in apoLP-Ala in the absence of the phospholipid as indicated by the lower curve of Figure 1; without phospholipid apoLP-Ala shows only a gradual reversible decrease in α-helix as the temperature is increased.

Temperature Induced Spectral Shifts of the Fluorescence of ApoLP-Ala:DMPC Mixtures - The fluorescence maximum of apoLP-Ala in the presence of DMPC liposomes exhibits a large blue shift in the fluorescence maximum above T_c relative to that observed below T_c. This effect is depicted in Figure 2 where

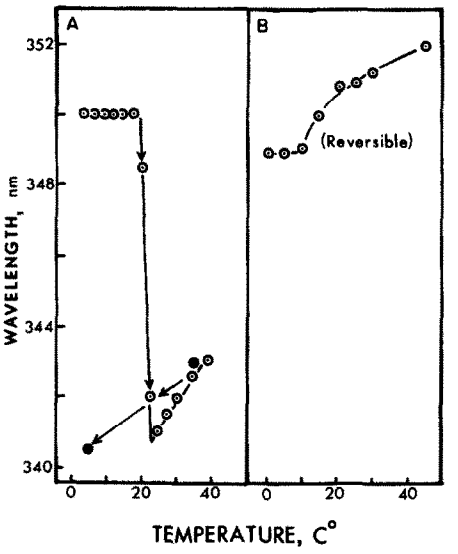

Figure 2: Changes in the wavelength of maximum fluorescence of apoLP-Ala with and without DMPC as a function of temperature. Left-handed panel, DMPC + apoLP-Ala, 50:1. (Empty and filled circles represent increasing and decreasing temperature, respectively.) Right-handed panel, apoLP-Ala without DMPC; heating and cooling curves are superimposable.

λ_{max} versus T plots of apoLP-Ala with and without DMPC are shown. The maximum fluorescence shift is observed at 23°. No such change is observed in the fluorescence spectrum of apoLP-Ala in the absence of phospholipid. Above T_c, the fluorescence maximum increases slightly but the changes in λ_{max} are not reversed by cooling to the original temperature.

Density Gradient Ultracentrifugation - The density gradient ultracentrifugation of apoLP-Ala with DMPC further emphasizes the importance of the physical state of the phospholipids in regulating the lipid binding of apoLP-Ala. The following two experiments were performed simultaneously. First, a "5° complex"

of DMPC:apoLP-Ala was produced by mixing DMPC and apoLP-Ala (50:1), applying the mixture to a linear density gradient and centrifuging for 72 hrs. at 234,000 x g. All procedures were done at 5°. A DMPC:apoLP-Ala complex (66:1) floats at d = 1.115 (Fig. 3 a,b). Not all of the lipid and protein appear in

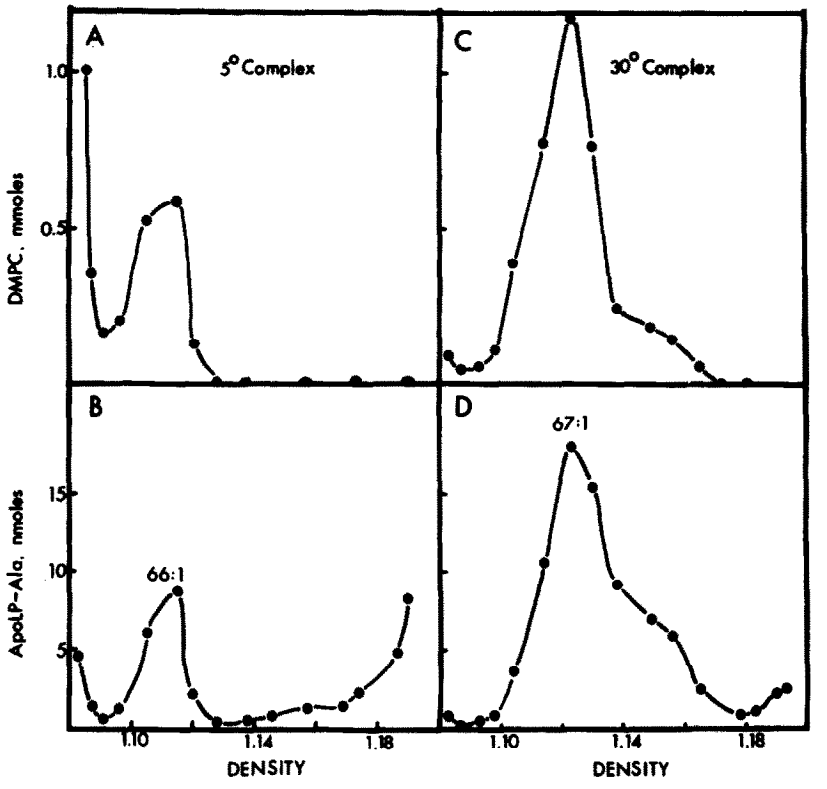

Figure 3: Lipid (A) and protein (B) density profiles of apoLP-Ala and DMPC mixed and centrifuged at 5°. DMPC (C) and apoLP-Ala (D) mixed at 30° and centrifuged at 5°. Nearly twice as much protein and lipid are isolated as a complex in the sample mixed at 30° relative to that combined at 5°.

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the complex; a substantial amount of the lipid floats while much of the protein sediments to the more dense fractions of the tube. The complex constitutes only 48 and 40% of the total quantities of DMPC and apoLP-Ala, respectively, applied to the density gradient. When the above experiment is repeated with all mixing procedures done at 30°, 90-95% of the lipid is incorporated into a complex with d = 1.123 and a DMPC:apoLP-Ala ratio of 67:1. A shoulder

contributing to this complex at d = 1.15 is preferentially enriched with protein having a DMPC:apoLP-Ala molar ratio of 25:1.

DISCUSSION

Previous studies with egg phosphatidylcholine (egg PC) have shown that the binding of this phospholipid to apoLP-Ala produces an increase in the α-helicity and a blue shift in the intrinsic tryptophanyl fluorescence of the protein (1). These changes were attributed to the formation of a phospholipid: protein complex. These results, however, did not reveal the effect of temperature since egg PC is in a liquid crystalline state above -10° (12). The temperature dependence described in this communication suggests that fluidity is important in regulating the lipid binding of the apolipoproteins. The absence of a reversible cooling pattern in the CD and fluorescence spectra indicates that the complex formation is not a simple temperature dependent change in equilibrium but that a new molecular species, loosely specified as a lipid:protein complex, has been produced. Comparison of the helicity and fluorescence maximum of apoLP-Ala at 5° (α-helix = 25%, λ_{max} = 350 ± 0.75 nm) with that of the complex cooled to 5° (α-helix = 70%, λ_{max} = 340 ± 0.75 nm) provides a dramatic illustration of the constant in the spectral properties of the free apoprotein with those of the apoprotein complex with phospholipid.

The spectral studies are corroborated by the density gradient ultracentrifugation experiment which shows that the formation of the complex (66-67:1) is slower below T_c (on the order of days) than above T_c from which we conclude that the complex formation is kinetically controlled; i.e. in the gel phase of the phospholipid, the complex forms more slowly than in the liquid crystalline phase. For this reason no changes in the CD or fluorescence spectra are observed at 5° over the 1 h time period in which these experiments are conducted. We assign the structure of the 66-67:1 complex to that of a DMPC vesicle with protein penetrating its surface.

Additional studies are underway to confirm this assignment and to determine the structure of the 25:1 DMPC:apoLP-Ala complex which is isolated only from the sample which has been incubated above T_c. Tentatively, we assign the structure of this complex to an aggregate consisting of a protein core surrounded by boundary lipid.

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