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Cholesterol Determines and Limits rHDL Formation from Human Plasma Apolipoprotein A-II and Phospholipid Membranes

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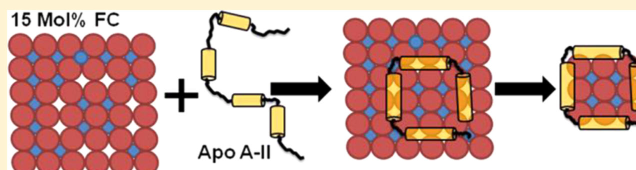
Supporting Information

ABSTRACT: Apolipoprotein (apo) A-II, the second most abundant protein after apo A-I of human plasma high-density lipoproteins (HDL), is the most lipophilic of the exchangeable apolipoproteins. The rate of microsolubilization of dimyristoylphosphatidylcholine (DMPC) membranes by apo A-I to give rHDL increases as the level of membrane free cholesterol (FC) increases up to 20 mol % when the level of reaction decreases to nil. Given its greater lipophilicity, we tested the hypothesis that apo A-II and its reduced and carboxymethylated monomer (rcm apo A-II) would form rHDL at a membrane FC content of >20 mol %. According to turbidimetric titrations, the DMPC/apo A-II stoichiometry is 65/1 (moles to moles). At this stoichiometry, apo A-II forms rHDL from DMPC and FC. Contrary to our hypothesis, apo A-II, like apo A-I, reacts poorly with DMPC containing ≥20 mol % FC. The rate of formation of rHDL from rcm apo A-II and DMPC at all FC mole percentages is faster than that of apo A-II but nil at 20 mol % FC. In parallel reactions, monomeric and dimeric apo A-II form large FC-rich rHDL coexisting with smaller FC-poor rHDL; increasing the FC mole percentage increases the number and size of FC-rich rHDL. On the basis of the compositions of coexisting large and small rHDL, the free energy of transfer of FC from the smallest to the largest particle is approximately −1.2 kJ. On the basis of our data, we propose a model in which apo A-I and apo A-II bind to DMPC via surface defects that disappear at 20 mol % FC. These data suggest apo A-II-containing HDL formed intrahepatically are likely cholesterol-rich compared to the smaller intracellular lipid-poor apo A-I HDL.



A high human plasma low-density lipoprotein cholesterol concentration is a risk factor for cardiovascular disease (CVD), which causes ~400000 deaths per year in the United States,[1] and its lowering by the statin class of hypolipidemic drugs reduces the number of CVD events. In contrast, the plasma concentration of high-density lipoprotein cholesterol (HDL-C) is negatively correlated with the number of CVD events. However, this correlation is imperfect because the number of CVD events is also determined by HDL functionality.[2] Thus, the mechanisms by which various HDL subclasses are formed are important in identifying their functional determinants.

Apo A-I and apo A-II, the most abundant HDL apolipoproteins (~50 and ~25 μM, respectively, in human plasma), microsolubilize macrophage phospholipids (PL) and free cholesterol (FC) via ABCA1, giving nascent HDL.[3,4] FC loading of macrophages increases the rate of efflux of FC to apo A-I (~5-fold), the size of the resulting nascent HDL, their FC/PL ratio, and the fraction of apo A-I on large particles.[3] Various nascent HDL are also formed from apolipoproteins by their intrahepatic ABCA1-independent lipidation in the endoplasmic reticulum followed by ABCA1-dependent lipidation in Golgi and at the plasma membrane.[5] Half of apo A-I is secreted lipid-free and later remodeled by lecithin:cholesterol acyltransferase (LCAT), which mediates the transition from discoidal to spherical HDL.[6,7] Human apo A-II, unlike most mammalian apo A-IIs, contains Cys6 and in plasma is present primarily as the homodimer. In contrast to apo A-I, apo A-II is fully lipidated and dimeric early during its intrahepatic processing on particles without apo A-I or apo E, and only after secretion does discoidal apo A-II HDL fuse with apo A-I- and apo E-containing particles.[7,8]

In vitro microsolubilization of dimyristoylphosphatidylcholine (DMPC) multilamellar vesicles (MLV) by apo A-I produces rHDL, the in vitro analogue of cellular apo lipidation. This mechanism has been verified in other systems in which DMPC was replaced by more physiological lipids representative of the plasma membrane.[9] FC has a profound effect on the dynamics of formation of rHDL from DMPC and apo A-I. The kinetics of rHDL formation is fastest at ~12.5 mol % cholesterol,[10] a composition that produces the maximal number of defects between lipid clusters where apo A-I inserts.[10] As with ABCA1-mediated apo A-I lipidation,[3] FC increases the size and number of rHDL species formed from apo A-I and DMPC.[11] The formation of rHDL from DMPC and apo A-I is rapid up to 20 mol % FC, above which the rate decreases to nil.[11]

Less is known about the effects of FC on the formation of rHDL from apo A-II. Ample data show that apo A-II is more lipophilic than apo A-I. Prolonged centrifugation of HDL sheds apo A-I but not apo A-II;[12] apo A-II displaces apo A-I from

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HDL,[13] and denaturants or heat disrupts HDL structure with the release of lipid-free apo A-I but not apo A-II.[14] Many proteins that target HDL (LCAT, hepatic lipase, lipid transfer proteins,[15−18] and streptococcal serum opacity factor[19]) disrupt HDL with the concomitant release of lipid-free apo A-I but not apo A-II. Given its greater lipophilicity versus that of apo A-I, we hypothesized apo A-II would form rHDL from MLV with >20 mol % FC, and given its smaller size, the rate of association of rcm apo A-II would be faster than that of apo A-II at all FC mole percentages in DMPC. We addressed these hypotheses by studying the kinetics of rHDL formation and measuring the compositions of rHDL formed from monomeric and dimeric apo A-II as a function of the initial FC mole percentage in DMPC.

■ MATERIALS AND METHODS

Multilamellar Vesicle Preparation and Labeling. Multilamellar vesicles (MLV) were prepared from [^{3}H]DMPC synthesized as described previously[20] and [^{14}C]FC (Perkin-Elmer, Inc.). The specific activity of DMPC was 0.110 ± 0.001 μCi/mg. Undiluted [^{14}C]FC, i.e., negligible FC mass, was added to the stock solution of [^{3}H]DMPC so that the activity of [^{3}H]DMPC was 12 times that of [^{14}C]FC and ^{14}C spillover into the ^{3}H channel of the scintillation counter was minimized. This solution was combined with various amounts of cold FC to give various FC mole percentages. DMPC and FC were dissolved in a chloroform/methanol mixture (2/1, v/v); the solvent was evaporated under nitrogen and the residue dried in vacuo for >30 min. The dried lipids were dispersed into Tris-buffered saline [TBS (10 mM Tris, 100 mM NaCl, 1 mM azide, and 1 mM EDTA)] by being vortexed, after which the lipids were subjected to more than three cycles of warming to >37 °C with vortexing and freezing to −20 °C. Radiochemical data were corrected for ^{14}C spillover into ^{3}H counts.

Determinations of rHDL Stoichiometry. rHDL containing apo A-II were prepared by several methods. DMPC was the phospholipid of choice because at its transition temperature it spontaneously forms rHDL, which can be easily separated from the MLV by centrifugation. rHDL are optically transparent at 325 nm, whereas DMPC MLV are turbid. Thus, turbidimetric titration of apo A-II with DMPC MLV was used to determine the rHDL stoichiometry, the point at which additional MLV are in stoichiometric excess over apo A-II and turbidity appears and increases. Apo A-II aliquots were stirred for >18 h with various amounts of DMPC MLV at the DMPC transition temperature of 24 °C, after which each mixture was analyzed for turbidity by right angle light scattering (325 nm) using a Jobin Yvon (Edison, NJ) Spex Fluorolog-3 FL3−22 spectrofluorimeter equipped with a Peltier heating−cooling device. The DMPC/apo A-II molar ratios in rHDL were determined by extrapolation.

Preparation and Analysis of rHDL Formed from DMPC and Apo-II. Apo A-II was isolated as described previously.[21] Reduced and carboxymethylated (rcm) apo A-II was prepared by reducing dimeric apo A-II with dithiothreitol and treating the monomeric product apo A-II with excess iodoacetic acid.[22] Apo A-II and rcm apo A-II concentrations were determined spectrophotometrically.[23] rHDL were prepared from apo A-II and DMPC (1—5 mg in 1 mL) containing 0−30 mol % FC under three conditions: spontaneous association of apolipoprotein with excess lipid (10 mg of DMPC and 1 mg of apo A-II, corresponding to a 256/1 molar ratio), with stoichiometric amounts of DMPC (65/1 molar ratio), or by dialysis of stoichiometric amounts of apolipoprotein and mixed DMPC/cholate micelles, a method that "catalyzes" apolipoprotein−lipid association.[24,25] For spontaneous rHDL formation with stoichiometric or excess DMPC (10/1, w/w), the apolipoprotein and [^{3}H]DMPC MLV containing 0−30 mol % [^{14}C]FC were mixed and incubated for >18 h at 24 °C, and the unbound DMPC sedimented at 16000*g* for 30 min in an Eppendorf 5415 C centrifuge at 4 °C. The rHDL in the supernatant were collected and separated by size exclusion chromatography (SEC) over tandem columns of Superose HR6 (GE Healthcare) and the collected fractions β-counted. The sedimented DMPC was solubilized in 0.5 mL of ethanol, and an aliquot thereof was β-counted. SEC analysis showed no MLV in the void volume, from which we conclude that MLV sedimentation is quantitative. In the third method, DMPC (2.5 mg) and various amounts of FC were reduced to dryness as described above, resolubilized in 9.5 mg of cholate in 0.5 mL of TBS to which 1 mg of apo A-II in 0.5 mL of TBS was added, and incubated while being stirred for >18 h at 24 °C. After exhaustive dialysis versus TBS, excess lipid, if any, was sedimented and the supernatant analyzed by SEC as described above.

Kinetics of rHDL Formation. The MLV were preincubated at the DMPC transition temperature, 24 °C, for 10 min, after which apo A-II was added to final concentrations of 0.83 mg of DMPC/mL and 0.33 mg of apo A-II/mL (65/1 DMPC/apo A-II molar ratio) in a final volume of 3 mL. The kinetics of rHDL formation were determined while the mixture was being stirred according to the change in turbidity (T) measured as above. The data were fit to the three-parameter exponential function $T = T_0 + ae^{-kt}$, where k is the rate constant.

Cross-Linking. rHDL particles (∼1 mg/mL) were cross-linked with BS^3 [bis(sulfosuccinimidyl) suberate] at 4 °C in 10 mM HEPES and 100 mM NaCl (pH 7.4) at a BS^3/apo A-II molar ratio of 100/1 for 24 h as previously described.[11] The reactions were terminated by adding 1 M Tris-HCl (pH 7.5) at a final concentration of 100 mM. The rHDL samples were analyzed on 4 to 20% gradient sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) gels and stained to visualize the apo A-II bands. The sizes of apo A-II oligomers were determined by comparison with standards of known molecular masses (Precision Plus Protein Standards, Bio-Rad) and imaged with the Gel Doc EZ System (Bio-Rad).

■ RESULTS

The stoichiometry of apo A-II−DMPC association was determined by incubating various concentrations of DMPC MLV with a fixed concentration of apo A-II for >18 h and measuring the turbidity of the samples by right angle light scattering at 325 nm (Figure 1A). At low DMPC/apo A-II ratios, the light scattering was low and decreased up to a molar ratio of 65/1, above which the turbidity increased. From the increased light scattering due to excess DMPC MLV, we inferred a maximal stoichiometry for rHDL formation from DMPC and apo A-II at 24 °C of ∼65/1. The kinetics of microsolubilization determined under similar conditions (Figure 1B) showed a decrease in the microsolubilization rate from 17.0 to 5.5 × 10^{-3} s^{-1} with an increase in the DMPC/apo A-II molar ratio from 20/1 to 70/1. The rate constants at 60/1 and 70/1 DMPC/apo A-II ratios were similar, 5.6 and 5.5 × 10^{-3} s^{-1}, respectively.

Differential Effects of FC Mole Percentage on the Partitioning of FC into rHDL and MLV. To investigate the

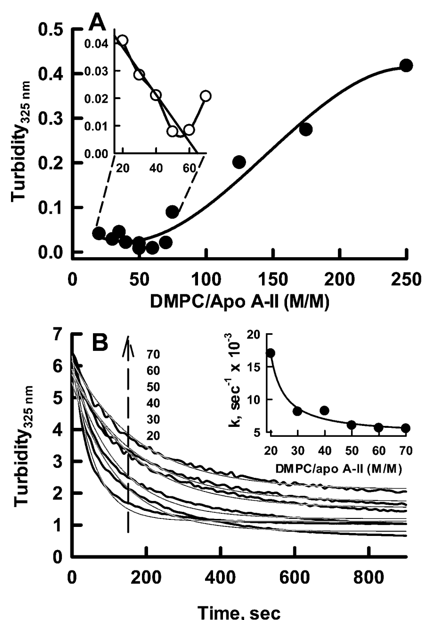

Figure 1. Apo A-II concentration dependence of rHDL formation. (A) Determination of the DMPC/apo A-II stoichiometry according to the increase in turbidity produced by excess MLV. The inset shows the maximal stoichiometry of 65/1 for solubilization of DMPC by apo A-II was determined by extrapolation. (B) Kinetics of formation of rHDL from DMPC as a function of added apo A-II according to the disappearance of MLV turbidity. The DMPC/apo A-II molar ratios for the top (70/1) to bottom (20/1) curves are given. The black and gray curves are the data and the fit of the data, respectively. The inset shows the first-order rate constant calculated from curve fits of Figure 1B as a function of DMPC/apo A-II molar ratio.

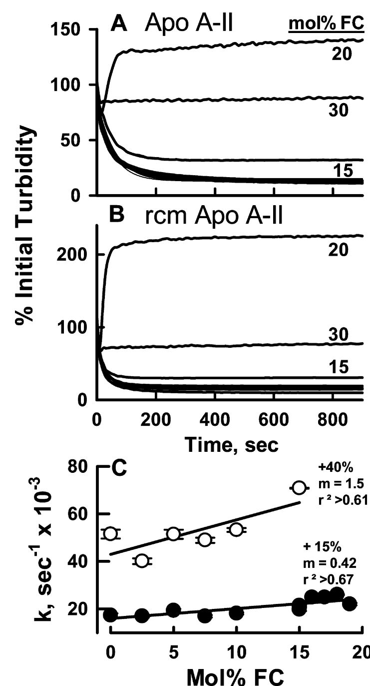

Figure 2. Kinetics of solubilization of DMPC MLV by apolipoproteins according to the reduction in MLV turbidity at 24 °C as a function of FC mole percentage. Experiments were conducted with stoichiometric amounts of DMPC and apo A-II, i.e., 65/1 molar ratio: (A) apo A-II and (B) rcm apo A-II. For both apolipoproteins, decay curves for 0–15 mol % FC are closely spaced and are distinct from curves at 20 and 30 mol % FC. (C) Plots of the rate constants for the solubilization by apo A-II (●) and rcm apo A-II (○) vs FC mole percentage.

effect of FC on the formation of rHDL from DMPC MLV, we conducted kinetic studies at stoichiometries of 65/1 for apo A-II and 32.5/1 for rcm apo A-II, reasoning the latter gives the same number of DMPC per helical regions as 65/1 for apo A-II. For both apo A-II and rcm apo A-II, the decay curves for 0–15 mol % FC are similar and clustered, and distinct from the curves at 20 and 30 mol % FC, at which solubilization essentially stops (Figure 2A,B). The rate constants for rHDL formation increase by 40% for rcm apo A-II and 15% for apo A-II as the starting MLV FC mole percentage increases from 0 to 15% (Figure 2C). The effects of FC mole percentage on the association of DMPC with apo A-II and rcm apo A-II were followed under three conditions: excess lipid, a stoichiometric lipid/apo A-II ratio (65/1 and 32.5/1 for dimeric and rcm apo A-II, respectively), and cholate dialysis of a stoichiometric lipid/apo A-II ratio. In the latter, cholate is a well-known "catalyst" of apolipoprotein–lipid association. The dependence of incorporation of DMPC into rHDL was a function of method but similar for apo A-II and rcm apo A-II (Figure 3A–C). Under stoichiometric conditions, with or without cholate dialysis (Figure 3A,B), the percent of the starting DMPC incorporated into rHDL was nearly quantitative at low FC mole percentages but decreased rapidly between 15 and 30 mol %. Under conditions of excess DMPC without FC, ~50% of the starting DMPC was incorporated into rHDL (Figure 3C). With increasing amounts of FC, up to 15 mol %, the amount of DMPC incorporated into rHDL increased to >85% and then declined at higher FC mol percentages, falling to just under 50% at 30 mol % FC in the initial MLV. Interestingly, the FC mole percentages in the supernatant (rHDL) and the pellet (unreacted DMPC MLV) were different, likely reflecting the preference of the apolipoprotein for domains with a specific FC mole percentage (Figure 3D,E). For both apo A-II and rcm apo A-II, the FC mole percentages in the supernatant (rHDL) were similar to that of the starting MLV up to 10 and 15 mol % for rHDL formed from stoichiometric (Figure 3D) and excess (Figure 3E) amounts of DMPC. However, above 10 and 15 mol %, respectively, the FC mole percentage in the products diverged to be higher for the MLV pellet and lower for the rHDL supernatant than the initial FC mole percentage. The product rHDL FC mole percentage plateaued at ~15 mol %. These effects were the same for apo A-II and rcm apo A-II.

Differential Effects of FC Mole Percentage on the Size of rHDL. SEC was used to determine the size and FC mole percentage of rHDL formed from DMPC and apo A-II as a function of the initial FC mole percentage. To increase the detection sensitivity for minor peaks, starting MLV were radiolabeled with [^{3}H]DMPC and [^{14}C]FC, and SEC elution profiles for absorbance at 280 nm, ^{3}H, and ^{14}C were determined. rHDL prepared from stoichiometric amounts of DMPC and apo A-II with 0 and 5 mol % FC eluted as a single SEC peak (Figure 4A,B). At 10 mol % FC, a second peak with a smaller elution volume (i.e., larger size) appeared (Figure 4C), and at 15 and 20 mol % FC, yet a third earlier eluting (still larger size) peak appeared (Figure 4D,E). The co-elution of radioactivity and absorbance at 15 and 20 mol % FC indicates rHDL is formed. At 30 mol % FC, very little rHDL was formed. As the FC mole percentage is increased, the amount of lipid-free apo A-II, which elutes at 36 mL, also increases. Strikingly, as shown by the increase in the ^{14}C/^{3}H ratio, the larger rHDL have larger amounts of FC.

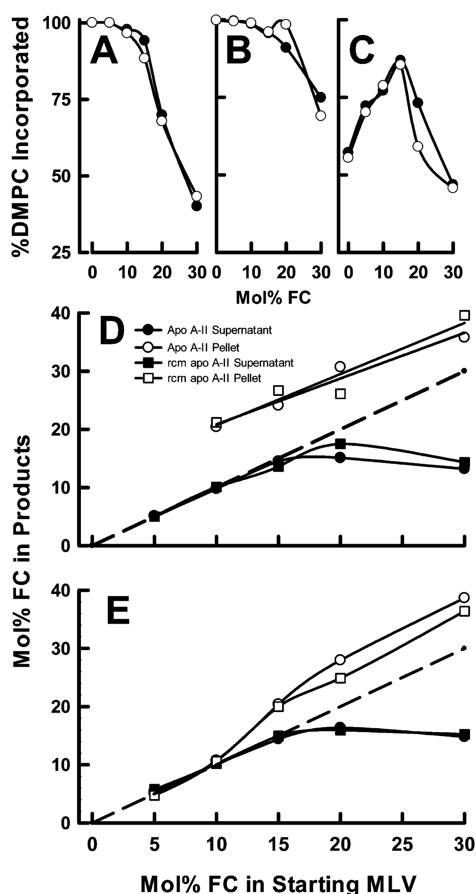

Figure 3. Distribution of FC between rHDL (supernatant) and MLV (pellet). (A–C) Fraction of DMPC incorporated into rHDL, calculated as the total counts in the sample minus the counts in the MLV pellet obtained by centrifugation. (A) rHDL formation with stoichiometric amounts of DMPC and apolipoproteins. (B) Cholate-catalyzed rHDL formation of stoichiometric amounts of DMPC and apolipoproteins. (C) rHDL formation in the presence of excess DMPC: (●) apo A-II and (○) rcm apo A-II. (D) Stoichiometric DMPC/apolipoprotein ratio and (E) excess DMPC. Both panels show the FC mole percentage in the supernatant (rHDL) and pellet (residual MLV), calculated from the ratio of ^{14}C to ^{3}H radioactivity in the supernatant and pellet, as a function of FC mole percentage in the starting MLV, as labeled in panel D. The dashed gray lines in D and E represent the expected FC mole percentage in rHDL if FC were incorporated in proportion to the initial FC mole percentage.

The effect of FC on the size and composition of rHDL prepared from excess DMPC was distinct from that prepared under stoichiometric conditions, with relatively more of the larger rHDL formed at each FC mole percentage. While at 0 mol % FC the rHDL eluted as a single SEC peak (Figure 4G), at 5 mol % a second peak appeared (Figure 4H), which was the dominant peak at 10 mol % FC (Figure 4I). At 15 mol %, the next larger peak predominated (Figure 4J), and at 20 mol % FC, this peak and a yet larger peak were detected (Figure 4K). With excess DMPC, these two larger peaks were also detected at 30 mol %, although much of the apo A-II was lipid-free (Figure 4K,L). The co-elution of radioactivity and absorbance at 15 and 20 mol % FC indicates rHDL is formed. At 30 mol %, very little rHDL was formed, as indicated by the amount of lipid-free apo A-II, which elutes at 36 mL. Under conditions of excess DMPC, relatively larger amounts of the larger rHDL products were formed at a given FC mole percentage compared to stoichiometric amounts of DMPC. With both stoichiometric and excess DMPC, increasing the level of FC to 15 mol % resulted in larger rHDL, enriched in FC. However, additional FC in the starting MLV (20–30 mol %) resulted in a decreased level of rHDL formation.

Finally, the effect of cholesterol on rHDL formation by cholate dialysis was determined. In contrast to spontaneous rHDL formation by the first two methods, which gave larger particles as a function of increasing FC level, the cholate method gave only one particle size irrespective of the initial FC mole percentage (Figure 4M–R). In addition, our data showed that the FC mole percentage incorporated into rHDL was lower than in the starting MLV. We conducted a control experiment with DMPC/apo A-II rHDL assembly at 10 mol % FC to address the possibility that FC escaped during dialysis. The resulting rHDL was centrifuged to pellet unreacted material. Our data showed that 96.5 ± 7.5% of the [^{3}H]DMPC remained in the supernatant, whereas a smaller amount of [^{14}C]FC, 85.7 ± 7.6%, was recovered. Moreover, the $^{3}H/^{14}C$ ratios of the predialysis sample, supernatant, and pellet were 12.6 ± 0.5, 11.7 ± 0.8, and 4.0 ± 0.4, respectively. Thus, FC-rich lipid domains are partially excluded from the (small) rHDL product obtained by the cholate dialysis method. The amount of rHDL formed was constant up to 15 mol % and then declined thereafter, while the amount of lipid-free apo A-II increased. Finally, as the FC in the initial mixture increased from 5 to 30 mol %, the fraction of initial cholesterol in the MLV that appeared in the product rHDL decreased. As in Figures 2 and 3, the SEC data of the rHDL formed from DMPC and rcm apo A-II were similar to those of apo A-II (Figure 1 of the Supporting Information).

The effects of FC mole percentage on rHDL formation are summarized in Figure 5, which shows the following. The FC mole percentage in the smallest rHDL (peak I) is similar to that of the starting mixture at 5 mol % FC and increases linearly with respect to the initial FC mole percentage with values that are lower than that in the respective starting MLV (Figure 5A, gray dashed line). In contrast, the FC mole percentage in the larger rHDL (peaks II and III) is greater than that of the initial mixture and increases linearly with an increasing initial FC mole percentage. The slopes of the lines for peaks I–III in Figure 5A are similar. FC preferentially associates with larger rHDL (peaks II and III). The relationship between initial FC mole percentages and FC compositions of the four different rHDL formed from excess lipid (Figure 5B) is qualitatively similar, i.e., parallel lines, to what was observed with stoichiometric amounts of DMPC (Figure 5A). However, at a given initial FC mole percentage, the compositions of rHDL in peaks I–III were more similar to each other when formed from excess versus stoichiometric amounts of lipid. At a given initial FC mole percentage, the FC mole percentages in rHDL formed from stoichiometric amounts of lipid were higher than that formed from excess lipid.

Free Energy of Transfer of FC among Various rHDL Species. Given that the various rHDL formed under each condition are at equilibrium, one can calculate a free energy of transfer of cholesterol from one rHDL$_i$ to another rHDL$_j$ as $\Delta G = -RT \ln(\text{mol \% FC}_i/\text{mol \% FC}_j)$, where mol % FC$_i$ and mol % FC$_j$ are the mole percentages for cholesterol in two differently sized rHDL formed concurrently at a given FC mole percentage. According to this calculation, differences in the free energies of transfer of FC between coexisting rHDL are small, with the greatest differences existing for the transfer from the

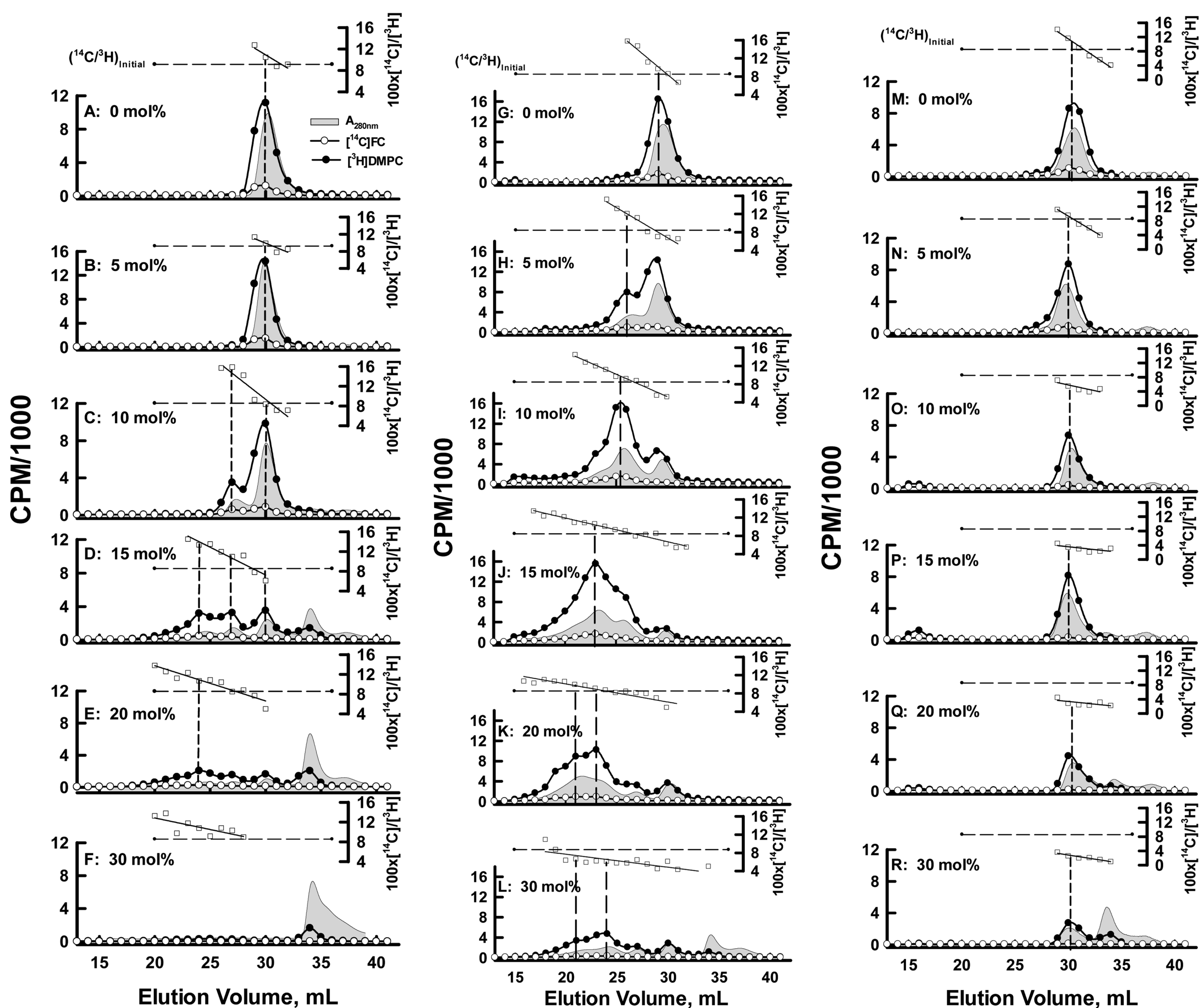

Figure 4. SEC analysis of the effect of FC on the formation of rHDL from apo A-II with DMPC. (A−F) Data collected with stoichiometric amounts of DMPC and apo A-II. (G−L) Data collected with an excess of DMPC. (M−R) Data collected using the cholate dialysis method. Panel A contains the legend for all plots. The inset above each panel is the $^{14}C/^{3}H$ ratio; the dashed line denotes the starting $^{14}C/^{3}H$ ratio.

smallest to the largest rHDL; e.g., at 20 mol % FC, the free energy of transfer of FC from the smallest to the largest particle is approximately −1.2 kJ. Given that the curves are nearly parallel, at any given initial MLV FC mole percentage, the differences in the free energies of transfer of FC between peaks are similar.

Cross-Linking rHDL. Cross-linking studies were conducted to determine the number of apo A-II molecules per rHDL particle as a function of size. rHDL were cross-linked with BS^3 and separated into fractions [peaks I−III (Figure 6A−D)], which were pooled, collected, and analyzed by native PAGE and SDS−PAGE. According to native PAGE, each peak comprises a homogeneous species (Figure 6E) with respective Stokes diameters of ∼11, 13, and 17 nm. After delipidation, SDS−PAGE (Figure 6F) showed that each rHDL peak contained two major cross-linked species as follows. Peak I had molecular masses of ∼60 and 71 kDa (3.5 and 4 apo A-II dimers, respectively) and peaks II and III had molecular masses of 97 and 124 kDa (∼6 and 7 apo A-II dimers, respectively) and 130 and 151 kDa (7 and 9 apo A-II dimers, respectively), respectively. The relationship between particle circumference, calculated from the Stokes diameter, and the number of apo A-II per rHDL is shown in Figure 6G.

■ DISCUSSION

Kinetics of rHDL Formation. Although microsolubilization of DMPC by apolipoproteins has been used as a model of HDL assembly via ABCA1, few studies have been conducted in the presence of FC, the lipid most central to reverse cholesterol transport (RCT). In a recent study, we showed the size of apo A-I rHDL increased as a function of FC mole percentage in MLV and at high FC mole percentages, the reaction was incomplete.[11] Given apo A-II is more lipophilic than apo A-I, we hypothesized apo A-II would associate efficiently even at high FC mole percentages and tested the effects of FC on the microsolubilization of DMPC by apo A-II and rcm apo A-II.

Although, as our SEC data show (Figure 4 and Figure 1 of the Supporting Information), the reaction products of apo A-II and rcm apo A-II with DMPC are remarkably similar at each FC mole percentage, there are differences in reaction kinetics.

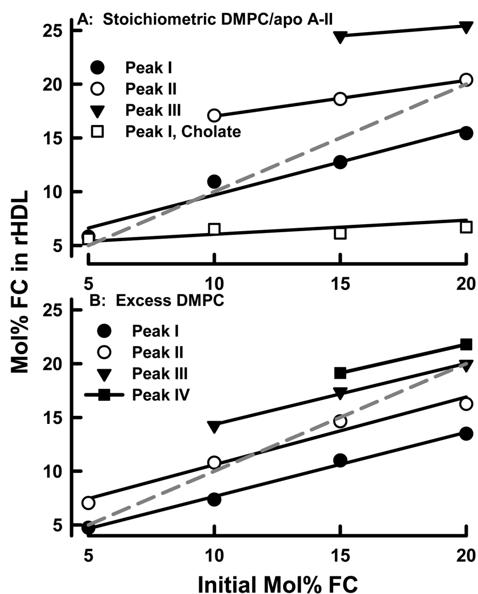

Figure 5. FC mole percentage in rHDL of various sizes as a function of initial FC mole percentage in MLV. rHDL were formed from stoichiometric ratios of DMPC and apo A-II (A) and excess DMPC (B). Compositions were calculated from the data depicted in Figure 4. The dashed gray line is a plot of the expected rHDL FC mole percentage vs the initial FC mole percentage if FC and DMPC were incorporated into rHDL at the ratio in the starting MLV.

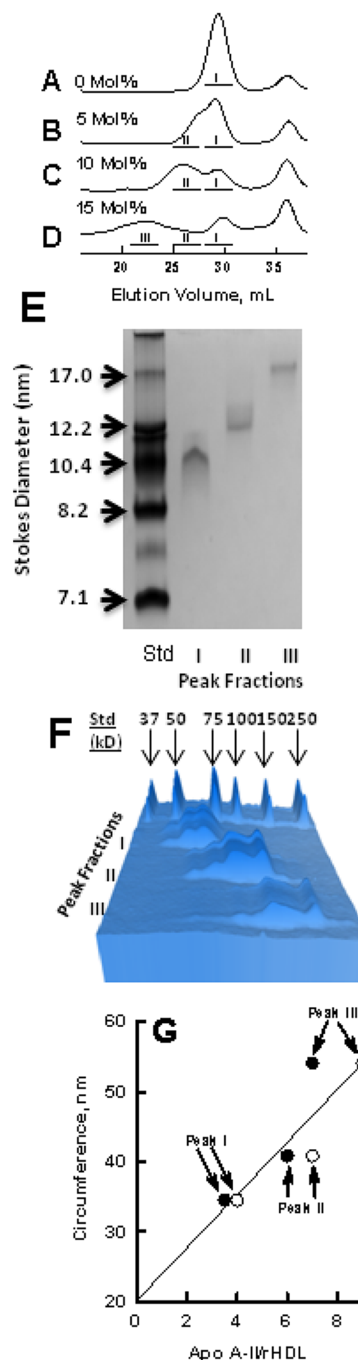

Figure 6. Analysis of cross-linked rHDL. rHDL were prepared from apo A-II and DMPC containing various FC mole percentages as labeled (A–D) and peaks I–III isolated by SEC. (E) Native gel of pooled fractions for each peak and standards for calculation of the Stokes diameter. (F) Scan of a 4 to 20% gradient SDS–PAGE gel of cross-linked delipidated proteins in each peak along with molecular mass standards. (G) From the Stokes diameters, the circumference of the rHDL in each peak was calculated and plotted vs the number of apo A-II per particle.

In the absence of FC, the rate of solubilization of rcm apo A-II with DMPC was ~2 times faster than that of apo A-II (Figure 2C). In the context of the cluster model of lipid–apolipoprotein association, this is likely due to the size distribution of defects in the DMPC surface; all defects that can accommodate the insertion of apo A-II can also accommodate rcm apo A-II. However, rcm apo A-II can fit into smaller surface defects that exclude 2-fold larger apo A-II molecule. The rate of rHDL formation increases linearly with the addition of up to 20 mol % FC, an increase that is greater for rcm apo A-II (+40%) than for apo A-II (+15%), suggesting that added cholesterol induces the formation of more small defects that accommodate rcm apo A-II than large ones that accommodate both apolipoproteins.

Kinetic measurements at each FC mole percentage between 15 and 20 mol % revealed a change in the "quality" of the DMPC interface exactly at 20 mol %, at and above which the reaction was slow and incomplete; i.e., unreacted apo A-II and rcm apo A-II remained at the end of the reaction. This was observed for apo A-II and rcm apo A-II, and similar studies of apo A-I also showed a decrease in the level of rHDL formation at 20 mol %, although a percent-by-percent titration was not performed.[11] The rates of association of apolipoproteins with DMPC decrease dramatically at 20 mol % FC for apo A-I, apo A-II, and rcm apo A-II, which have different sizes and lipophilicities, suggesting that the effect is intrinsic to the properties of DMPC at 20 mol % FC and independent of the properties of the apolipoproteins. There is some other evidence for this. The rate of penetration of a small fluorescent dye into DMPC peaks at 20 mol % FC and decreases ~200-fold between 20 and 30 mol % FC.[26] According to differential scanning calorimetry, as the FC mole percentage in DPPC is increased to 20 mol %, a mix of pure phospholipid and cholesterol-rich phases is replaced by a single FC–DPPC phase.[27,28] The rate of rHDL formation at 24 °C increases up to 20 mol % FC but above this drops precipitously (Figure 2). We propose that this effect is due to an increase in the number of defects with an increasing FC mole percentage at the DMPC surface (Figure 7A) until at 20 mol % FC the reaction does not occur. This FC mole percentage corresponds to a DMPC/FC ratio of 4/1. We propose that at this composition the failure of rHDL to form is due to close packing of the lipids and the near

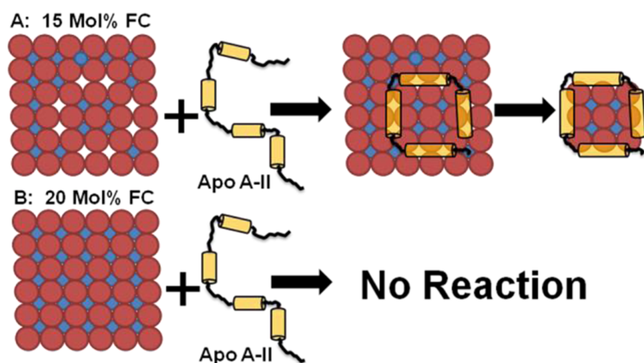

Figure 7. Models of lipid packing of FC in DMPC and rHDL formation. (A) Packing at ∼15 mol % FC showing defects as white space, which permit insertion of apo A-II leading to rHDL formation. (B) Close packing at 20 mol % FC (4/1 DMPC/FC), showing the absence of major defects and no reaction with apo A-II.

total loss of surface defects needed for the insertion of apo A-II; this is illustrated in Figure 7B, in which the relative surface areas of DMPC and FC are 7 and 4 nm^2, respectively. Thus, despite its greater lipophilicity, and contrary to our initial hypothesis, apo A-II cannot penetrate the lipid surface at 20 mol % FC because of the absence of defects, which are required for insertion of apolipoproteins. Even rcm apo A-II, which is half the molecular mass of apo A-II, does not penetrate the lipid efficiently, so defects, if present, must be small.

Apo A-II and rcm ApoA-II Select FC-Poor Domains for rHDL Formation. Comparison of the FC mole percentage in unreacted MLV and rHDL showed apolipoproteins preferentially associate with FC-poor domains for formation of small rHDL (peak I) and increasing FC mole percentages in larger rHDL (peaks II and III). At all initial FC mole percentages tested, the FC mole percentage in unreacted MLV in the sedimented pellet was always equal to or greater than that of the starting mixture; the opposite was true for rHDL found in the supernatant (Figure 3D,E). On the basis of the FC mole percentage in the rHDL isolated by SEC, we observed parallel increases in FC mole percentage in each rHDL (peaks I−III) with the FC mole percentage in the two or three rHDL formed at a given initial FC mole percentage increasing with rHDL size (in Figure 5A, compare ●, ○, and ▼ at a given FC mole percentage). These data show that large rHDL accommodate more FC than small rHDL. The simultaneous formation of rHDL of different sizes from the same reaction mixture likely begins in the context of coexisting large cholesterol-rich and smaller cholesterol-poor domains within the MLV. Those apolipoproteins that circumscribe cholesterol-rich and cholesterol-poor domains are destined to form large cholesterol-rich and small cholesterol-poor rHDL, respectively. In contrast, under stoichiometric conditions in which rHDL formation is catalyzed by cholate, only small rHDL form irrespective of the initial FC mole percentage and very little FC is incorporated into the rHDL, even at high FC mole percentages. According to differential scanning calorimetry, mixed DMPC/Na cholate SUV exhibit sharp low-temperature and broad high-temperature endotherms consistent with two coexisting immiscible phases. The sharp transition corresponds to a relatively pure PC phase, whereas the other, which like FC/DMPC MLV exhibits a broad endotherm, is a cholate/DMPC phase.[29] Thus, on the basis of our data, in the presence of cholate the apolipoproteins preferentially associate with the relatively pure DMPC phase that gives rise to the sharp transition and from which most FC is excluded.

Given that apolipoproteins associate with pure phospholipids and not directly with FC, under stoichiometric conditions, FC and apolipoproteins compete for binding to DMPC, a competition that is expected to be on the side of FC because of its much lower aqueous solubility. Thus, even at 5 mol % FC, a small amount of apo A-II is lipid-free, and this amount increases with an increasing FC mole percentage in the starting mixture (Figure 4B−F). In contrast, under conditions of excess DMPC (∼260/1), there is no lipid-free apo A-II until FC in the initial mixture reaches 20 mol %. Thus, with excess DMPC present, competition between FC and apo A-II for binding sites on the MLV is not apparent until very high FC mole percentages are reached.

FC Modifies the Stoichiometry of Apo A-II in rHDL. In the absence of FC, the major rHDL peak appears to contain 3.5 and 4 dimers of apo A-II per particle. In their studies of the configuration of apo A-II in rHDL containing an unsaturated PC, Silva et al. observed a stoichiometry of 4 apo A-II per rHDL.[30] Given the unlikely occurrence of a monomer of apo A-II, we opine that cross-linking causes abnormal migration of one of the species and that the actual stoichiometry of both is 4/1. Similarly, the likely stoichiometries for peaks II and III are 6 and 8, respectively, as suggested by a plot of measured stoichiometry versus rHDL circumference (Figure 7G). Thus, as with apo A-I,[11] as the FC mole percentage in the initial incubation is increased, the size of rHDL increases in a nearly quantized way, which accommodates dimers of 4, 6, and 8 apo A-II molecules per rHDL. As such, the even-numbered stoichiometry permits similar associations between each of the dimers so the simple model of cross-links reported by Silva et al.[30] would be preserved.

Physiological Relevance. Most studies indicate that the exchangeable apolipoproteins form similar structures with DMPC and unsaturated phospholipids that are found in vivo. Thus, the FC content of plasma membranes is likely to control nascent HDL size in vivo. Although most of the exchangeable apolipoproteins tested elicit FC efflux from macrophages via ABCA1, this process is likely not relevant to human apo A-II because unlike apo A-I, there have been no verified examples of lipid-free apo A-II being formed in plasma in response to physicochemical or biological perturbations. Intrahepatically, however, apo A-II forms HDL particles that contain no apo A-I or apo E. In studies with HepG2 cells, we found that the HepG2 cell lysate consists of lipoproteins containing apo A-II with no apo A-I or E, and according to their elution SEC volumes, these particles are larger than the intracellular lipid-poor apo A-I nascent HDL.[7] Larger HDL in media of HepG2 cells contain apo E. This suggests, but does not prove, that early intracellular forms of apo A-II-containing lipoproteins are FC-rich compared to nascent apo A-I HDL and convert to spherical HDL found in plasma after secretion and undergoing LCAT-mediated fusion with apo A-I-containing HDL.[8]

Two competing forces determine the size of sterol-containing rHDL. The first is domain size, and this is a function of the type of sterol, the kind of sterol−PC association, and the sterol concentration; the higher the free energy of association of the lipids, i.e., PL and sterol, the larger the domains. The second is the energy derived from the transfer of apolipoprotein helical and nonhelical segments to the surface of the bilayer where many of the nonhelical segments adopt a helical conformation. The observation of two or three different

sizes of rHDL from a given apolipoprotein/sterol/PL mixture illustrates small differences in the energy of formation of different rHDL and argues for the coexistence of different size domains. If our model is correct, the size of rHDL is an indication of the size of the domains from which it formed. Apolipoproteins do not remove domains with different sizes and/or compositions preferentially. If this were the case, one would expect that the composition of products when the lipids are in great excess would reflect the most "reactive" domains and differ from the product mixture obtained when the lipids are mixed at their stoichiometric ratios. Our previous studies with apo A-I demonstrated that apo A-I formed a series of rHDL of discrete sizes from both model DMPC/FC MLV and macrophage cell membranes.[11] This study indicates apo A-II is capable of forming a similar series of nascent discoidal HDL from interations with membranes. The striking observation that rHDL formation abruptly stops above 20 mol % FC in our model MLV system suggests a similar limit in vivo in macrophage intracellular or plasma membranes may preclude the export of FC from cholesterol-overloaded foam cells.

■ ASSOCIATED CONTENT

Supporting Information

SEC analysis of the effect of FC on rHDL formation from rcm apo A-II with DMPC (Figure 1). This material is available free of charge via the Internet at http://pubs.acs.org.

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