Apolipoprotein C-III/Sphingomyelin Recombinants: Formation, Isolation, and Characterization†

Tareq Y. Ahmad,‡ John R. Guyton,§,‖ James T. Sparrow,‖ and Joel D. Morrisett*,‡,‖

Departments of Biochemistry, Cell Biology, and Medicine, Baylor College of Medicine and The Methodist Hospital, Houston, Texas 77030

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ABSTRACT: The association of apolipoprotein C-III (apoC-III) from human very low density lipoprotein with sphingomyelin from egg yolk (EYSM) has been studied at the transition temperature (T_c) of the phospholipid. Upon incubation of aliquots of the apoprotein with increasing amounts of sphingomyelin, the α-helical content of the apoprotein increased from 20% in the absence of EYSM to a limiting value of 67% at a protein:lipid molar ratio of 1:200. The tryptophan fluorescence spectrum of the apoprotein exhibited a gradual blue shift from 356 nm in the absence of EYSM to 348 nm when the protein:lipid ratio in the complex had reached 1:50. Gel filtration chromatography of complexes formed by incubating the apoprotein and phospholipid at differing apoC-III:EYSM ratios demonstrated a disintegration of sphingomyelin vesicles into particles of decreasing size with increasing proportion of protein. This effect was confirmed by sedimentation velocity experiments in which the observed sedimentation coefficient of EYSM decreased from 14.0 S (for vesicles) to a limiting value of 7.0 S when the apoprotein:phospholipid ratio reached 1:50 in the complex. Electron micrographs of negatively stained EYSM vesicles showed spherical particles of 380-Å diameter. Addition of apoC-III led to the formation of disk-shaped structures whose diameter decreased to a limiting value of 204 ± 34 Å at a protein:lipid ratio of 1:50. In contrast, the disk thickness was relatively constant at 51 ± 2 Å for all isolated complexes. Differential scanning calorimetry of apoC-III/EYSM complexes of increasing protein content showed only a small elevation of the transition temperature from 37.0 °C for vesicles alone to a limiting value of 40.5 °C for the 1:50 complex; the transition enthalpy decreased only slightly from 3.83 to 3.20 kcal/mol. Nitroxide-induced quenching of tryptophan fluorescence from apoC-III/EYSM complexes (1:110 mol/mol), containing 2–10% spin-labeled phosphatidylcholine, was the same when the doxyl ring was located at carbon 5 or 12 of the fatty acyl chain. However, quenching was lower when the doxyl ring was located at carbon 16. These results indicate that apoC-III binds to EYSM vesicles, inducing their structural transition to much smaller particles whose lipids exhibit highly cooperative thermal properties. The data are consistent with a model featuring a cylindrical 51-Å-thick bilayer whose outermost rings of acyl and alkenyl chains are covered by apoC-III whose helical axis runs perpendicular to the long axis of the chains.

The interaction of apolipoprotein C-III (apoC-III)[1] with various synthetic and naturally occurring phosphatidylcholines has been studied by various physicochemical methods. The α-helical content of the protein has been shown to increase by 2–3-fold and the intrinsic tryptophan fluorescence to undergo a 13-nm blue shift upon binding to egg yolk phosphatidylcholine vesicles (Morrisett et al., 1973). The structure of the resulting protein/lipid complex has been shown to depend on several factors such as the initial mixing protein:lipid ratio (Morrisett et al., 1974; Aune et al., 1977; Pownall et al., 1981) and the fatty acyl chain composition of the phosphatidylcholine (Pownall et al., 1977). When present at a protein:lipid mass ratio >0.08 g/g, apoC-III can induce structural transformation of DMPC vesicles into much smaller particles. The carboxyl-terminal half of apoC-III (residues 41–79) also binds to DMPC vesicles and causes their conversion to much smaller particles. In contrast, the amino-terminal half of the apoprotein is unable to bind or bring about this conversion (Sparrow et al., 1977).

Sphingomyelin is an important component of many biological systems. Its structure, synthesis, physical properties, and distribution in biological membranes and lipoproteins have been reviewed recently (Barenholz & Thompson, 1980; Barenholz & Gatt, 1982). The sphingomyelin found in egg yolk contains about 85% palmitic acid. This relatively high level of molecular homogeneity for EYSM allows efficient packing and cooperative behavior for bilayer membranes comprised of it (Calhoun & Shipley, 1979). In model membranes, sphingomyelin has been shown to undergo significant inter-

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* Correspondence should be addressed to this author at the Department of Medicine.

‡ Department of Biochemistry.

§ Department of Cell Biology.

‖ Department of Medicine.

[1] Abbreviations: EYSM, egg yolk sphingomyelin; BBSM, bovine brain sphingomyelin; PC, phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; SL, spin-label; 5-DP-PC, 12-DS-PC, and 16-DS-PC, 5-doxylpalmitate, 12-doxylstearate, and 16-doxylstearate esterified at the *sn*-2 position of egg phosphatidylcholine; apoC-III-1, apolipoprotein C-III (bearing one sialic acid residue) isolated from human very low density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; DEMA, diethylmalonic acid; DSC, differential scanning calorimetry; T_c, transition temperature; R_s, Stokes radius; TLC, thin-layer chromatography; CD, circular dichroism; EPR, electron paramagnetic resonance; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; EM, electron microscopy.

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action with other phospholipids and cholesterol (Stoffel et al., 1974a; Untracht & Shipley, 1977; Pal et al., 1981). Sphingomyelin also interacts with membranes and plasma proteins. Reconstitution experiments have demonstrated that membrane proteins isolated from sheep erythrocytes bind more strongly to sphingomyelin than those isolated from human erythrocytes (Kramer et al., 1972). This high binding specificity has been attributed to the high content of sphingomyelin in the former membranes. Early lipoprotein recombinant studies suggested that sphingomyelin binds preferentially to apoA-II over apoA-I (Stoffel et al., 1974b). More recently, however, apoA-I has been shown to form complexes with bovine brain sphingomyelin at the phase transition temperature of the lipid (Swaney & Schwartz, 1983). ApoC-III from human plasma has been shown to increase the substrate efficacy of sphingomyelin for lysosomal sphingomyelinase by 14-fold (Alpert & Beaudet, 1981). Studies on the mechanism of this effect are the subject of a separate paper (Ahmad et al., 1986). In this paper, we describe the interaction of apoC-III with EYSM vesicles. The study has been designed to elucidate the effect of sphingomyelin on apoprotein structure and, conversely, the effect of apoprotein on sphingomyelin structure. The resulting protein/phospholipid complexes have been rigorously characterized, and models of their structure have been proposed.

Materials and Methods

Egg yolk sphingomyelin (EYSM) was purchased from Sigma Chemical Co. (St. Louis, MO), and its purity was verified by thin-layer chromatography using chloroform/methanol/10% NH_4OH (65:25:4). Its fatty acyl composition as determined by Calhoun and Shipley (1979) is as follows: 16:0, 86.2%; 18:0, 6.0%; 22:0, 3.0%; 24:1, 4.9%. Its long-chain base composition as determined by Karlsson (1970) is predominantly dihydroxyl 18:1 (97%) with traces of dihydroxy 18:0 (3%). [^{3}H]Methyl iodide was obtained from Amersham (Arlington, IL). Diethylmalonic acid (DEMA) and its dipotassium salt were purchased from Calbiochem (La Jolla, CA). Fluram was obtained from Roche (Nutley, NJ). Protein assay reagent (Coomassie Blue G-250) was purchased from Pierce (Rockford, IL). 1,4-Diazabicyclo[2.2.2]octane was obtained from Aldrich Chemical Co. (Milwaukee, WI). Silica gel 60 was obtained from Merck (Darmstadt, West Germany). Unless otherwise specified, all experiments were conducted in a buffer of 10 mM DEMA, 1 mM EDTA, 1 mM NaN_3, and 150 mM NaCl, pH 7.2 (specified as DEMA buffer). Spin-labeled phosphatidylcholine isomers were prepared as described previously (Novosad et al., 1976).

Preparation of [choline-(C^3H_3)$_3$]Sphingomyelin. Sphingomyelin was demethylated by using 1,4-diazabicyclo-[2.2.2]octane (Stoffel, 1975) and purified on a silica gel column eluted with chloroform/methanol/water (65:25:2) and then remethylated with [$^3H_3$]methyl iodide (Patel et al., 1979). The product was partially purified by silica gel column chromatography and then completely on preparative TLC using chloroform/methanol/10% NH_4OH (65:25:4). The purified material was dissolved in chloroform/methanol (9:1) and stored under argon at −20 °C until used.

Preparation of Sphingomyelin Vesicles. About 100 mg of unlabeled EYSM and 1 mg of [^{3}H]SM (~10 000 cpm/mg) were dissolved in chloroform/methanol (9:1) in a 15-mL Corex tube and evaporated to dryness under a stream of dry nitrogen. Residual traces of solvent were removed under reduced pressure in a vacuum desiccator for 2 h. To the dry phospholipid was added 5 mL of DEMA buffer. This mixture was initially bath sonicated for 10 min at 45 °C and then subjected to probe sonication with a Heat Systems sonifier (w-350) equipped with a microtip. The sample temperature was maintained at ~10 °C with an ice/water bath during sonication at a power setting of 40% for 45 min. The resulting dispersion was centrifuged at 18 000 rpm for 45 min to sediment undispersed phospholipids and titanium particles released from the probe during sonication. The opalescent supernatant contained no degradation products of EYSM as determined by thin-layer chromatography. The dispersion was chromatographed (6 mL/h) on a column (1.6 × 100 cm) of Sepharose CL-4B equilibrated with DEMA buffer to obtain single-bilayer vesicles. The concentration of SM was estimated by phosphorus analysis using the procedure of Bartlett (1959). Unilamellar vesicles of EYSM containing 2–10% spin-labeled phosphatidylcholines (SL-PC) were prepared in the same manner as described above. Incorporation of 10% SL-PC in EYSM vesicles did not change their gel filtration elution pattern nor that of apoC-III/EYSM complexes formed from them. The concentration of spin-label in each vesicle preparation was determined by treatment of an aliquot with an equal volume of ethanol to disrupt intermolecular interactions and obtain narrow line spectra whose center field line height could be reproducibly measured and used for spin counting.

Purification of ApoC-III-1. ApoC-III was obtained from the plasma of subjects with type V hyperlipoproteinemia as described previously (Morrisett et al., 1973) except that the final step for separation of the different apoC proteins was accomplished on a Spectra Physics 8100 liquid chromatograph equipped with an AX 300 column (9.4 × 250 mm). To minimize its aggregation, the apoprotein was stored in 6 M guanidine. Immediately prior to use, the protein was either dialyzed exhaustively against DEMA buffer or desalted over a Bio-Gel P-2 column. Protein concentration was determined by the method of Bradford (1976) or with a fluorescamine assay (Weigele et al., 1972).

Preparation of ApoC-III/EYSM Recombinants. Prior to mixing, solutions of apoC-III and EYSM were separately maintained at 40 °C for 10–20 min. In all cases, the protein solution was added to the gently stirred phospholipid vesicles and the mixture incubated in a water bath at 40 °C for 2 h, conditions found to allow complete interaction of apoprotein with the phospholipid. Incubation mixtures of apoC-III and EYSM were fractionated by chromatography on Sepharose CL-4B as described above for vesicles. Fractions of 2 mL were collected and analyzed for protein by the absorbance at 280 nm and for lipid by liquid scintillation counting of ^{3}H. Recovery of labeled phospholipids and protein from the column (presaturated with EYSM) was greater than 90%. The Stokes radii of EYSM vesicles and apoC-III/EYSM complexes with different molar ratios were estimated from elution volumes by the method of Ackers (1967) and McGuinness (1973). The column was calibrated with unilamellar egg yolk PC vesicles (R_s = 106 Å; Newman & Huang, 1975), LDL, HDL_2, and HDL_3 (R_s = 110, 46, and 39 Å, respectively; Scanu, 1979).

Ultracentrifugation. Incubation mixtures of the vesicles and apoprotein (2 h at 40 °C) were diluted to a final volume of 5 mL with DEMA buffer. Their densities were adjusted to 1.07 g/mL with a solution of CsCl (22.16 g/100 mL of DEMA buffer). Centrifugation of each sample in a Beckman SW 50.1 rotor at 10 °C for 62 h caused the formation of a salt gradient in which the protein/lipid complex formed a visible, white band. Fractionation of the complexes was performed from the top using a Densi-Flow II gradient fractionator (Buchler Instruments). The density of each fraction (0.25 mL) was determined by refractive index measurements (Abbe refractometer) on a blank gradient (Weast, 1974). The

Table I: Properties of EYSM Unilamellar Vesicles and ApoC-III/EYSM Complexes

incubn mixture, apoC-III/EYSM (mol/mol)	α-helicity (%)	elution vol (mL)	obsd s (S)	complexes isolated by[e] DG (mol/mol)	complexes isolated by[e] GF (mol/mol)	disk thickness by EM[a] (Å)	particle diameter by EM (Å)	Stokes radius[b] (Å)
EYSM unilamellar vesicles	0[d]	104.5	14.0				380 ± 14[c]	175 ± 5
1:200	67	109.7	14.1	1:206	1:216	49 ± 2	305 ± 15[a]	132 ± 5
1:150	64		13.0	1:160		53 ± 2	254 ± 20[a]	
1:110	62	113.4			1:120	51 ± 2	240 ± 27[a]	120 ± 5
1:100	61	114.0	8.8	1:118	1:110	51 ± 2	231 ± 30[a]	118 ± 5
1:50	50	126.9	7.0	1:102	1:110	52 ± 2	204 ± 32[a]	80 ± 5

[a] Determined from 100 disks in rouleaux. [b] Computed by the method of Ackers (1967) and McGuinness (1973) from the elution volumes and known Stokes radii of several lipoproteins and sonicated vesicles. Further details are given under Materials and Methods. [c] Determined on 100 free-standing vesicles. [d] At the concentrations of EYSM used for these measurements (≤4.25 mg/mL), its contribution to the CD spectrum was negligible. [e] DG, density gradient; GF, gel filtration.

protein and phospholipid contents of each fraction were measured as described above.

Analytical ultracentrifugal measurements were performed on a Beckman Model E instrument as described previously (Morrisett et al., 1974; Aune et al., 1977). To obtain the desired protein:lipid ratios, a precisely measured volume from a stock solution of apoC-III (4.1 mg/mL) at 40 °C was added to an appropriate aliquot from a stock solution of EYSM vesicles (2.76 mg/mL) equilibrated at 40 °C in the centrifuge cell. The solution was mixed by manually rotating the closed cell and then incubated at 40 °C for 2 h before ultracentrifugation.

Optical Measurements. Circular dichroic spectra were acquired at room temperature (~23 °C) on a Jasco Model J-500 C spectropolarimeter, using a 2-mm cell and 20 mM Tris buffer, pH 7.4. The helical content of protein in each protein/lipid complex was determined from θ_{222} as described previously (Morrisett et al., 1973). Although sphingomyelin made a significant contribution to the CD spectrum below 210 nm, the contribution at 222 nm was insignificant (Litman & Barenholz, 1975).

Fluorescence spectra were recorded on an SLM 8000 spectrofluorometer. Measurements on apoC-III alone, apoC-III/EYSM complexes with differing stoichiometries, and apoC-III/SL-PC/EYSM complexes were recorded at room temperature (~23 °C).

Thermodynamic Measurements. ApoC-III/EYSM mixtures of differing ratios were incubated for 2 h at 40 °C. After incubation, each mixture was concentrated in a collodion bag (Schleicher & Schuell) under reduced pressure to a final lipid concentration of 50–80 mg/mL. An aliquot of 70 μL from a mixture or from a stock preparation of unilamellar or multilamellar EYSM vesicles was transferred to a 75-μL stainless-steel pan and hermetically sealed. Differential scanning calorimetry was performed on a Perkin-Elmer DSC-2 instrument equipped with a subambient cooling unit. The calorimeter was calibrated with an indium standard to an accuracy of ±0.3 °C. Scans were repeated at least 3 times at a heating and cooling rate of 2.5 °C/min. After the last scan of a sample, the pan was opened, and the contents were transferred to a known volume of chloroform/methanol/water (6:4:1). The actual amount of lipid in each pan was determined by phosphorus analysis (Bartlett) and its chemical integrity checked by TLC on silica gel. The transition temperature, T_c, was determined from the center peak of the endotherm. Transition enthalpy, ΔH, was determined from the area under the endotherm by weighing the trace bounded by the peak and base line. The ΔH was computed by comparison of the experimental area with the area generated by the indium standard. At least five determinations were used to compute the mean enthalpy of transition. Transition entropy was determined by assuming that the free energy $\Delta G = 0$ so that $\Delta H = T\Delta S$.

Electron Microscopy. Samples of EYSM vesicles (0.5–1.0 mg/mL) obtained from gel filtration and apoC-III/EYSM complexes isolated by density gradient ultracentrifugation or gel filtration chromatography (0.5–1.0 mg/ml) were dialyzed against 0.15 M ammonium acetate/ammonium carbonate, pH 7.4. Formvar- and carbon-coated copper grids were pretreated briefly with 0.001% bovine serum albumin, incubated for 1–2 min with vesicles or complexes, and then stained with 2% potassium phosphotungstate, pH 6.8. Grids were viewed in a Philips 201 electron microscope.

EPR spectra were obtained on a Varian E-12 spectrometer interfaced to a Data Translation Lab Datex system based on an LSI 11/2. The spectrometer was operated at 9.1 GHz and the cavity temperature governed by a variable-temperature controller.

Results

To determine the effect of apoC-III binding to EYSM vesicles on the secondary structure of the protein, the CD spectra of individual apoprotein/phospholipid complexes of differing stoichiometries were recorded. The CD spectrum of apoC-III alone at 23 °C indicated that the apoprotein contained about 20% α-helical structure. When the proportion of phospholipid present in the recombinant particle was increased, a concomitant increase in α-helical structure was observed until a protein:lipid ratio of about 1:100 was reached, where the α-helicity had risen to about 61%. Beyond this ratio, only modest increases in α-helicity were observed, so that at a protein:lipid ratio of 1:200, the α-helicity had risen further to only 67% (Table I).

In a separate set of experiments designed to evaluate the effect of sphingomyelin binding on apoC-III structure, the wavelength of maximum tryptophan fluorescence was measured as a function of protein:lipid ratio (Figure 1). Individual mixtures of apoC-III and EYSM which had been incubated under standard conditions were each measured at room temperature. The apoprotein alone gave an emission spectrum with a maximum at 356 nm. As the proportion of sphingomyelin in the recombinant particle was increased, there was a concomitant decrease (blue shift) in λ_{max} until a protein:lipid ratio of 1:50 was reached, at which point the λ_{max} reached a limiting value of 348 nm. Complexes formed by mixtures containing a higher proportion of sphingomyelin did not cause a further decrease in λ_{max}.

The effect of apoprotein on the structure of single-bilayer sphingomyelin vesicles was initially studied by gel filtration chromatography. When chromatographed on a column of Sepharose CL-4B, a sonicated dispersion of sphingomyelin eluted as two populations of differing size. The lesser abundant

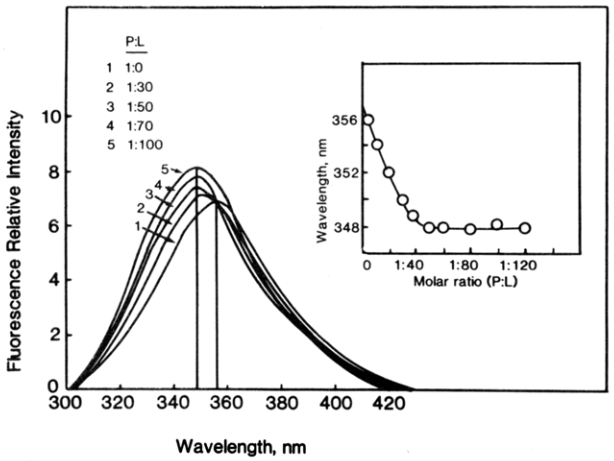

FIGURE 1: ApoC-III intrinsic fluorescence dependence on the amount of EYSM present in apoC-III/EYSM recombinants. Each data point represents an individual apoC-III/EYSM mixture incubated at 40 °C for 2 h in 10 mM DEMA, 1 mM EDTA, 150 mM NaCl, and 1 mM NaN_3 at pH 7.2. The inset indicates the fluorescence wavelength shift as a function of protein:lipid molar ratio. Full details are given under Materials and Methods.

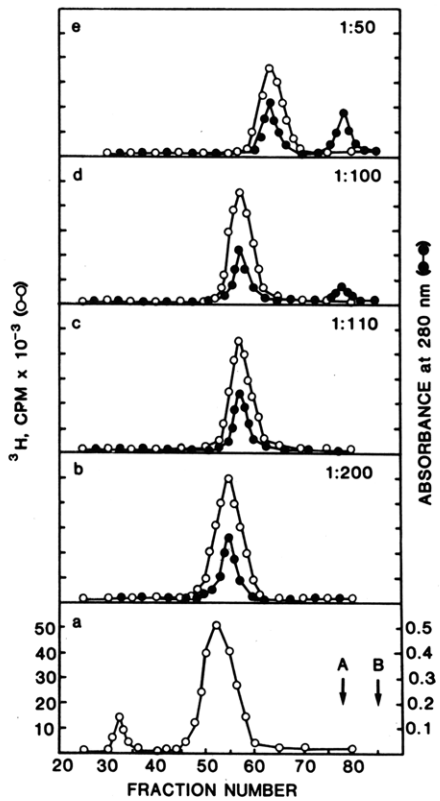

FIGURE 2: Elution profiles of EYSM vesicles and apoC-III/EYSM recombinants on Sepharose CL-4B: (a) 16 mg of EYSM vesicles; (b) recombinants formed by mixing 1 mg of apoC-III with 15.7 mg of EYSM (1:200) in a total volume of 2 mL; the mixture was incubated for 2 h at 40 °C and then chromatographed at room temperature (c–e). Same as (b) except for different protein:lipid molar ratios. Arrows A and B designate the elution volumes for apoC-III alone and [^{14}C]ethanolamine, respectively. Fraction volumes = 2 mL.

population eluted as multilamellar vesicles in the void volume of the column, while the much more abundant fraction of material eluted as a single symmetrical peak centered at an included volume of 104.5 mL (Figure 2a). This corresponded to an apparent Stokes radius of 175 Å. When EYSM vesicles were incubated with increasing proportions of apoC-III-1 before chromatography, the volume at which the resulting complex eluted from the column also increased. At a protein:lipid ratio of 1:50, a limiting volume for elution of the complex was reached (126.9 mL). At this ratio, excess unbound apoC-III was present as evidenced by its elution at 152 mL (Figure 2e).

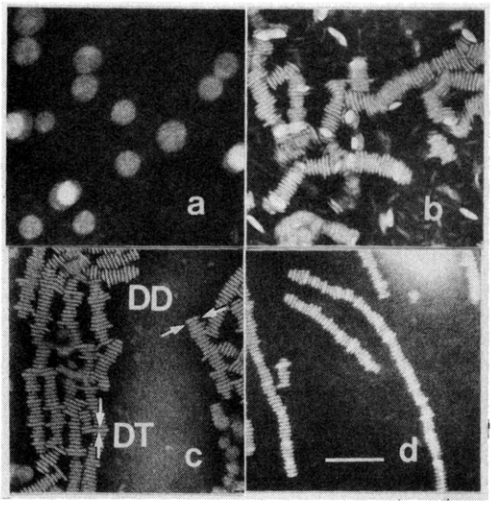

FIGURE 3: Electron micrographs of negatively stained EYSM vesicles (a) and apoC-III/EYSM complexes with protein:lipid ratios of 1:200 (b), 1:100 (c), and 1:50 (d). DD and DT designate the disk diameter and disk thickness, respectively. Magnification for EYSM vesicles is 99225× and for apoC-III/EYSM complexes is 102052×. The bar in panel d is equivalent to 756 Å.

Negative-stain electron microscopy was used to compare the dimensions of the recombinant particles isolated by gel filtration with the sizes computed on the basis of their elution volumes. Sphingomyelin vesicles alone appeared in the micrographs as uniform spherical particles with diameters of about 380 Å (Figure 3a). The micrographs of the 1:200 complex isolated by gel filtration (Figure 2b) contained disk-shaped particles which associated to form stacks or rouleaux. Individual disks had an average thickness of about 49 Å and a longest dimension of about 305 Å (Figure 3b, Table I). The particle population produced by gel filtration of a 1:100 mixture also appeared as rouleaux. The thickness of individual disks was not altered significantly (53 Å), but their longest dimension (diameter) was reduced to about 254 Å (Table I). Gel filtration of the 1:50 mixture produced a highly homogeneous population of particles whose disk thickness was not decreased (51 Å) but whose disk diameter was reduced to a limiting value of 204 Å (Figure 3d, Table I). In general, increasing the proportion of apoprotein in the incubation mixture produced disk-shaped particles whose longest dimension decreased with increasing protein content but whose shortest dimension (disk thickness) remained unaltered.

Additional quantitative hydrodynamic data characterizing the recombinants formed by interaction of apoC-III and EYSM vesicles were sought in a series of sedimentation velocity experiments (Table I). Sphingomyelin vesicles alone had an *s* value of about 14.0 S. When these vesicles were incubated at 40 °C in mixtures containing increasing proportions of apoC-III, the value of *s* decreased sharply until a protein:lipid ratio of about 1:100 was reached. At this ratio, the resulting complexes exhibited an *s* value of about 8.8 S. Separate incubation mixtures containing larger proportions of apoprotein (e.g., 1:50) afforded particle populations which exhibited almost no further decrease in *s* value (Table I).

The above analytical ultracentrifugation experiments suggested that the apoC-III/EYSM recombinants of differing densities might be isolatable by density gradient centrifugation. This possibility was evaluated by centrifugation of incubation mixtures containing different protein:lipid ratios on cesium

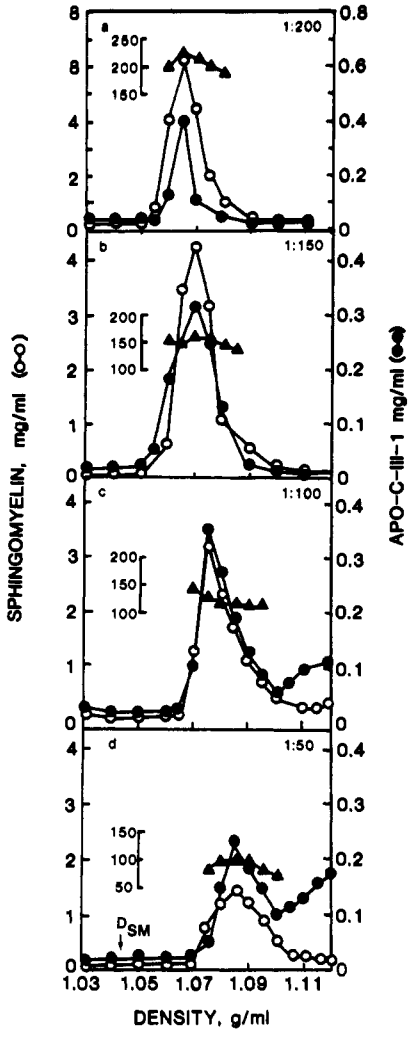

FIGURE 4: Density gradient ultracentrifugation of apoC-III/EYSM complexes. The density of each incubated protein/lipid mixture was adjusted to 1.07 g/mL with CsCl (total volume = 5 mL). The mixtures were centrifuged at 45 000 rpm for 62 h, 10 °C. Protein (closed circles) was estimated by the fluorescamine assay and EYSM (open circles) by the phosphomolybdate assay. The final molar ratios are indicated by closed triangles. Initial mixing protein:lipid ratios are indicated in the upper right-hand corner of each panel. The arrow in panel d indicates the hydrated density of EYSM vesicles.

chloride gradients spanning a density range from 1.03 to 1.12 g/mL (Figure 4). Addition of a relatively small amount of apoprotein to give an apoC-III:EYSM ratio of 1:200 produced a complex which banded as a single species centered at a hydrated density of 1.065 g/mL (Figure 4a). Mixtures containing increasing proportions of apoprotein formed complexes which banded at increasing densities until a mixing ratio of 1:100 was reached, where the apoprotein was clearly in excess as indicated by its accumulation in lipid-free form at the high-density end of the gradient (Figure 4c). This recombinant banded at a density of 1.075 g/mL and had a stoichiometry of 1:118, which compares favorably to a stoichiometry of 1:110 for the complex formed from a mixture of the same mixing ratio but isolated by gel filtration (Table I). Even though all of the apoprotein present in the 1:100 incubation mixture was not incorporated into an isolated complex, increasing the proportion of apoprotein in the initial apoprotein mixture to 1:50 produced a recombinant which banded at an even higher hydrated density (1.085 g/mL) because of its greater protein content (Figure 4d, Table I). However, incubation mixtures containing greater proportions of apoprotein than that in the 1:50 mixture did not yield complexes of greater density or greater apoprotein content (data not shown).

Table II: Thermotropic Properties of EYSM and ApoC-III/EYSM Complexes

sample	T_c[a] (°C)	ΔH[b] (kcal/mol)	ΔS (cal mol^{-1} deg^{-1})
EYSM multilamellar liposomes	40.0	7.30	23.4
EYSM unilamellar vesicles	37.0	3.83	12.35
1:200	38.0	3.80	12.22
1:150	39.5	3.75	12.00
1:100	40.5	3.51	11.20
1:50	40.5	3.20	10.21

[a] Values represent the averages of three determinations; standard deviation = ±0.3 °C. [b] Values represent the average of five measurements on a single sample; standard deviation = ±0.2 kcal/mol.

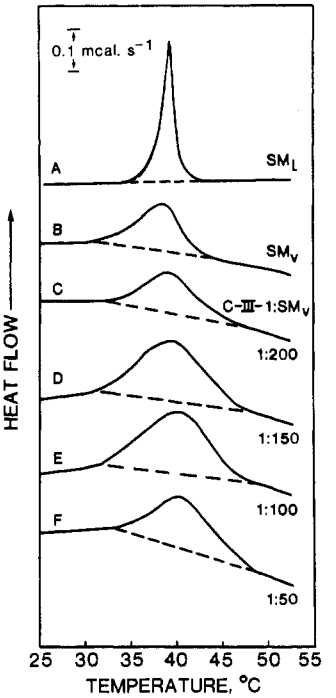

FIGURE 5: DSC heating curves of EYSM as a function of apoC-III:EYSM molar ratio: (A) 5.7 mg of multilamellar EYSM liposomes; (B) 3.9 mg of unilamellar EYSM vesicles; (C–F) apoC-III/EYSM complexes whose initial mixing ratios are shown to the right of each endotherm. All samples were 70 μL in DEMA buffer and were run against an equal volume of the same buffer at a scan rate of 2.5 °C/min and a sensitivity of 1.0 mcal/s.

The effect of apoC-III-1 binding on the thermal properties of sphingomyelin was studied by DSC. Multilamellar liposomes exhibited a sharp, highly cooperative transition that occurred at 40.0 °C with an enthalpy of 7.30 kcal/mol (Figure 5A, Table II). The somewhat smaller unilamellar vesicles exhibited a somewhat less cooperative transition at lower temperature (37.0 °C) and with lower enthalpy (3.83 kcal/mol). Apoprotein binding to EYSM vesicles and subsequent disintegration to much smaller particles were attended by a small increase in transition temperature, which increased further with increasing protein:lipid ratio of the incubation mixture. For example, as the apoprotein:phospholipid ratio was changed from 1:200 to 1:50, the transition temperature rose from 38.0 to 40.5 °C. This series of recombinants had transition enthalpies that were moderately dependent on stoichiometry of the complex. Whereas vesicles alone had a transition enthalpy of 3.83 kcal/mol, this decreased to 3.20 in the complex formed from the 1:50 incubation mixture.

The fluorescence quenching of tryptophan in apoC-III/EYSM complexes containing 2–10% SL-PC was studied. In these experiments, the protein and phospholipids (1:110

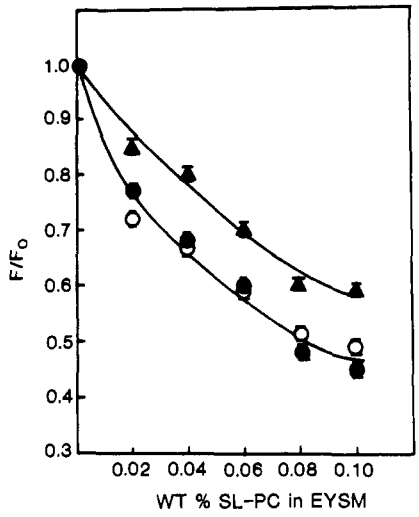

FIGURE 6: Quenching of tryptophan fluorescence in apoC-III/EYSM complexes by spin-labeled phosphatidylcholine. F represents the fluorescence intensity at 348 nm in the presence of spin-label; F_0 represents the intensity in the absence of spin-label. Closed circles, 5-DP-PC; open circles, 12-DS-PC; closed triangles, 16-DS-PC. The apoC-III:total phospholipid molar ratio was 1:110. Results are the mean ± SD of three measurements.

mol/mol) were incubated at 40 °C for 2 h and then used directly. The fluorescence intensity in the recombinant containing 10% 5-DP-PC or 12-DS-PC was diminished to about 50% of the intensity observed in the absence of spin-labeled phospholipid. For 16-DS-PC, quenching to about 60% of the control intensity was observed. This lower level quenching for 16-DS-PC was observed not only at the 10% SL-PC level but also at all the other lower levels tested down to 2% (Figure 6).

Discussion

In previous reports, we have described the interaction of apoC-III with two well-characterized types of phospholipid vesicles. When apoC-III is added in excess to egg yolk phosphatidylcholine single-bilayer vesicles, a complex is formed in which about 46 apoC-III molecules have been bound to the vesicle surface without loss of vesicular structure (Morrisett et al., 1974). In contrast, addition of excess apoC-III to DMPC single-bilayer vesicles resulted in the loss of vesicular structure and the formation of much smaller complexes which contained 9 apoC-III molecules and 454 DMPC molecules (Aune et al., 1977). The reason for the striking difference in the stoichiometry and the structure of complexes formed by these two phospholipids has been attributed to the difference in the stability of their hydrated bilayers. In the present study, we have demonstrated that the interaction of apoC-III with single-bilayer vesicles of EYSM results in the collapse of vesicle structure and formation of smaller complexes. The size of these complexes is highly dependent upon the apoC-III:EYSM ratio in the initial incubation mixture (Table I). When that ratio is 1:200, a particle with a diameter of 305 Å is produced as judged by negative-stain electron microscopy. When this ratio is 1:50, this diameter is reduced to 204 Å. Significantly, the thickness of each complex does not depend on the apoprotein:phospholipid ratio but remains constant at about 51 Å, which is about the width of a sphingomyelin bilayer. Each of the apoC-III/EYSM complexes, spontaneously produced by incubation for 2 h at the thermal transition temperature of EYSM, has a protein:lipid ratio that reasonably approximates that of the initial incubation mixture from which it originated, with the exception of the 1:50 mixture which produced complexes with a stoichiometry 1:102–1:111 (Table I). Clearly, the 1:50 incubation mixture contains excess apoprotein which is not incorporated into the isolated complex as judged either by density gradient ultracentrifugation (Figure 4d) or by gel filtration (Figure 2e). However, the presence of excess apoC-III in the incubation mixture appears to be essential for obtaining the smallest possible size population. These had a disk diameter of 204 Å as determined by electron microscopy (Table I). These particles are somewhat larger than the smallest apoC-III/DMPC complex formed, which had a corresponding dimension of 170 Å (Laggner et al., 1979).

The observation that the thickness for apoC-III/EYSM complexes of widely differing protein:lipid ratio does not vary but remains constant at about 51 Å strongly suggests that bilayer structure has been retained and that the complexes have a discoidal shape. Some dimensional aspects of this discoidal model were tested by computing the number of sphingomyelin molecules that could be accommodated within the volume of a right cylinder and the number of apoC-III molecules that could be accommodated around its outermost hydrophobic surface. These calculations are based on a 40.5-Å cross-sectional area for sphingomyelin (Yedgar et al., 1981), a 1194 $Å^3$ molecular volume for sphingomyelin (Luzzati et al., 1979), an α-helix width of 15 Å (including amino acid side chains) and length of 1.5 Å per residue (Laggner et al., 1979), and a helix length determined by the fraction of helical residues present within apoC-III as judged by the CD spectrum (Table I). On the basis of the above assumptions, we compute that the smallest (204-Å diameter) particle can accommodate 9.4 apoC-III molecules and 1015 sphingomyelin molecules. This protein:lipid ratio agrees to within 2% of the 1:106 average composition of this complex (Table I). For the largest (305-Å diameter) particle, 10.6 apoC-III molecules and 2536 sphingomyelin molecules can be accommodated. This corresponds to a protein:lipid ratio of 1:230 which is within 9% of the 1.211 average composition of this complex (Table I). Hence, the dimensional and compositional properties of these complexes are reasonably consistent with a disk-shaped model.

The recombination of human apoA-I with bovine brain sphingomyelin (Swaney & Schwartz, 1983) yielded complexes which differ significantly from those obtained in the present study. For example, an apoA-I/BBSM incubation mixture weight ratio of 1:7.5 produced a discoidal complex with a thickness of 78 Å and a diameter of 217 Å. In the present study, a comparable incubation mixture of 1 g of apoC-III/7.8 g of EYSM (1 mol:100 mol) produced a complex with a thickness of 51 Å and diameter of 231 Å (Table I). The greater thickness of the complex formed by BBSM is most probably due to the longer acyl chains present in this more heterogeneous sphingomyelin preparation than in EYSM whose acyl chains are about 85% C16:0. The difference in disk diameter for the two complexes may well reflect the difference in molecular weights of apoA-I (~27 000) and apoC-III (~9000) and the lengths of helical segments each is capable of developing. Similar differences exist between their 1:15 (grams of apoA-I per gram of BBSM) complex and our 1:16 (grams of apoC-III per gram of EYSM complex) mixtures, i.e., the 1:200 mol/mol complex listed in Table I.

The DSC data (Figure 5) indicate that complexes formed from apoC-III/EYSM incubation mixtures containing an increasing proportion of protein exhibit slightly increasing thermal transition temperatures and slightly decreasing thermal transition enthalpies. These results are consistent with the discoidal model presented in Figure 7. Similar results have been obtained for complexes formed by DMPC vesicles

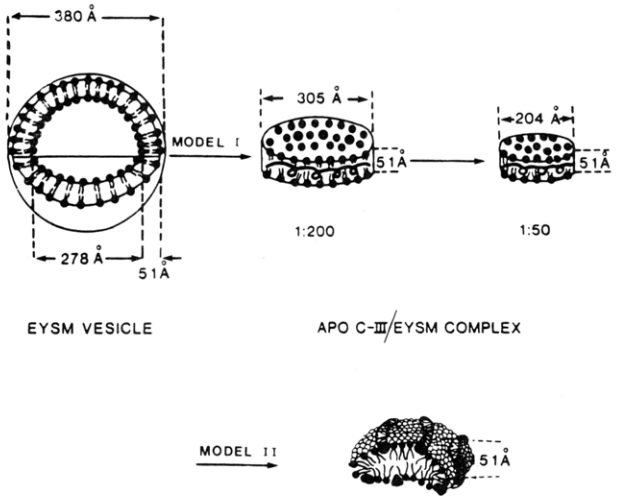

FIGURE 7: Possible models for complexes formed by interaction of apoC-III and EYSM vesicles. In model I, apoC-III induces transformation of EYSM vesicles into disk-shaped structures. The diameter, but not the thickness, of the disk is determined by the protein:lipid molar ratio until it reaches 1:50. In model II, apoC-III induces the vesicles to form ellipsoidal structures with the apoprotein randomly distributed among the phospholipid polar head groups. A basic difference between these two models is the more definitive location of the apoprotein in the discoidal structure.

and apoA-I or apoA-II with different molar ratios (Gilman et al., 1981; Massey et al., 1981). Complexes containing an increasing proportion of boundary apoprotein will have an increasing fraction of sphingomyelin molecules interacting with that apoprotein at the periphery of the disk. The effect of this interaction will be to decrease the rate and/or amplitude of boundary acyl and alkenyl chain motion(s) at a given temperature, thereby elevating the transition temperature. Similarly, complexes of decreasing diameter will have lipid domains of decreasing size which will have decreasing transition enthalpy. On the basis of this model, one might predict that two populations of sphingomyelin molecules could be observed: those interacting with the apoprotein on the outer rim of the disk and those inner molecules not interacting with the apoprotein. Observation of these two populations will, of course, require that the rate of exchange between the two environments is slow on the time scale of the technique being used. Experiments designed to detect and characterize these two populations are currently in progress.

Experimental measurements (Atkinson et al., 1980; Tall et al., 1977; Wlodawer et al., 1979) and theoretical calculations (Segrest, 1977) have provided strong arguments for recombinant models featuring a tire of protein circumscribing a wheel of bilamellar phospholipid. However, little if any experimental evidence has been presented that indicates the orientation of the apoprotein's amphipathic helix with respect to the phospholipid acyl chains. Two extreme possibilities exist in which the long axis of the helix is either parallel or perpendicular to the long axis of these chains. In the present study, evidence has been obtained from spin-label-induced tryptophan fluorescence quenching experiments that suggests discrimination between these two orientations in the discoidal model. Space-filling model studies of the apoC-III amphipathic helix suggest that the indole side chains of tryptophan residues 42, 54, and 65 all lie at the outermost periphery of the hydrophobic half of the cylindrical surface (Sparrow & Gotto, 1980). If this helix is superimposed on a 51-Å sphingomyelin bilayer (*perpendicular* to its chains), the indole side chains are about equidistant from carbons 5 and 12, but more distant from carbon 16 which is near the middle of the bilayer and hence near the center of the helix. On the basis of this perpendicular orientation, one would then predict that a paramagnetic doxyl group at C5 or C12 would more strongly quench apoC-III fluorescence than would a group at C16. If the helix axis were oriented *parallel* to the acyl/alkenyl chains of the bilayer, there would be no unambiguous spacial relationship between the indole side chains and acyl/alkenyl carbon positions, and differential quenching by doxyl groups at different positions would not be expected. Hence, the quenching data obtained (Figure 6) are consistent with the perpendicular helix orientation in the discoidal model. Other amphipathic apoproteins (e.g., apoA-I), whose helices contain fractions of hydrophobic and hydrophilic surface differing from that of apoC-III, may orient their indole side chains at relative locations on their amphipathic helices quite different from those of apoC-III. Hence, the pattern of quenching would then be different.

A second possible model for the apoC-III/EYSM complex is the ellipsoidal structure (model II, Figure 7). This model is similar to that proposed previously from X-ray data for the apoC-III/DMPC recombinant (Laggner et al., 1979). In this model, the protein is randomly interspersed among the polar head groups of the particle surface. Its appearance is similar to that of a flattened micelle, a structure which has also been proposed for the Triton X-100/sphingomyelin mixed micelle (Barenholz & Gatt, 1982). If the protein were located on the surface of the ellipsoidal micelle, then one would expect to observe the greatest quenching of tryptophan fluorescence when the doxyl group is located nearest the polar head groups. However, the same extent of quenching is observed when the doxyl group is located at positions 5 and 12, a result suggesting that the indole rings are equidistant from these carbons. The similarity of the thermal transition enthalpies for the apoC-III/EYSM complexes and EYSM bilayer vesicles suggests that the lipids in the complexes possess bilayer structure; by reason of its curved edges, an ellipsoidal structure would be expected to exhibit a significantly altered transition enthalpy. Hence, these results favor the discoidal model I over the ellipsoidal model II (or flattened micelle) but do not prove it.

The present study of the interaction of apoC-III with sphingomyelin developed from an earlier observation that apoC-III strongly enhanced the hydrolysis of sphingomyelin by preparations of lysosomal sphingomyelinase (Alpert & Beaudet, 1981). Moreover, of all the human plasma apolipoproteins isolated and characterized to date, apoC-III is clearly the most effective. We have examined each of the apoC-III/EYSM complexes characterized in this study and have found a clear correlation between their size and substrate efficacy (Ahmad et al., 1986). Sphingomyelin in the small 204-Å-diameter particle is hydrolyzed more than twice as rapidly as that in the large 305-Å-diameter particle. A possible reason for the greater substrate efficacy of the smaller complex is that a greater fraction of sphingomyelin molecules would be present on the periphery of the disk and presumably more accessible to hydrolysis by the enzyme. Calculations based on the assumptions enumerated above indicate that the outer ring (or.) of sphingomyelin molecules occupies 9.9% of the total (tot) area of the two polar faces of the 305-Å-diameter complex. This fractional area, $A_{or.}/A_{tot}$, increases gradually with decreasing disk diameter: 12.1%, 12.9%, 14.4%, and 15.4% for disks of 254, 240, 231, and 204 Å, respectively (Table I). This increase in fractional area is not of the same magnitude as the increase in sphingomyelin hydrolysis: 15%, 17%, 26%, and 33%, respectively (Figure 3; Ahmad et al., 1986). Hence, the enzymatic data may be more consistent with the ellipsoidal

model in which the surface-located apoprotein disrupts interactions between sphingomyelin molecules, making them more accessible to the enzyme. The following paper considers the implications of these results in fuller detail.

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