Effect of Apolipoprotein A-I Lipidation on the Formation and Function of Pre-β and α-Migrating LpA-I Particles†

Daniel L. Sparks,* Philippe G. Frank, Sylvie Braschi, Tracey A.-M. Neville, and Yves L. Marcel

Lipoproteins and Atherosclerosis Group, University of Ottawa Heart Institute, 40 Ruskin Street, Ottawa, Ontario K1Y 4W7, Canada

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ABSTRACT: A unique class of lipid-poor high-density lipoprotein, pre-β_1 HDL, has been identified and shown to have distinct functional characteristics associated with intravascular cholesterol transport. In this study we have characterized the structure/function properties of poorly lipidated HDL particles and the factors that mediate their conversion into multimolecular lipoprotein particles. Studies were undertaken with homogeneous recombinant HDL particles (LpA-I) containing apolipoprotein (apo) A-I and various amounts of palmitoyloleoylphosphatidylcholine (PC) and cholesterol. Complexation of apoA-I with small amounts of PC and cholesterol results in the formation of discrete lipoprotein structures that have a hydrated diameter of about 6 nm but contain only one molecule of apoA-I (Lp1A-I). While the molecular charge and α-helix content of apoA-I are unaffected by lipidation, the thermodynamic stability of the protein is reduced significantly (from 2.4 to 0.9 kcal/mol of apoA-I). Evaluation of apoA-I conformation by competitive radioimmunoassay with monoclonal antibodies shows that addition of small amounts of PC and cholesterol to apoA-I significantly increases the immunoreactivity of a number of domains over the entire molecule. Increasing the ratio of PC:apoA-I to 10:1 in the Lp1A-I complex is associated with increases in the α-helix content and stability of apoA-I. However, incorporation of 10−15 mol of PC destabilizes the Lp1A-I complex and promotes the formation of more thermodynamically stable (1.8 kcal/mol of apoA-I) bimolecular structures (Lp2A-I) that are approximately 8 nm in diameter. The formation of an Lp2A-I particle is associated with an increased immunoreactivity of most of the epitopes studied, with the exception of one central domain (residues 98−121), which becomes significantly less exposed. This structural change parallels a significant increase in the net negative charge on the complex. Characterization of the ability of these lipoproteins to act as substrates for lecithin:cholesterol acyltransferase (LCAT) shows that unstable Lp1A-I complexes stimulate a higher rate of cholesterol esterification by LCAT than the small but more stable Lp2A-I particles (V_{max} values are 5.8 and 0.3 nmol of free cholesterol esterified/h, respectively). The ability of LCAT to interact with lipid-poor apoA-I suggests that LCAT does not need to bind to the lipid interface on an HDL particle but that LCAT may directly interact with apoA-I. The data suggests that lipid-poor HDL particles may be metabolically reactive particles because they are thermodynamically unstable.

A unique class of small lipid-poor lipoprotein, pre-β_1 HDL,[1] has been shown to be generated by the dissociation of apoA-I molecules from abnormally unstable HDL particles in hypertriglyceridemic patients (*1*, *2*). An increased formation and amount of this lipoprotein appears to reflect abnormalities in cholesterol metabolism and is directly related to low HDL levels (*3*−*5*). Observations from in vitro studies with the lipolytic enzymes lipoprotein lipase and hepatic lipase suggest that these lipid-poor HDL species may be generated from lipolysis of either larger HDL particles or their triglyceride-rich precursors (*6*, *7*). Other studies suggest that these particles may in fact represent nascent newly secreted apoA-I (*8*, *9*). Most of these studies show that the stability of this unique class of HDL is very low in plasma, as it appears that these particles are quickly converted into α-migrating species in the presence of lecithin:cholesterol acyltransferase (LCAT) (*10*) or some other transforming factor (*11*). This metastability has made structure/functional characterizations of pre-β HDL particles difficult.

Structural characterization of both native and reconstituted lipoprotein complexes has shown that the physicochemical properties that define HDL particles are associated with unique composition-dependent properties of the primary protein on HDL, apoA-I. We and others have shown that the conformation of apoA-I on HDL is highly variable and

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* Corresponding author: Telephone (613) 761-4822; Fax (613) 761-5281; E-mail dsparks@heartinst.on.ca.

[1] Abbreviations: apoA-I, apolipoprotein A-I; appK_m, apparent K_m; CD, circular dichroism; CE, cholesteryl ester; FC, free (unesterified) cholesterol; DMS, dimethyl suberimidate; ED_{50}, particle concentration required for 50% inhibition of the maximal antibody binding; GdnHCl, guanidine hydrochloride; HDL, high-density lipoproteins; LCAT, lecithin:cholesterol acyltransferase; Lp2A-I, reconstituted particles containing 2 molecules of apolipoprotein A-I; Lp1A-I, reconstituted particles containing 1 molecule of apolipoprotein A-I; mAb, monoclonal antibody; PC, palmitoyloleoylphosphatidylcholine; TG, triglyceride.

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sensitive to both the lipid and apoprotein components of HDL (*12*−*19*). Changes in both surface and core lipid constituents of HDL directly affect the structural integrity or stability of the lipoprotein and confer unique electrostatic features on different classes of HDL particles (*18*, *20*). Of particular interest is the observation that a reduction in core contents and cholesteryl ester/triglyceride (CE/TG) ratio in HDL significantly reduces the thermodynamic stability of the lipoprotein and promotes the dissociation of partially lipidated monomeric molecules of apoA-I (*18*). This observation appears to explain why apoA-I can dissociate from HDL after lipolysis by hepatic or lipoprotein lipase (*6*, *7*), and why the apoprotein can then reassociate with HDL during incubations with LCAT (*6*, *10*). The data supports the view of a cyclic dissociation/reassociation pathway for apoA-I on HDL particles (*21*), one that appears to be regulated by changes in the core lipid composition of the lipoprotein particles. This pathway appears to be perturbed in hypertriglyceridemic patients, as it appears that when HDL-TG levels rise, the lipoprotein stability falls and apoA-I is shed (*7*). Previous work has suggested that this dissociation of apoA-I may promote HDL clearance by enhancing the renal filtration of the apoprotein (*3*).

Investigations from different laboratories have shown that the metabolism of pre-β HDL may be unique and very different from that of α-migrating HDL particles (*4*, *11*, *22*, *23*). Most notably, work by Fielding and co-workers has shown that pre-β_1 HDL may be the preferred initial acceptors of cellular cholesterol (*22*, *24*). To further investigate this functional property of poorly lipidated HDL particles, we developed a method for the preparation of well-defined Lp1A-I structures and have compared and contrasted the efflux-promoting ability of these particles to free apoA-I and to lipoproteins containing 2 molecules of apoA-I (Lp2A-I) (*25*). We showed that poorly lipidated Lp1A-I are much better acceptors of cholesterol from fibroblasts than lipid-free apoA-I and that progressive lipidation of Lp1A-I and Lp2A-I enhances this process. In the present study, we have attempted to show that the distinct physical characteristics of Lp1A-I particles confer their unique functional properties. Structural analyses show that these poorly lipidated lipoprotein particles have unique charge and physical properties that render them both structurally and functionally distinct from the multimolecular α-migrating species of HDL. In addition, we show that Lp1A-I are also good substrates for LCAT and can promote greater rates of cholesterol esterification than some Lp2A-I complexes.

EXPERIMENTAL PROCEDURES

Materials

Free cholesterol (FC) was purchased from Sigma Chemical Co. (St. Louis, MO). 1-Palmitoyl-2-oleoylphosphatidylcholine was obtained from Avanti Polar Lipids (Birmingham, AL). Monoclonal antibodies 2G11 and 4A12 were purchased from Sanofi (France); A16, A51, A05, A17, A11, A03, and A44 were obtained from the Institut Pasteur (Lille, France); and 4H1, 2F1, 3G10, and 5F6 were produced in our laboratory (*26*). All other reagents were analytical grade.

Table 1: Effect of Lipid Composition on the Size and Charge of LpA-I Complexes[a]

particle composition PC:FC:apoA-I[b] (mol:mol:mol)		EM	hydrodynamic diameter[d]	surface potential[e]	valence (/apoA-I)[f]
initial	final	shape[c]	(nm)	(−mV)	(−*e*)
apoA-I		nd	7.0	8.3	3.2
5:10:1	1:2:1	nd	6.0*	8.3	3.6**
10:15:1	2:4:1	nd	6.0*	8.2	3.5**
15:10:1	9:4:1	nd	6.0*	8.1	3.5**
30:10:1	30:6:2	sphere	6.0/7.5[g]	8.1/10.2[g]	3.5/3.1[g]
35:6:1	48:4:2	sphere	7.6***	10.2***	3.2
50:4:1	64:4:2	sphere	7.7**	10.1***	3.3
75:6:1	134:4:2	sphere	7.8***	10.0***	3.3
40:2:1	62:6:2	disc	9.4***	9.5***	4.2***

[a] Significance of difference from the value for apoA-I: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$. [b] PC, FC (cholesterol), and apoA-I molar composition before and after preparation and re-isolation. Particles were prepared by sonication (sphere) or by a cholate dispersion/Bio-Bead removal technique (disc). Apoprotein stoichiometries were determined from SDS−polyacrylamide gels after cross-linking with DMS. Values are representative of three different preparations of LpA-I. [c] Particle structural morphology determined by negative staining electron microscopy (EM) as described previously (*30*). Lp1A-I morphology could not be determined (nd) as preparations contained no disc rouleaux or spherical complexes. [d] Particle diameters from nondenaturing gradient gel electrophoresis ±0.5 nm (SD). [e] Charge potential at the particle surface ±0.2 (SD). [f] Number of excess negative charges in electronic units ±0.1 (SD). [g] Two distinct bands are present on the gel.

Methods

Preparation of Reconstituted LpA-I Complexes. Purified apoA-I was isolated from delipidated HDL by size-exclusion chromatography on a Sephacryl S-200 HR column (*18*). Discoidal Lp2A-I complexes were prepared by the cholate−Bio-Bead removal technique previously described (*15*). Reconstituted Lp1A-I and Lp2A-I were prepared by cosonication of PC, FC, and apoA-I (*18*). Specific amounts of PC and FC in chloroform (see Table 1 for initial concentrations) were dried under nitrogen into a 12 × 75 mm glass test tube and 800 μL of 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 0.02% NaN_3 (TBS) was added. The lipid−buffer mixture was sonicated for 1 min in a 15 °C water bath, incubated for 30 min at 37 °C, and sonicated again for 5 min. ApoA-I (2 mg of a 1.4 mg/mL TBS solution) was added to the lipid suspension and the protein−lipid mixture was sonicated for 4 × 1 min punctuated by 1 min cooling periods. All LpA-I complexes were filtered through a 0.22 μm syringe-tip filter and reisolated by size-exclusion chromatography on a Superose 6 column (*18*).

Determination of LpA-I Physical and Structural Characteristics. The size and homogeneity of apoA-I complexes were estimated by both electron microscopy and nondenaturing gradient gel electrophoresis on precast 8−25% acrylamide gels. The number of molecules of apoA-I per particle was determined by apoprotein cross-linking with dimethyl suberimidate (DMS) as described by Swaney and O'Brien (*27*) and SDS−PAGE on 8−25% acrylamide gels to determine the extent of oligomer formation. Lipoprotein surface charge characteristics were determined by electrophoresis on precast 0.5% agarose gels (Beckman, Paragon Lipo kit) as previously described (*28*). FC and PC contents were determined enzymatically with Boehringer Mannheim (Indianapolis, IN) kits. Protein concentrations were deter-

mined by the Lowry method as modified by Markwell et al. (*29*).

Circular Dichroism. The average secondary structures of LpA-I and apoA-I were monitored by circular dichroism (CD) spectroscopy on a Jasco J40A spectropolarimeter calibrated with a 0.1% (w/v) *d*-10-camphorsulfonic acid solution (*30*). CD spectra were measured at 24 °C in a 0.1 cm path length quartz cell with a sample protein concentration of 66.7 μg/mL in 5 mM phosphate buffer, pH 7.2. The percent α-helix in apoA-I was calculated from the molar ellipticity at 222 nm, using a mean residue weight of 115.3. The effect of GdnHCl concentration on the secondary structure of apoA-I in various LpA-I particles was monitored by the changes in molar ellipticity at 222 nm. Aliquots of each complex (33 μg of protein/mL of buffer) were incubated with 0−6 M GdnHCl in 50 mM phosphate buffer (pH 7.2) for 72 h at 4 °C. The free energy of unfolding of apoA-I on the surface of LpA-I complexes was calculated as described previously (*30*).

Immunoreactivity of LpA-I. Immunoreactivity measurements were performed as previously described (*14*). Briefly, Removawells (Immulon 2, Dynatech Laboratories, MA) were coated with 100 μL of apoHDL (0.2 μg in 15 mM Na_2CO_3, 35 mM $NaHCO_3$, and 0.02% NaN_3, pH 6.9), washed with PBS and 0.02% NaN_3, pH 7.2 (buffer A), and saturated with 0.5% gelatin in buffer A. Anti-apoA-I antibodies (at a predetermined dilution) were mixed with serial dilutions of the antigen (in buffer A with 0.1% gelatin) and transferred to the previously coated and saturated Removawells for 1 h of incubation at room temperature. After three washes (in buffer A with 0.05% Tween 20), the Removawells were incubated for 1 h with an ^{125}I-labeled anti-mouse antibody in buffer A with 0.1% gelatin (200 000 cpm/well). Wells were washed 3 times and their radioactivity was measured. Results were graphed as B/B_0, where B and B_0 represent the cpm bound in the presence and absence of competitor, and were used to calculate the particle concentration required for 50% inhibition of the maximal antibody binding (ED_{50}) and the affinity (slope) of the antibody for each LpA-I complex. Lipid-free apoA-I was diluted at 100 μg/mL for 48 h before the assays to eliminate any oligomerization.

Purification and Assay of LCAT Activity. LCAT was purified as previously described (*31*). LCAT activity was quantitated into units of enzyme activity, where 1 unit = 1 nmol of cholesterol esterified (ng of protein)$^{-1}$ h^{-1}. Reconstituted LpA-I were characterized as substrates for purified LCAT by a standard assay system, as we have described previously (*31*). Each enzyme assay contained the lipoprotein substrate, 0.3 unit of purified LCAT, bovine serum albumin (2.5 mg), β-mercaptoethanol (2.5 mM), and Tris/NaCl buffer, pH 8.0 (final volume = 500 μL). Incubations were carried out for 10 min at 37 °C and were terminated with the addition of 2 mL of ethanol. Reaction products were extracted in 4 mL of hexane and then the amount of ^{3}H associated with cholesteryl ester was determined by thin-layer chromatography on ITLC−SG plates (Gelman) with a solvent system composed of hexane/diethyl ether/acetic acid, 90:10:1 (v/v/v). Under these conditions, initial rates were estimated with minimal substrate conversion; less than 5% of Lp2A-I cholesterol was esterified at enzyme saturation.

Statistical Methods. Group standard deviation values reported (see Table legends) are maximum estimates from eight different measurements in representative experiments with different LpA-I particles.

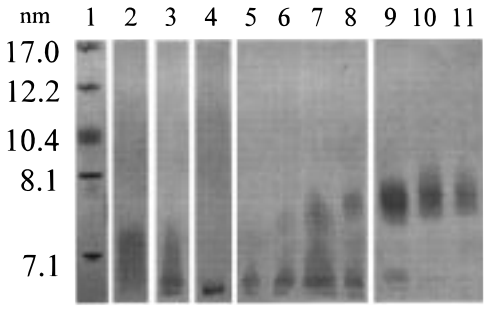

FIGURE 1: LpA-I subjected to electrophoresis in 8−25% nondenaturing gradient gels. Original gel profiles are shown for high molecular weight standards (lane 1), purified apoA-I at 1 mg/mL (lane 2), apoA-I at 0.5 mg/mL (lane 3), apoA-I at 0.1 mg/mL (lane 4), and seven different reconstituted LpA-I at 1 mg/mL with PC:FC:apoA-I compositions of 1:2:1 (lane 5), 2:4:1 (lane 6), 9:4:1 (lane 7), 30:6:2 (lane 8), 48:4:2 (lane 9), 64:4:2 (lane 10), and 134:4:2 (lane 11). Stokes' diameters for Lp2A-I were determined by comparison to high molecular weight standards.

RESULTS

Effect of LpA-I Composition on the Size and Charge of the Lipoprotein Particles. Sonicated LpA-I particles were prepared to contain various amounts of PC while maintaining a constant amount of cholesterol (see Table 1 for starting concentrations). Recoveries of cholesterol in the LpA-I complexes were low at low PC concentrations and required higher starting cholesterol levels to give similar final concentrations to particles made with high amounts of PC. Nondenaturing gradient gel electrophoresis shows that complexation of 1−15 molecules of PC with apoA-I results in the formation of particles that exhibit a hydrodynamic diameter of approximately 6 nm (Figure 1), while incorporation of greater than 15 mol of PC resulted in the formation of larger complexes, about 8 nm in diameter. As we had reported in a previous study (*25*), chemical cross-linking of poorly lipidated apoA-I shows a significant reduction in the propensity of apoA-I to form oligomeric structures. This is evident on the nondenaturing gel shown in Figure 1. Addition of a small amount of lipid to apoA-I prevents apoA-I migrating as a dimeric molecule under nondenaturing conditions (Figure 1, lane 2) (*25*), and instead, discrete complexes are formed that have one molecule of apoA-I (Lp1A-I) (Figure 1, lane 5).

Similar to what was observed by others (*27, 32*), SDS−polyacrylamide gels of the DMS cross-linked protein show that lipid-free apoA-I primarily exists as an oligomeric structure, at concentrations greater than 1 mg/mL. Nondenaturing acrylamide gels of apoA-I at concentrations >1 mg/mL, however, show that the basic building block of these oligomers is a dimeric molecule about 7 nm in diameter (Figure 1, lane 2). As the protein concentration is decreased, apoA-I exists in equilibrium between dimers and monomers (Figure 1 lane 3), until the monomer predominates at a protein concentration of <0.1 mg/mL (Figure 1, lane 4). Lipidation of apoA-I therefore appears to reduce the particle diameter by approximately 1 nm (Figure 1, lane 5). Negative staining electron microscopy of the minimally lipidated apoA-I shows no evidence of spherical or discoidal Lp2A-I structures. Electrophoresis of the lipid-poor apoA-I in 0.5%

Table 2: α-Helix Content and Denaturation Characteristics of LpA-I Complexes[a]

complex (PC:FC:apoA-I)	α-helix content (%)[b]	$D_{1/2}$ (M GdnHCl)[c]	ΔG_D^0 (kcal/mol of A-I)[d]	Δn (mol of GdnHCl/mol of A-I)[e]
apoA-I	49	1.0	2.4	15.4
1:2:1	50	1.1	0.7***	5.7
2:4:1	50	1.2	0.9***	6.3
9:4:1	52	1.4	1.5***	7.3
30:6:2	52	1.7	1.4***	6.1
48:4:2	53*	1.3	2.2*	10.0
64:4:2	57**	1.2	1.7***	8.3
134:4:2	63***	1.4	1.7***	7.4
62:6:2 (disc)	73***	2.3	2.2*	7.2

[a] Significance of difference from the value for apoA-I: *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.005$. [b] ApoA-I α-helix content determined from molar ellipticities at 222 nm ± 4% (SD). [c] Midpoint of GdnHCl denaturation curve (Figure 2) ±0.03 M (SD); values are representative of three different preparations of LpA-I. [d] Free energy of denaturation at zero GdnHCl concentration ±0.2 kcal (SD). [e] GdnHCl bound during denaturation ±1.0 SD.

agarose gels also shows that increases in PC have negligible effects on electrophoretic mobilities. To maintain a relatively constant surface potential, as particle size increases, apoA-I valence on the Lp1A-I complex appears to increase with increasing amounts of PC (Table 1).

Incorporation of more than 15 molecules of PC into apoA-I causes the formation of a much larger Lp2A-I complex (contains 2 molecules of apoA-I) that has a hydrated diameter of close to 8 nm (Table 1). As we have previously shown (*31*), electron micrographs of these particles show that they differ from discoidal structures and are spherical in appearance. This large increase in size, going from an Lp1A-I to an Lp2A-I, parallels a significant increase of almost 2 mV in the negative surface potential of the complex. Further increasing the PC content of these Lp2A-I, from 48 to 134 molecules/particle, slightly increases the particle size and decreases the negative surface charge on the particle (Table 1). It is of note that the net valence of apoA-I on the Lp2A-I complexes is the same as that for lipid-free apoA-I.

Effect of LpA-I Composition on the Secondary Structure and Stability of ApoA-I. Differences in apoA-I organization on the LpA-I particles are evident as PC content is increased, particularly in terms of the amount of amphipathic α-helical structure in apoA-I. Sonicated PC/apoA-I particles exhibit a significant increase in α-helix content in apoA-I as PC content is increased but have a much lower α-helix content as compared to a discoidal particle with a similar protein and lipid composition (Table 2). While increasing PC content in the Lp1A-I conferred only a small increase in helix content, increased PC in Lp2A-I brought about much larger increases, of up to 25%, in the α-helix content of apoA-I. This is similar to what has been observed for discoidal Lp2A-I particles (*33*).

Variations in the PC composition of LpA-I particles have significant effects on the denaturation of lipid-bound apoA-I by GdnHCl. Figure 2A shows that addition of only a couple of molecules of lipid to apoA-I has a major effect on the ability of GdnHCl to unfold the α-helical structure of apoA-I. Lipidated apoA-I exhibits a more gradual unfolding of secondary structure in the presence of GdnHCl than the lipid-free molecule (Figure 2). Table 2 illustrates this point clearly and shows that the midpoint of denaturation ($D_{1/2}$) for apoA-I on the Lp1A-I complexes increases as PC content is increased but then falls when Lp2A-I particles are formed. While this increased $D_{1/2}$ suggests that the molecule is more stable in the presence of small amounts of lipid, analysis of denaturation curves with the binding model of Aune and Tanford suggests that this is not the case. The binding model helps to disassociate the two factors that contribute to the efficiency of unfolding by GdnHCl (*30*), notably (1) the accessibility of guanidinium ions to the peptide backbone (Δn) and (2) the thermodynamic stability of the protein (ΔG_D^0). As we have reported earlier (*18*), analysis of LpA-I denaturation curves shows that the more gradual unfolding of apoA-I in the presence of lipids is due to a major decrease in the accessibility of GdnHCl to the peptide backbone. In addition, this analysis shows that the free energy of stability of apoA-I is profoundly compromised in the presence of lipid. Only a couple of molecules of lipid are sufficient to reduce the ΔG_D^0 for apoA-I by over 70%. Further lipidation of apoA-I appears to progressively increase the thermodynamic stability of apoA-I (Figure 3, Table 2), until Lp2A-I formation begins, at which point the ΔG_D^0 values fall slightly and then again begin to rise. The ΔG_D^0 for Lp2A-I appears to peak for the 48:4:2 particle, at 2.2 kcal/mol of apoA-I, and then falls to an apparent plateau at 1.7 kcal/mol. Since this value is higher than that observed for an Lp2A-I particle containing only PC, it appears that, similar to what was observed in discoidal Lp2A-I (*15*), small amounts of cholesterol slightly increase the thermodynamic stability of apoA-I in sonicated particles.

Effect of LpA-I Composition on the Conformation of ApoA-I. ApoA-I conformation was probed on the different LpA-I complexes by competitive solid-phase radioimmunometric assay, with a panel of 13 anti-apoA-I mAbs specific for epitopes that range from the amino terminus to the carboxyl terminus of apoA-I (*14*). Competitive binding curves were parallel for all mAbs and therefore ED_{50} values were determined and are presented in Figure 4. While apoA-I competed effectively with immobilized apoHDL for binding to most of the mAbs, some mAbs were less effective competitors (2F1 and A11) or were unable to compete with the HDL apoproteins (A16, A51, 3G10, A03, and 4A12). The ED_{50} values shown in Figure 4 show clear differences between the mAb immunoreactivity/conformation of apoA-I when lipid-free and lipid-associated. The data are consistent with earlier studies (*14*, *17*) and show that most of the antibodies utilized (A16, A51, 3G10, A03, and 4A12) exhibit a greatly increased immunoreactivity upon association with lipid. It is of note that the immunoreactivity of most of these antibodies changed with the addition to apoA-I of as little as 2 molecules of PC and 4 molecules of cholesterol. It

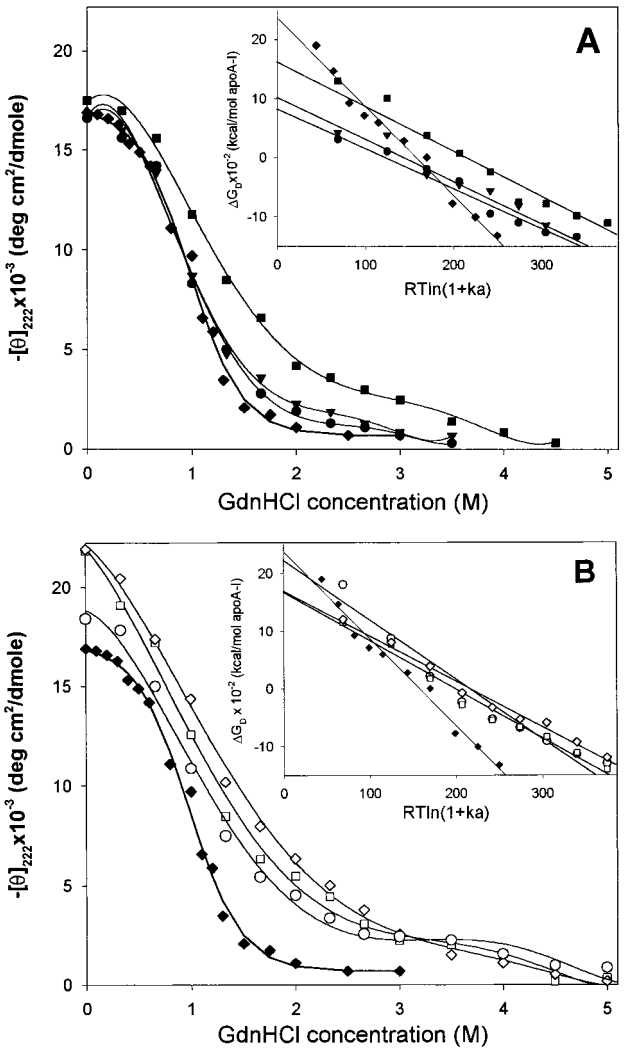

FIGURE 2: Effect of GdnHCl concentration on the molar ellipticity of apoA-I on LpA-I particles. Aliquots of apoA-I (◆) or LpA-I particles [PC:FC:apoA-I molar ratios were (●) 1:2:1, (▼) 2:4:1, and (■) 30:6:2; in panel A and (○) 48:4:2, (□) 64:4:2, and (◇) 134:4:2 in panel B] were incubated with 0−6 M GdnHCl in 0.05 M phosphate buffer for 72 h at 4 °C. CD spectra were measured at 24 °C in a 0.1 cm path length quartz cell with sample protein concentrations of 33.3 μg/mL phosphate buffer, and 4 scans from 230 to 200 nm were collected and averaged at each GdnHCl concentration. Insets: Linear regression plots are shown of the observed free energies of denaturation (ΔG_D) against RT ln(1 + k_a) for discoidal LpA-I. The free energy of denaturation at zero GdnHCl concentration (ΔG_D^0) is estimated from the y intercept (*30*).

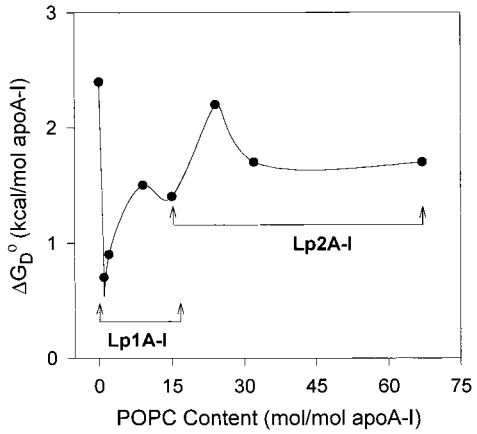

FIGURE 3: Effect of the PC content of LpA-I on the thermodynamic stability of apoA-I. The free energy of denaturation ΔG_D^0 for the various LpA-I particles was determined as shown in Figure 2 (Table 2) and is plotted relative to the complex PC content. ApoA-I undergoes cyclic changes in thermodynamic stability that parallel lipid-apoprotein reorganization and lipoprotein formation.

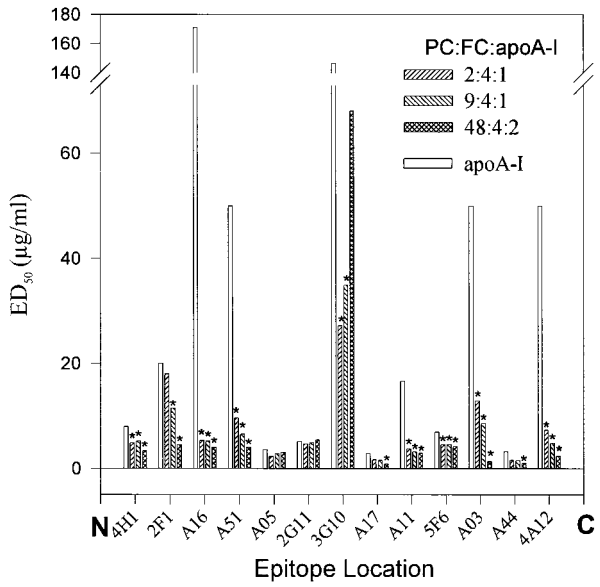

FIGURE 4: Effect of the PC content of Lp1A-I and Lp2A-I particles on the immunoreactivity of different epitopes in apoA-I. Immunoreactivity measurements were performed on Removawells coated with apoHDL as previously described (*14*). Wells were washed with PBS and saturated with 0.5% gelatin. Different anti-apoA-I mAbs were mixed with serial dilutions of LpA-I and transferred to the Removawells for 1 h of incubation at room temperature. After three washes, the Removawells were incubated for 1 h with an ^{125}I-labeled anti-mouse antibody. Wells were washed three times and their radioactivity was measured. Results were graphed as B/B_0 and were used to calculate the immunoreactivity (ED_{50}) of the antibody for each LpA-I complex. Significance of difference from the ED_{50} value for the apoA-I: $^*p < 0.05$.

appears that major changes in apoA-I conformation occur with only trace lipidation. Indeed, for the smallest Lp1A-I there is a major increase in the exposure of several epitopes (3G10, A11, and A03) located in a central domain of apoA-I, at residues 98−148. This result is in agreement with previous reports that show an increased accessibility for central apoA-I epitopes in lipid-poor pre-β HDL particles (*34*, *35*). In contrast, progressive lipidation of apoA-I decreases the immunoreactivity of one mAb, 3G10, which is essentially unreactive with the Lp2A-I particle. The increased immunoreactivity of A16, A51, A05, and 2G11 with increases in PC is consistent with that observed previously for discoidal Lp2A-I (*17*). However, while epitopes for mAbs 4H1, A11, and 5F6 are only partially exposed on discoidal Lp2A-I (*17*), these mAbs are highly immunoreactive with the sonicated Lp1A-I and Lp2A-I and show an increase in immunoreactivity with increasing PC content. These mAbs have also been shown to be highly immunoreactive with native HDL particles (*26*, *36*). These lipoprotein morphology-dependent differences in apoA-I conformation are similar to what we have previously reported (*37*) and show that major differences exist in the conformation of both N-terminal and central domains between apoA-I on discoidal and spherical Lp2A-I particles. In addition, comparison of these immunochemical measurements with previous investigations shows that the immunoreactivity/

Table 3: Effect of ApoA-I Lipidation on LCAT Kinetic Parameters[a]

complex (PC:FC:apoA-I)	V_{max}[b] (nmol of CE/h)	appK_m-I[b] (μM apoA-I)	appK_m-II[b] (μM PC)	appK_m-III[b] (μM FC)	$V_{max}/K_{m_{apoA-I}}$ [nmol/(h·μM apoA-I)]
2:4:1	5.8 ± 0.4***	22.0	44	88	0.26
9:4:1	0.4 ± 0.2	0.9	14	3	0.44
48:4:2	0.3 ± 0.2	1.0	24	2	0.30
64:4:2	7.3 ± 0.5***	22.1	707	44	0.33
134:4:2	10.0 ± 0.5***	18.7	1253	37	0.53

[a] Significance of difference from the value for the Lp2A-I, 48:4:2: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$. [b] V_{max} (±SD) and appK_m for apoA-I (±0.1 μM, SD), phospholipid (PC; ±1.5 μM, SD) and cholesterol (FC; ±0.5 μM, SD) values were estimated from the Lineweaver−Burk plots of the data shown in Figure 5. Values are representative of three different preparations of Lp2A-I.

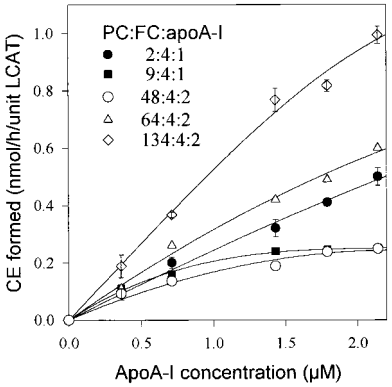

FIGURE 5: Effect of the PC content of LpA-I on LCAT activation. The esterification of [^{3}H]cholesterol by LCAT in different LpA-I particles [PC:FC:apoA-I molar ratios were (●) 2:4:1, (■) 9:4:2, (○) 48:4:2, (△) 64:4:2, and (◇) 134:4:2] is shown. LpA-I were prepared by cosonication as described in the text and were incubated with purified LCAT. Values are the mean ± SD of triplicate determinations and are representative of three different preparations of HDL particles.

conformation of apoA-I on sonicated Lp1A-I and Lp2A-I is much closer to that observed for apoA-I on native pre-β (*34*, *35*) and α-HDL (*26*, *36*) than to that for this apoprotein on discoidal Lp2A-I particles (*14*, *37*).

Effect of LpA-I Phosphatidylcholine Content on Cholesterol Esterification by LCAT. Increasing the PC content in LpA-I particles has a significant effect on the lipoprotein structural characteristics and therefore would be expected to also affect LpA-I functional properties. The effect of apoA-I lipidation on the rate of cholesterol esterification by LCAT was investigated and is shown in Figure 5. Addition of only a couple of molecules of lipid to apoA-I is sufficient to generate a very reactive substrate for LCAT. Figure 5 shows that Lp1A-I particles readily promote cholesterol esterification by LCAT. It is of note that apoA-I can activate LCAT equally well whether it is complexed into an Lp1A-I or Lp2A-I particle. Lp1A-I containing 2 molecules of PC and 4 molecules of cholesterol displays a similar rate of cholesterol esterification to an Lp2A-I containing 64 molecules of PC and 4 molecules of cholesterol. Increasing the PC content in the Lp1A-I complex inhibits cholesterol esterification by LCAT, while increasing PC content in the Lp2A-I particles has the opposite effect and stimulates the enzyme. This is particularly clear in Table 3, which illustrates reaction kinetic values that were determined from Lineweaver−Burk plots of the data shown in Figure 5. Increasing the PC content in the Lp1A-I complexes is associated with a 14-fold decrease in the maximum velocity (V_{max}) of cholesterol esterification by LCAT. In contrast, increasing the PC content of the sonicated Lp2A-I particles causes an almost 30-fold increase in V_{max}. This observation is exactly the opposite of what we observed with discoidal Lp2A-I particles; where increasing PC in discs is inhibitory to LCAT (*38*). It is of note that major changes in LCAT V_{max} values parallel changes in apparent K_m values. This is consistent with what we have observed with discoidal Lp2A-I, i.e., low V_{max} values are usually concomitant with low appK_m apoA-I values. Nonetheless, the overall catalytic efficiency of LCAT (V_{max}/appK_m) appears to increase slightly when LpA-I PC content is increased.

DISCUSSION

Effect of Phospholipidation on the Structural Properties of ApoA-I. We have previously shown that the structural integrity of an HDL particle is related to the thermodynamic stability of its primary constituent apoA-I (*39*). In certain dyslipidemic states, HDL composition is modified to an extent that destabilizes the lipoprotein and leads to the reorganization of its constituents into more thermodynamically stable structures (*40*, *41*). The thermodynamic stability of lipidated apoA-I is highly sensitive to the lipid composition of a lipoprotein particle and, interestingly, is usually less than that for the lipid-free protein (*28*). This unique propensity of apoA-I to maintain a thermodynamically unfavored conformation in the presence of lipid appears central to the structure/function properties of an HDL particle (*39*). We have shown that when the difference between the free energy of denaturation of apoA-I (ΔG_D) in the lipid-free and lipid-bound states ($\Delta G_D^{free} - \Delta G_D^{bound}$) is positive, spontaneous dissociation of apoA-I from LpA-I particles is thermodynamically favored. Spherical Lp2A-I particles are thermodynamically unstable because they exhibit ΔG_D^{bound} values less than that for lipid-free apoA-I (Table 2). These unstable Lp2A-I particles can reorganize and form new, more thermodynamically stable complexes and, in the process, liberate a molecule of lipid-poor apoA-I (*39*). Several reports suggest that the rate of this process may be enhanced by the actions of a variety of different plasma proteins (*6*, *41*−*43*) and, as such, suggest that this process may reflect the *de novo* synthesis of lipid-poor, pre-β_1 HDL (*22*).

Numerous studies have suggested that pre-β_1 HDL is a unique class of lipid-poor HDL with distinct and potentially important functions (*44*−*50*). However, their characterization has been hampered by the instability of these native particles in plasma (*24*, *51*−*54*). We have developed a novel method to produce Lp1A-I particles that have composition and structural properties similar to those of native pre-β_1 HDL (*25*). Of all the lipoprotein complexes that this laboratory has characterized, Lp1A-I are by far the most thermodynamically unstable. These studies suggest that while lipid-

free apoA-I readily self-associates, it is unlikely that lipid-free apoA-I structures would exist in vivo, because even small amounts of lipid have the ability to dissociate the aggregates into monomeric species. It is possible that the low thermodynamic stability of the Lp1A-I may be partially due to the reduced ability of one molecule of lipidated apoA-I to associate with another and protect its hydrophobic domains from aqueous exposure. Indeed, with such low ΔG_D^0 values it is surprising that these structures do not spontaneously reorganize into more thermodynamically stable Lp2A-I. Partial lipidation appears to block distinct domains in apoA-I that may facilitate dimerization and thereby directly interfere with protein−protein interactions between two molecules of apoA-I.

The binding of apoA-I to lipid is thought to be mediated by its amphipathic α-helices (*55*). Since the protein is soluble in aqueous solutions, it appears that apoA-I may be able to form a globular structure, wherein the hydrophobic domains on the α-helical segments may be brought together to be hidden/protected from molecules of water. X-ray crystallography of the N-terminal fragment of apoE shows that in the absence of lipid this molecule adopts a helical bundle conformation comprising four main α-helices arranged with their hydrophobic faces brought together to the center of the molecule (*56*). Several lines of evidence suggest that apoA-I may adopt a structure similar to that of apoE in the absence of lipid (*57*). Amino acid sequence comparisons show that apoE and apoA-I share substantial structural homology (*58, 59*). Both apoA-I and apoE readily self-associate and are primarily associated with HDL particles in the plasma but can exist as a lipid-poor complex dissociated from an HDL particle (*60, 61*). ^{13}C NMR studies show that the Lys titration profiles for apoA-I and apoE are similar and undergo significant but similar changes when bound to different kinds of particles (*62, 63*). In the present study, we show that apoA-I is smaller and more compact when monomeric and lipid-free. Immunoreactivity studies of apoA-I shows that three large domains, which are generally highly immunoreactive on lipoprotein particles, are essentially inaccessible to the mAbs in the absence of lipids. Together these data suggest that, in the absence of lipid, apoA-I may also form a globular helical bundle to protect its hydrophobic structure. Phospholipids appear to unfold this helical bundle in apoA-I and open the molecule to a more elongated structure.

Addition of PC to apoA-I has been shown to promote a conformational change in apoA-I that parallels an increased exposure of almost all epitopes on apoA-I and a concomitant increase in hydrodynamic diameter (Figures 1 and 4). Lund-Katz et al. (*63*) showed that when the N-terminal fragment helical bundle of apoE associates with lipid, the molecule opens up to form a larger, expanded structure of antiparallel helices. It appears that apoA-I may reorganize similarly in the presence of lipid. In the present study we show that addition of a small amount of lipid causes the formation of an Lp1A-I structure that is slightly larger than a monomer of apoA-I. This increase in size parallels an opening up of the molecule in a manner that profoundly increases the immunoreactivity of the three hidden domains on apoA-I and all other epitopes as well. With this extensive change in tertiary structure of apoA-I in the presence of a small amount of lipid, we observe no change in the α-helix content in this apoprotein. This indicates that the secondary structure of apoA-I remains intact but that its tertiary structure undergoes a major rearrangement in the presence of a few moles of PC.

Effect of ApoA-I Phospholipid Content on Lp2A-I Formation. Data from the present study suggest that there is a minimal lipid requirement for the formation of a dimeric lipoprotein particle; Lp2A-I formation can only occur when greater than 10 molecules of PC are bound to the apoprotein. At this point, apoA-I exhibits a similar α-helical content and stability to that for apoA-I on an Lp2A-I particle (*13, 28*). Therefore, the formation of a dimeric particle appears to be related to the conformational stability of the amphipathic α-helices in apoA-I. Dimerization may be catalyzed by a unique conformation, within which apoA-I has a similar structure and stability to the apoprotein in a multimolecular complex. This is consistent with what we have observed in NMR (*62*), circular dichroic (*13*), and epitope expression studies (*37*). Lipidation of apoA-I appears to progressively increase the thermodynamic stability of apoA-I (Figure 3) until Lp2A-I formation begins. When apoA-I dimerizes as an Lp2A-I complex, a much more stable complex is formed. Lipid-bound apoA-I appears to adopt its most thermodynamically favorable conformation when complexed to about 24 molecules of PC. At this point, apoA-I exhibits a ΔG_D^0 very similar to that of lipid-free apoA-I (Table 2). When more molecules of PC are added to LpA-I, thermodynamic stability values fall to an apparent plateau at 1.7 kcal/mol. In so doing, it appears that apoA-I undergoes cyclic changes in thermodynamic stability that parallel lipid−apoprotein reorganization and lipoprotein formation.

It is of note that the difference in hydrodynamic diameter between dimeric lipid-free apoA-I and monomeric Lp1A-I is only about 1 nm. Earlier chromatographic investigations by Scanu and co-workers (*32, 64*) and others (*65, 66*) have shown that the lipid-free monomeric apoA-I has an expanded ellipsoidal shape with an estimated hydrated Stokes diameter of about 6 nm. Chemical cross-linking of apoA-I shows that, when stored at a concentration above 0.5 mg/mL, this molecule exists predominantly as a dimer. As we (*25*) and others (*66*) have shown, dimeric lipid-free apoA-I appears much larger, ∼7 nm, on nondenaturing gradient gels. Therefore, dimerization of apoA-I only modifies the hydrated size by about 20%, even though the molecular weight doubles. The data suggest that monomers of apoA-I are asymmetric molecules, with a long axis length of 6 nm and a short axis length of about 2.5 nm (*64*). Dimerization appears to be associated with the close association of two elongated apoA-I structures in a manner that has little effect on the long axis dimensions but essentially doubles the short axis length (*64, 67*). This appears consistent with that observed with a mutant of apoA-I (with a deletion of residues 1−43) in a recent report by Borhani et al. (*68*). In this study, the mutant protein exhibited a lipid-free conformation similar to that of lipid-bound apoA-I and appeared as an elliptical ringlike structure consisting of multiple dimers of the apoprotein. The data suggest that, when sufficiently lipidated, apoA-I may adopt a fundamental unit structure that is highly asymmetric but similar in size to that of a small HDL particle. In essence, this complex appears to represent a building block morphology that allows for progressive lipidation and acquisition of other molecules of apoA-I. In previous studies, we have shown that the conformation and structural proper-

ties of apoA-I on sonicated Lp2A-I particles is very similar to that observed in much larger lipoprotein particles, containing as many as 5 molecules of apoA-I (*13*, *28*). This appears to indicate that the structure of apoA-I in a multimolecular lipoprotein particle is relatively constant and similar to the asymmetric basic unit that defines the Lp2A-I complex.

Effect of LpA-I Phospholipid Content on the Lipoprotein Functional Properties. Previous studies in this laboratory have shown that sonicated Lp2A-I particles promote similar rates of cholesterol esterification as native, ultracentrifugally isolated HDL particles but are much poorer LCAT substrates than discoidal Lp2A-I with a comparable stoichiometry (*31*). In the present study, it is evident that Lp1A-I particles are also viable as substrates for LCAT. Indeed, it is striking that a complex of apoA-I, 2 molecules of PC, and 4 molecules of cholesterol is a better substrate for the enzyme than an Lp2A-I particle containing the same amount of cholesterol but 48 mol of PC. This indicates that complex phospholipid content must not be rate-limiting to the LCAT reaction. In addition, the data suggest that LCAT does not require a large lipid interface to bind and interact with on a lipoprotein, such as has been proposed from studies with discoidal lipoprotein particles (*69*). In contrast, it appears that the enzyme may interact either with apoA-I specifically or with the small amount of lipid associated with the apoprotein.

Increasing the PC content of Lp1A-I appears to inhibit the rate of cholesterol esterification by LCAT. While Figure 5 only shows two representative particles, we have evaluated a number of different complexes ranging from 2 to 15 mol of PC/particle (not shown) and we clearly see a reduction in the estimated V_{max} for LCAT as the Lp1A-I PC content is increased. It should be noted, however, that this reduction in V_{max} parallels a concomitant reduction in appK_m values and thereby actually gives rise to a subtle increase in the catalytic efficiency (V_{max}/K_m or k_{cat}) for the enzyme. The significance of this estimate for an interfacial enzyme such as LCAT, however, appears limited, as numerous studies have shown that the higher the affinity that an interfacial enzyme has for its substrate, the lower its catalytic rate (*31*, *38*, *70*). The data are consistent with our view that maximal rates of esterification require low substrate affinity to facilitate optimal intersubstrate movement and catalysis (*38*). The reduction in V_{max} observed is also similar to that observed when discoidal PC content is increased (*38*). Further, as with discoidal particles, this reduction in the initial rate of LCAT appears to be associated with a reduction in negative charge and stability of apoA-I (see Tables 1−3) and with a reduced exposure of a central domain in the molecule (Figure 4). However, in contrast to that observed with Lp1A-I, increasing the PC content in the sonicated Lp2A-I has the opposite effect on LCAT and is associated with an increase in cholesterol esterification. While a similar relationship is apparent between apoA-I structure on Lp2A-I and LCAT activation, increases in Lp2A-I PC content apparently have unique and very distinct effects on both the structure and function of apoA-I, relative to that observed with Lp1A-I particles.

This investigation shows how the lipidation of apoA-I and speciation of HDL particles is controlled by a complex interplay between the hydrophobic and electrostatic properties of apoA-I. Phospholipid has the ability to reorganize the tertiary structure of apoA-I and generate an unstable building block with structure and functional properties similar to those of apoA-I on large HDL particles. This Lp1A-I or pre-β_1 HDL particle is uniquely reactive as a result of its very low thermodynamic stability. Further speciation of HDL and formation of larger particles is then governed by the acquisition of new lipid molecules and resultant effects on the structural stability of apoA-I.

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