Lipid-free apolipoproteins A-I and A-II promote remodeling of reconstituted high density lipoproteins and alter their reactivity with lecithin:cholesterol acyltransferase

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Abstract **We examined the effect of lipid-free apolipoprotein A-I (apoA-I) and apoA-II on the structure of reconstituted high density lipoproteins (rHDL) and on their reactivity as substrates for lecithin:cholesterol acyltransferase (LCAT). First, homogeneous rHDL were prepared with either apoA-I or apoA-II using palmitoyloleoylphosphatidylcholine (POPC) and cholesterol. Lipid-free apoA-I and apoA-II were labeled with the fluorescent probe dansyl chloride (DNS). The binding kinetics of apoA-I-DNS to A-II-POPCrHDL and of apoA-II-DNS to A-I-POPCrHDL were monitored by fluorescence polarization, adding the lipid-free apolipoproteins to the rHDL particles in a 1:1 molar ratio. For both apolipoproteins, the binding to rHDL was rapid, occurring within 5 min. Next, the effect on rHDL structure and particle size was determined after incubations of lipid-free apolipoproteins with homogeneous rHDL at 37°C from 0.5 to 24 h. The products were analyzed by nondenaturing gradient gel electrophoresis followed by Western blotting. The effect of apoA-I or apoA-II on 103 Å A-II-POPCrHDL was a rearrangement into 78 Å particles containing apoA-I and/or apoA-II, and 90 Å particles containing only apoA-II. The effect of apoA-I or apoA-II on 98 Å A-I-POPCrHDL was a rearrangement into complexes ranging in size from 78 Å to 105 Å containing apoA-I and/or apoA-II, with main particles of 78 Å, 88 Å, and 98 Å. Finally, the effect of lipid-free apoA-I and apoA-II on rHDL as substrates for LCAT was determined. The addition of apoA-I to A-II-POPCrHDL increased its reactivity with LCAT 24-fold, reflected by a 4-fold increase in apparent V_{max} and a 6-fold decrease in apparent K_m, while the addition of apoA-II to A-II-POPCrHDL had no effect on its minimal reactivity with LCAT. In contrast, the addition of apoA-II to A-I-POPCrHDL decreased the reaction with LCAT by about one-half. The inhibition was due to a 2-fold increase in apparent K_m; there was no significant change in apparent V_{max}. Likewise, the addition of apoA-I to A-I-POPCrHDL inhibited the reaction with LCAT to about two-thirds that of A-I-POPCrHDL without added apoA-I. In summary, both lipid-free apoA-I and apoA-II can promote the remodeling of rHDL into hybrid particles of primarily smaller size. Both apoA-I and apoA-II affect the reactivity of rHDL with LCAT, when added to the reaction in lipid-free form. These results have important implications for the roles of lipid-free apoA-I and apoA-II in HDL maturation and metabolism.**—Durbin, D. M., and A. Jonas. **Lipid-free apolipoprotein A-I and A-II promote remodeling of reconstituted high density lipoproteins and alter their reactivity with lecithin:cholesterol acyltransferase.** ***J. Lipid Res.*** **1999.** 40: **2293–2302.**

Supplementary key words HDL rearrangements • lipid-binding • phospholipid • fluorescence polarization • preβ-HDL

High density lipoproteins (HDL) comprise a heterogeneous population of particles that vary in size, density, electrophoretic mobility, apolipoprotein composition, morphology and very likely, function. This variability reflects the multiple stages of maturation and remodeling that HDL undergo during their metabolism. This complex process can be described using a simplified cyclic model that begins with the secretion of newly synthesized lipid-free or lipid-poor apolipoprotein A-I (apoA-I) and apoA-II from hepatocytes and enterocytes (1, 2). The lipid-free or lipid-poor apolipoproteins bind to existing lipoproteins and lipoproteins undergoing lipolysis, and also, in particular apoA-I, recruit phospholipids and cholesterol from cell membranes, forming preβ-1 HDL. In the latter process, nascent preβ-1 HDL are transformed into discoidal preβ-2 HDL, then preβ-3 HDL, and finally, into mature spherical alpha-migrating HDL_2 and HDL_3 upon exposure to lecithin:cholesterol acyltransferase (LCAT) (3). Remodeling of mature HDL continues in the plasma with the exchange of cholesteryl esters for triglycerides by cholesteryl ester transfer protein (CETP) and phospholipid

Abbreviations: apoA-I, apolipoprotein A-I; apoA-II, apolipoprotein A-II; HDL, high density lipoprotein; rHDL, reconstituted HDL particles; LCAT, lecithin:cholesterol acyltransferase; CETP, cholesteryl ester transfer protein; PLTP, phospholipid transfer protein; HL, hepatic lipase; POPC, l-α-palmitoyloleoylphosphatidylcholine; BS^3, bis(sulfosuccinimidyl)suberate; DAB, diaminobenzidine tetrahydrochloride; DNS, dansyl chloride; PVDF, polyvinylidene difluoride; GGE, gradient gel electrophoresis.

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transfer by phospholipid transfer protein (PLTP) (4–6). Finally, there is a shrinking of HDL_2 due to triglyceride hydrolysis and the phospholipase action of hepatic lipase (HL), producing HDL_3 in the process, and regenerating lipid-free or lipid-poor apoA-I and apoA-II to repeat the cycle (7, 8).

It has been proposed that the anti-atherogenic properties of HDL are due, in part, to the participation of lipid-free or lipid-poor apoA-I in the initial step of reverse cholesterol transport: the recruitment of phospholipid and cholesterol from extra-hepatic cells, and the formation of preβ-HDL particles. These nascent HDL particles are the most efficient acceptors of free cholesterol from cells and other lipoproteins (9, 10). In contrast, much less is known about the role of apoA-II in reverse cholesterol transport. ApoA-II has a higher affinity for lipids than does apoA-I, and in vitro work with cultured Chinese hamster ovary (CHO) cells has shown that lipid-free apoA-II can recruit cellular membrane lipids to the same extent as apoA-I (11). Although it has not been shown that apoA-II recruits cellular lipids in vivo, there are isolated reports of HDL particles purified from normolipidemic human plasma that contain apoA-II but not apoA-I. Although not extensively characterized, these A-II–lipid complexes contain a minimal nonpolar lipid core and may represent nascent HDL particles formed by apoA-II, or, alternatively, remnants of lipoprotein lipolysis (12, 13).

The remodeling of HDL described above resulting from the actions of LCAT, CETP, PLTP, and HL has been extensively studied. It involves the primary modification of the lipid composition of HDL followed by release or uptake of lipid-poor apoA-I, fusion of destabilized particles, and consequent changes in the morphology and size of the HDL (8, 14–16). Previous work by Jonas, Bottum and Kézdy (17) has shown that rHDL particle rearrangement can occur upon the addition of LDL or from incubations at 37° or 50°C. Lipid-free apolipoproteins added to HDL can also promote particle rearrangements. Labeur et al. (18) have recently reported that high proportions of lipid-free apoA-II (1/1 to 4/1, apoA-II/apoA-I) or a C-terminal peptide of apoA-II, displace apoA-I from rHDL particles prepared with 1-palmitoyl-2-linoleoylphosphatidylcholine (PLPC) and result in marked rearrangements of particle sizes as well as inhibition of LCAT activation. In a previous study (19) we had shown that lipid-free apoA-II binds rapidly to defined discoidal rHDL containing apoA-I and dipalmitoylphosphatidylcholine (DPPC). For molar ratios of apoA-II/apoA-I in the physiological range (0.5/1), we found that apoA-II did not displace apoA-I from the rHDL or result in particle rearrangement. Instead, it modified the structure and stability of a lipid-bound apoA-I molecule and decreased the reactivity of the rHDL with LCAT, probably by interfering with the binding of the enzyme. In this study our objective was to examine the effects of lipid-free apoA-I or apoA-II binding on discoidal rHDL particles containing a physiological phospholipid, 1-palmitoyl-2-oleoyl PC (POPC), at molar ratios of apolipoproteins to rHDL that may be found in vivo. We report on the kinetics of binding of lipid-free apolipoproteins to rHDL, the subsequent rearrangements of the rHDL and the time course of rearrangement, as well as on the initial effects of apolipoprotein binding on the LCAT reaction.

METHODS

Materials

Human apoA-I, apoA-II, and LCAT were prepared from blood plasma purchased from the Champaign County Blood Bank, Regional Health Resource Center. The purifications of apoA-I, apoA-II, and LCAT were performed as described previously (19–21). l-α-palmitoyloleoylphosphatidylcholine (POPC), chrystalline cholesterol, sodium cholate, and diaminobenzidine tetrahydrochloride (DAB) were purchased from Sigma. Radiolabeled [^{14}C]cholesterol was obtained from DuPont NEN Life Science Products. Bis(sulfosuccinimidyl)suberate (BS^3), polyvinylidene difluoride membranes (0.2 μm), and ImmunoPure ABC Peroxidase Staining kit were purchased from Pierce. Rabbit anti-human apoA-I polyclonal antibodies were purchased from Calbiochem; goat anti-human apoA-II polyclonal antibodies were purchased from Rockland, Gilbertsville, PA. Instant Thin-Layer Chromatography (ITLC) plates were obtained from Gelman Sciences, Ann Arbor, MI. Dansyl chloride (DNS) was purchased from Aldrich. All experiments (unless noted otherwise) were performed in a 10 mm Tris buffer (pH 8.0) containing 150 mm NaCl, 1.0 mm NaN_3, 0.01% EDTA; this buffer will be referred to as the standard buffer.

Preparation of rHDL

The sodium cholate dialysis method (22, 23) was used in the preparation of rHDL complexes. A molar ratio of 100:5:1, POPC:cholesterol:apoA-I, was used when preparing rHDL. The dimeric apoA-II (M.W. 17,400) particles contained the same mass of phospholipid and cholesterol per mg of apoA-II protein as rHDL prepared with apoA-I. The size and homogeneity of the rHDL particles were assessed by non-denaturing gradient gel electrophoresis (GGE) on 8–25% gradient gels using the Pharmacia Phast System. After staining the gels with 0.1% Coomassie Blue R-350 and appropriate destaining, the gels were scanned with a Pharmacia LKB UltroScan laser densitometer, and the size of the rHDL particles was determined by their mobility relative to that of protein standards of known Stokes diameters (High Molecular Weight Standards, Pharmacia). The A-I–POPC–rHDL preparation typically contained 98 Å (±2 Å, n = 6 preparations) and 120 Å species; isolation of the 98 Å particles was carried out by gel filtration chromatography on two Superdex 200 columns in tandem (1 × 30 cm, each) with the Pharmacia FPLC System using a flow rate of 0.3 mL per min with the standard buffer. The homogeneous A-I–POPC–rHDL species contained two molecules of apoA-I, determined by chemical cross-linking with BS^3. The A-II–POPC–rHDL was 103 Å in diameter (±1 Å, n = 5 preparations). The A–IIrHDL contained four molecules of dimeric apoA-II. Protein concentration was determined as the average of concentrations obtained from the method of Markwell et al. (24) and from absorbance at 280 nm, using an extinction coefficient of 1.13 mL/mg·cm (31,720 M^{-1} cm^{-1}) for apoA-I; and at 276 nm, using an extinction coefficient of 0.69 mL/mg·cm (12,000 M^{-1} cm^{-1}) for dimeric apoA-II (25).

Labeling of apolipoproteins with dansyl chloride

The labeling of apoA-I and apoA-II with dansyl chloride was done in 0.1 m phosphate buffer (pH 8.0) as published previously (19) using a molar ratio of 2:1, DNS:apolipoprotein. Molar extinction coefficients used for DNS determination were 4,300 M^{-1}

cm^{-1} and 1,850 M^{-1} cm^{-1} at 340 and 280 nm, respectively (26). The DNS:apolipoprotein molar ratios (labeling efficiencies) were 0.8:1 (±0.4, n = 4) for apoA-I, and 1.3:1 (±0.4, n = 11) for apoA-II. This extent of labeling did not appear to affect the ability of apoA-I or apoA-II to bind lipid, based on the similarity in size and composition of rHDL prepared with labeled or unlabeled apoA-I or apoA-II, or change the stability of labeled or unlabeled apoA-II associated with rHDL in the presence of GuHCl (19).

Fluorescence experiments

Fluorescence measurements were carried out on an ISS PC1 photon-counting spectrofluorometer at the Laboratory for Fluorescence Dynamics (LFD), University of Illinois, Urbana, IL. Fluorescence polarization was used to monitor the binding kinetics of lipid-free apoA-I–DNS or apoA-II–DNS when added to rHDL. Polarization values were acquired from 0.5 to 60 min after the addition of homogeneous rHDL particles to a solution of labeled apolipoprotein in the standard buffer. The samples were maintained at 20°C in a thermostated cell compartment. A constant "g" factor was applied to each experiment to compensate for the sensitivity of the detector for vertically and horizontally polarized emitted light (27). The excitation and emission wavelengths were 340 nm and 500 nm, respectively, and 1 mm excitation and emission slits were selected.

Remodeling of rHDL by lipid-free apolipoproteins

The effect of lipid-free apolipoproteins on rHDL structure and particle size distribution was examined by adding lipid-free apoA-I or apoA-II to A-II–POPCrHDL or A-I–POPCrHDL, in a molar ratio of 1:1, lipid-free apolipoprotein per rHDL particle. This ratio was selected for three reasons. *1*) The normal molar ratio of apoA-II to apoA-I in circulation is 0.5:1 (28) which corresponds to 1:1, apoA-II per rHDL particle containing two apoA-I molecules. *2*) In our previous study this ratio resulted in the complete binding of apoA-II to rHDL particles, containing apoA-I and DPPC, without displacement of apoA-I (19). *3*) Studies of the transformations of HDL or rHDL by PLTP, CETP, or LCAT have indicated that usually one molecule of lipid-poor apoA-I is released or taken up in these processes per HDL particle (6, 14, 15).

The remodeling reaction was performed on at least five different preparations of A-I–POPCrHDL and A-II–POPCrHDL. At least two remodeling experiments were done on each rHDL prep. The calculated size of the remodeled rHDL was based on their mobility relative to that of protein standards of known Stokes diameter (High Molecular Weight Standards, Pharmacia) and the variance in the reported size is ± 2 Å. The reaction mixtures usually contained 10 μg of rHDL protein and the appropriate lipid-free apolipoprotein to achieve the 1:1 molar ratio described above. The samples were mixed by pipetting, put under a stream of N_2, capped and sealed with parafilm, and incubated at 37°C in a shaking water bath from 30 min up to 24 h. The reactions were stopped by placing the samples on ice. The reaction products were analyzed by non-denaturing 8–25% gradient gels using the Pharmacia Phast System followed by Western blotting. The gels were blotted for 10 min with PVDF membranes saturated with MeOH to allow transfer of protein by diffusion. The gels were subsequently stained with 0.1% Coomassie Blue R-350, and the PVDF membranes were subjected to Western blotting with antibodies specific for apoA-I or apoA-II (1:7,000 dilution), following the protocol for the ImmunoPure ABC peroxidase staining kit from Pierce. Horseradish peroxidase-conjugated secondary antibodies of goat anti-rabbit and rabbit anti-goat secondary antibodies were used to visualize the apoA-I and apoA-II primary antibodies, respectively. DAB in 0.08% H_2O_2 was added as the horseradish peroxidase substrate.

LCAT reaction kinetics

Radiolabeled rHDL were prepared by the sodium cholate dialysis method (22, 23) with POPC, apoA-I or apoA-II, and cholesterol in molar ratios of 100:1:5, including 7500 cpm [^{14}C]cholesterol per nmol of cholesterol. To assess the effect of lipid-free apolipoproteins on LCAT kinetics, the radiolabeled rHDL were incubated with lipid-free apoA-I or apoA-II for 20 min at 37°C and subsequently reacted with LCAT for 30 min as described previously (21). The lipids extracted from the organic phase were spotted on ITLC plates developed in petroleum ether–ethyl ether–acetic acid 85:15:1 (v/v). The cholesteryl ester product was isolated from unesterified cholesterol and the percent conversion of [^{14}C]cholesteryl ester from free cholesterol was determined by scintillation counting. In some cases it was necessary to adjust the amount of LCAT added to the reactions to achieve initial velocities of 5–15% conversion of cholesterol. Therefore, the kinetic data are normalized to a constant amount of enzyme. The reactions were performed in triplicate in at least two different experiments. Data analysis was done using Microsoft Excel 5.0, Microsoft Corp., and kinetic constants were obtained using non-linear curve fitting with KaleidaGraph 3.0.5, Synergy Software, Reading, PA.

RESULTS

Binding kinetics of lipid-free apoA-I–DNS or apoA-II–DNS to rHDL monitored by fluorescence polarization

Fluorescence polarization was used to monitor the binding kinetics of lipid-free apoA-I and apoA-II to rHDL. To this end, the lipid-free apolipoproteins were labeled with dansyl choloride to allow the detection of a broad range of rotational motions not accessible by the intrinsic fluorescence from Trp. Homogeneous rHDL were added to the labeled lipid-free apolipoproteins, adding 1 mol of rHDL particles per mol of each apolipoprotein. **Figure 1** shows the results of a time course from 0.5 to 60 min for lipid-free apoA-I–DNS binding A-II–POPC–rHDL (panel A), and of apoA-II–DNS binding to A-I–POPCrHDL (panel B). In both cases the kinetics of binding of lipid-free apolipoproteins to rHDL are rapid, reflected by an increase in fluorescence polarization as the rotational motions of the lipid-free apolipoproteins are reduced upon binding to the large rHDL particles. For both apoA-I–DNS as well as apoA-II–DNS the polarization values reach an equilibrium polarization value within 5 min that remains unchanged for at least 1 h. The maximum polarization values are 0.24–0.25, considerably lower than the limiting value of 0.36 expected for particles of the size of the rHDL. Therefore, local motions of the probe or its immediate polypeptide environment contribute to the observed fluorescence polarization.

Effect of lipid-free apolipoproteins on the structure and particle distribution of rHDL

The effect of lipid-free apolipoproteins on the size and particle distribution of rHDL was evaluated over time from 0.5 h up to 24 h at 37°C. The lipid-free apolipoproteins were added to homogeneous rHDL in a 1:1 molar ratio. The products of the incubations were analyzed by

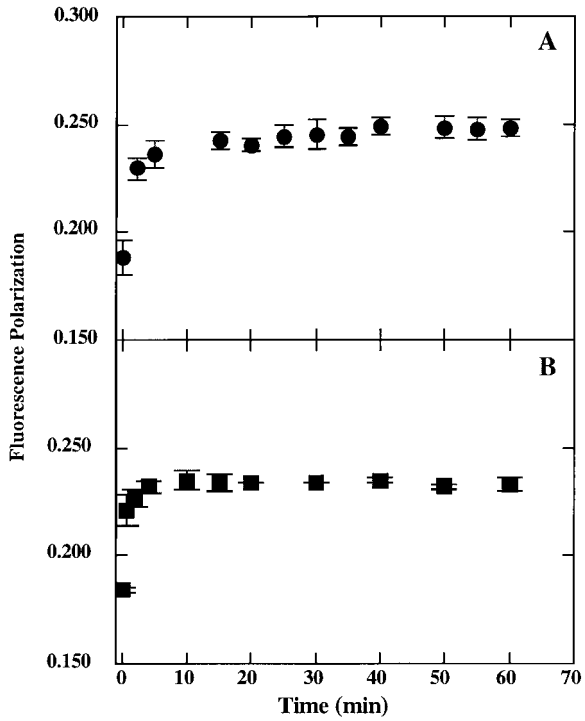

Fig. 1. Binding kinetics of lipid-free apoA-I–DNS or apoA-II–DNS to POPCrHDL. The binding of lipid-free apoA-I–DNS to A-II–POPCrHDL (panel A) and apoA-II–DNS to A-I–POPCrHDL (panel B) was monitored by fluorescence polarization. The rHDL were added to the DNS-labeled lipid-free apolipoproteins in a molar ratio of 1:1, rHDL particles:apolipoprotein. Polarization values were acquired from 0.5 to 60 min after the addition of rHDL. The polarization value for the lipid-free apolipoproteins in solution is indicated at time zero.

non-denaturing GGE followed by Coomassie staining and the protein components of the particles were identified by Western blotting. **Figure 2** shows the kinetics of rearrangement that occurred when lipid-free apoA-I was added to homogeneous 103 Å A-II–POPCrHDL particles. Within the first half-hour of incubation, rearrangement of A-II–POPCrHDL started, with the formation of 90 Å and 78 Å particles. There is a significant amount of lipid-free apolipoprotein present in the first few time points, but less in the samples incubated for a longer time, i.e., 8–18 h. After the initial particle rearrangement, the only visible changes are in the relative amounts of the species present. By 18 h, the initial 103 Å particles are reduced by about 70% and the 90 Å particles are the primary product (50% of protein stain). **Figure 3**, panel A, shows the initial 103 Å apoA-II–POPCrHDL and the particle distribution after 24 h at 37°C. From the immunoblots reacted with antibodies to apoA-I, panel B, or apoA-II, panel C, it can be seen that both apoA-I and apoA-II are present on 78 Å particles, but the 90 Å particles contain only apoA-II. A diffuse band between the 103 Å and 90 Å particles, which may represent the remnants of the initial 103 Å rHDL, appears to contain both apoA-I and apoA-II. In the 24-h samples some lipid-free apoA-I and apoA-II are present.

To see whether lipid-free apoA-II could also effect particle rearrangement of A-II–POPCrHDL, a similar experiment was performed, adding lipid-free apoA-II to A-II–POPCrHDL in a 1:1 mole ratio. **Figure 4** shows the result of a 24-h incubation at 37°C. The initial 103 Å A-II–POPCrHDL rearranged into the same distinct-sized particles, 90 Å and 78 Å, as with the addition of apoA-I; but there appears to be less of the 78 Å species and of lipid-free apoA-II in this reaction than seen with the addition of apoA-I.

Next, a similar experiment was performed with lipid-free apoA-II added to homogeneous 98 Å A-I–POPC–rHDL and incubated from 0.5 to 18 h at 37°C (**Fig. 5**). As seen with the addition of lipid-free apoA-I to A-IIrHDL, there is an initial fast rearrangement that occurs at 0.5 h, forming some 78 Å particles and lipid-free apolipoproteins. However, the rest of the rearrangement time course for A-I–POPOCrHDL was distinctly different from what was seen with A-II–POPCrHDL. From 0.5 to 8 h there is little change, but after 10 h further rearrangement occurs, producing intermediate-size and larger particles ranging from 78 Å to 105 Å. After 24 h at 37°C, about 50% of the

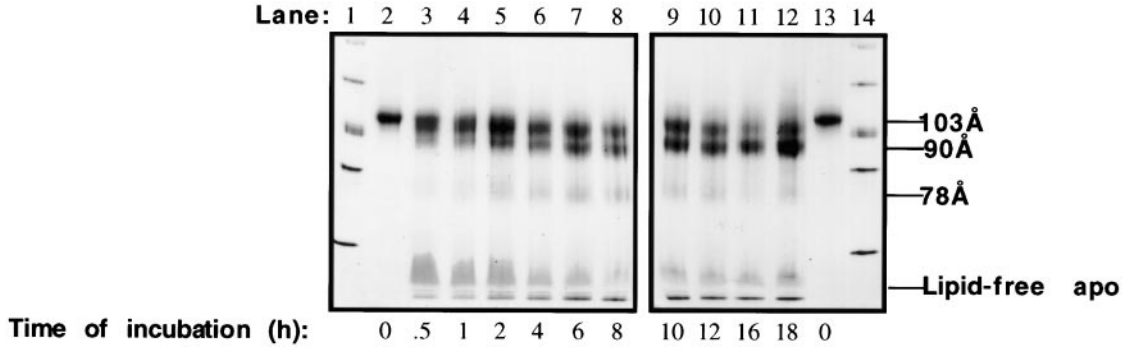

Fig. 2. ApoA-I added to A-II–POPCrHDL, time course. Coomassie-stained gels after non-denaturing GGE of A-II–POPCrHDL before and after addition of lipid-free apoA-I and incubation from 0.5 to 18 h at 37°C. Lipid-free apoA-I was added to the A-II–POPCrHDL in a molar ratio of 1:1, apolipoprotein:rHDL particles. The gels shown are representative of 4 experiments performed on three different rHDL preparations. Lanes 1 and 14: high molecular weight standards; lanes 2 and 13: initial A-II–POPC–rHDL without apoA-I; lanes 3–12: A-II–POPC–rHDL plus apoA-I incubated for the time indicated.

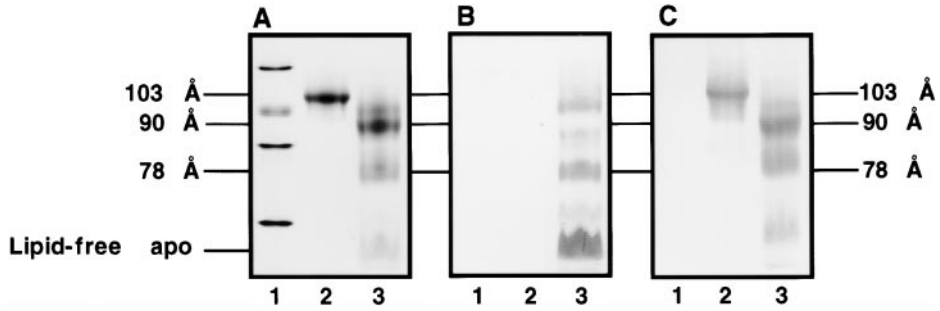

Fig. 3. ApoA-I added to A-II–POPCrHDL, 24 h. Coomassie-stained gel after non-denaturing GGE (panel A) followed by Western blotting with antibodies to apoA-I (panel B) and antibodies to apoA-II (panel C). A-II–POPCrHDL is shown before (lane 2) and after addition of lipid-free apoA-I in a molar ratio of 1:1, rHDL particles:apolipoprotein, and incubation for 24 h at 37°C (lane 3). To achieve optimal visualization of protein bands on the gel and blots, 50% more sample was loaded in lane 3 than in lane 2. The gel and blots shown are representative of 7 experiments performed on five different rHDL preparations. For all panels, lane 1: high molecular weight standards; lane 2: initial A-II–POPC–rHDL without apoA-I; lane 3: A-II–POPC–rHDL plus apoA-I.

initial 98 Å apoA-I–POPC–rHDL is remodeled into a series of smaller and larger complexes which include 78 Å and 88 Å particles (**Fig. 6, panel A**). Western blotting shows the presence of both apoA-I and apoA-II in the particles (Fig. 6, panels B and C). There is a significant amount of lipid-free apoA-I generated by the binding of apoA-II; some lipid-free apoA-II is also present.

Finally, we examined the effect of apoA-I on A-I–POPCrHDL in a 24-h incubation at 37°C, when added in a 1:1 molar ratio. **Figure 7** is a Coomassie-stained gel that shows the rearrangement of the initial 98 Å rHDL into distinct particles after the addition of lipid-free apoA-I. The 78 Å, 88 Å, and 105 Å particles are present that were formed upon the addition of apoA-II. There is also a band representing larger particles of 114 Å, as well as a considerable amount of lipid-poor or lipid-free apoA-I. **Table 1** summarizes the size rearrangements produced by the binding of the lipid-free apolipoproteins to POPCrHDL.

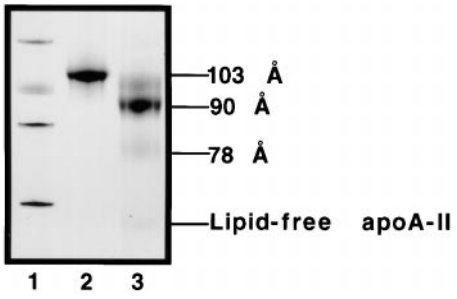

Fig. 4. ApoA-II added to A-II–POPCrHDL, 24 h. Coomassie-stained gel after non-denaturing GGE of A-II–POPCrHDL before and after addition of lipid-free apoA-II and incubation for 24 h at 37°C. Lipid-free apoA-II was added to A-II–POPCrHDL in a molar ratio of 1:1, apolipoprotein:rHDL particles. To achieve optimal visualization of protein bands on the gel, 50% more sample was loaded in lane 3 than in lane 2. The gel shown is representative of 4 experiments performed on three different rHDL preparations. Lane 1: high molecular weight standards; lane 2: initial A-II–POPC–rHDL without apoA-II; lane 3: A-II–POPC–rHDL plus apoA-II.

It is interesting to note that in incubations that contained both apoA-I and apoA-II (Fig. 3 and Fig. 6) the 78 Å products visualized by Western blotting contain apoA-I and apoA-II. In the experiments reported by Labeur et al. (18) addition of apoA-II to native HDL particles also produces smaller particles; however, after separation by 2-dimensional electrophoresis, the small, preβ-1-migrating particles contain only apoA-I. In contrast, Calabresi et al. (29) report that HDL_3 particles with reduced apoA-II, remodeled in the presence of intralipid and lipoprotein-depleted plasma, form small particles (75 Å in diameter). These particles contain both apoA-I and apoA-II and have α-mobility, in addition to the preβ-1-migrating species that only contain apoA-I. Thus, it is possible that certain small particles detected by gel filtration or GGE include apoA-II but have alpha rather than beta mobility on agarose gels.

Effect of lipid-free apolipoproteins on the reaction kinetics of LCAT

We evaluated the effect of lipid-free apoA-I or apoA-II on the reaction kinetics of LCAT with the various rHDL substrates. In each case, lipid-free apolipoprotein was added to A-II–POPCrHDL or A-I–POPCrHDL in a 1:1 molar ratio of apolipoprotein to rHDL particle, and incubated for 20 min at 37°C before the addition of LCAT. The reaction time in the presence of the enzyme was 30 min; therefore, some particle rearrangement (10–25%) could be expected. Based on the data shown above in Fig. 2 and Fig. 5, the remodeling of the rHDL begins within 30 min of adding lipid-free apolipoproteins, but the primary species at the 1 h time point is the intial rHDL particle with bound apolipoprotein. **Figure 8**, panel A, shows the effect of lipid-free apoA-I on the reaction of A-II–POPCrHDL with LCAT. The control A-II–POPC–rHDL is virtually unreactive with LCAT. However, upon the addition of lipid-free apoA-I there is a substantial increase in reactivity with LCAT. Even though this increase in reactivity is significant, the sample of apoA-I plus A-II–POPCrHDL is not as reactive as another control included in the experi-

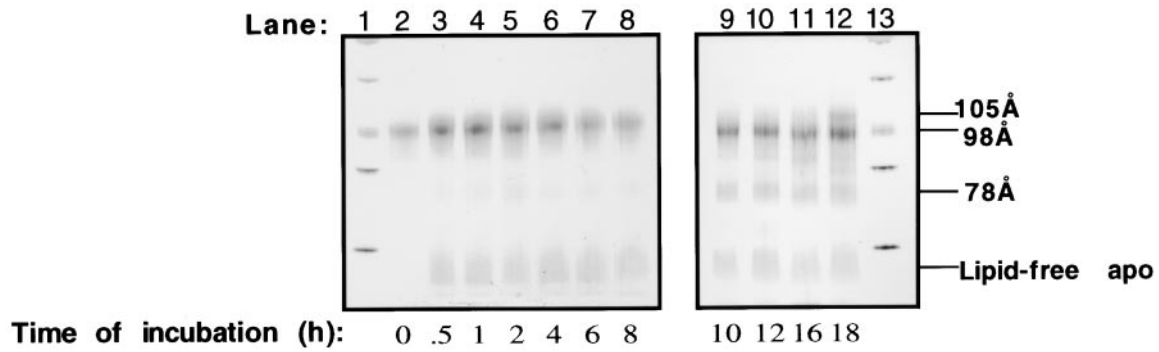

Fig. 5. ApoA-II added to A-I–POPCrHDL, time course. Coomassie-stained gels after non-denaturing GGE of A-I–POPCrHDL before and after addition of lipid-free apoA-II and incubation from 0.5 to 18 h at 37°C. Lipid-free apoA-II was added to A-I–POPCrHDL in a molar ratio of 1:1, apolipoprotein:rHDL particles. The gels shown are representative of 3 experiments performed on two rHDL preparations. Lanes 1 and 13: high molecular weight standards; lane 2: initial A-I–POPC–rHDL without apoA-II; lanes 3–12: A-I–POPC–rHDL plus apoA-II incubated for the time indicated.

ment, A-I–POPCrHDL (98 Å species), shown as the top curve in panel B. This result indicates that the activation that is seen in the presence of added apoA-I is different from the activation of LCAT that occurs when the 98 Å rHDL is prepared with apoA-I.

The effect of apoA-II on A-II–POPCrHDL reactivity with LCAT was also evaluated in a similar experiment. However, the addition of lipid-free apoA-II in a 1:1 molar ratio to A-II–POPCrHDL and LCAT produced velocities of CE conversion that were almost identical to that of A-II–POPCrHDL without added apoA-II. Unlike the activation seen by the addition of lipid-free apoA-I, adding lipid-free apoA-II neither enhanced nor inhibited the reactivity of A-II–POPCrHDL with LCAT, under the conditions used in these experiments. Consequently, these data are not shown in Fig. 8.

Table 2 summarizes the apparent kinetic constants for the reaction of apoA-I plus A-II–POPCrHDL with LCAT. The apparent V_{max} value which reflects the LCAT activation and catalytic steps of the reaction (30) is increased 4-fold in the presence of apoA-I. Furthermore, there is a 6-fold decrease in apparent K_m compared to A-II–POPCrHDL without apoA-I. In terms of relative reactivity, compared to the control A-I–POPCrHDL substrates (100%), the mixture of A-II–POPCrHDL plus apoA-I is only 17% as reactive, but it is 24-fold more reactive than A-II–POPCrHDL without apoA-I.

Next, we evaluated the effect of lipid-free apoA-II on A-I–POPCrHDL in its reaction with LCAT shown in Fig. 8, panel B. The kinetic constants for the reaction, listed in Table 2, indicate that there is no significant change in apparent V_{max} in the presence of apoA-II. However, there is a reduction in reactivity due to a 2-fold increase in apparent K_m, which reflects the enzyme-binding step of the reaction (30). In terms of relative reactivity, in the presence of apoA-II, A-I–POPCrHDL is only one-half as reactive with LCAT as A-I–POPCrHDL alone. These results agree with previous work done by Scanu, Lagocki, and Chung (31) who reported a decrease in LCAT activity with apoA-I-containing vesicles upon the addition of lipid-free apoA-II. The results of Labeur et al. (18) also show a modest inhibition of the reaction of A-I–PLPCrHDL with LCAT in

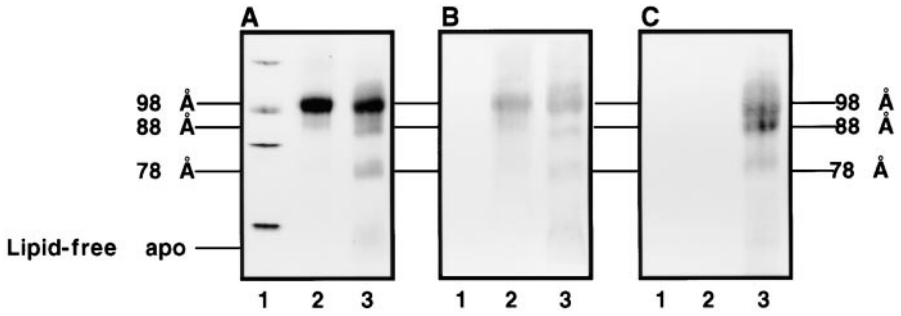

Fig. 6. ApoA-II added to A-I–POPCrHDL, 24 h. Coomassie-stained gel after non-denaturing GGE (panel A) followed by Western blotting with antibodies to apoA-I (panel B) and apoA-II (panel C). A-I–POPCrHDL is shown before (lane 2) and after addition of lipid-free apoA-II in a molar ratio of 1:1, rHDL particles:apolipoprotein, and incubation for 24 h at 37°C (lane 3). To achieve optimal visualization of protein bands on the gel, 50% more sample was loaded in lane 3 than in lane 2. The gel shown is representative of 7 experiments performed on six different rHDL preparations. For all panels, lane 1: high molecular weight standards; lane 2: initial A-I–POPC–rHDL without apoA-II; lane 3: A-I–POPC–rHDL plus apoA-II.

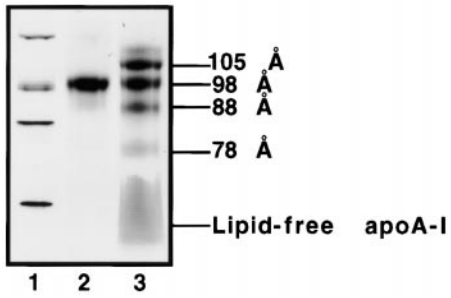

Fig. 7. ApoA-I added to A-I–POPCrHDL, 24 h. Coomassie-stained gel after non-denaturing GGE of A-I–POPCrHDL before and after addition of lipid-free apoA-I and incubation for 24 h at 37°C. Lipid-free apoA-I was added to A-I–POPCrHDL in a molar ratio of 1:1, apolipoprotein:rHDL particles. To achieve optimal visualization of protein bands on the gel, 50% more sample was loaded in lane 3 than in lane 2. The gel shown is representative of 6 experiments performed on five different rHDL preparations. Lane 1: high molecular weight standards; lane 2: initial A-I–POPC–rHDL without apoA-I; lane 3: A-I–POPC–rHDL plus apoA-I.

the presence of added apoA-II. However, their kinetic constants are affected differently, possibly because of the high apoA-II/apoA-I ratio and the major particle rearrangements.

Finally, we looked at the effect of lipid-free apoA-I on A-I–POPCrHDL with LCAT. From Fig. 8, panel B, it is apparent that there is a decrease in LCAT activity similar to that seen with the addition of apoA-II to A-I–POPCrHDL. From the apparent kinetic constants listed in Table 2, the presence of added apoA-I results in a slight decrease in apparent V_{max} and a slightly greater apparent K_m, producing an overall decrease in reactivity of approximately one-third, compared to that of A-I–POPCrHDL with LCAT without added apoA-I.

DISCUSSION

This work has demonstrated that lipid-free apoA-I and apoA-II bind rapidly to discoidal rHDL, promote the remodeling of the rHDL, and also affect their reactivity with LCAT. The main conclusions from this work are the following. *1*) Both lipid-free apoA-I and apoA-II bind to homogeneous rHDL rapidly, within 5 min. *2*) Binding of the lipid-free apolipoproteins results in significant rearrangements in rHDL particle sizes and distributions that begin within 30 min and continue for over 24 h. *3*) Lipid-free apolipoproteins added to rHDL affect their reactivity with LCAT. ApoA-I binding to A-II–POPCrHDL enhances their reaction dramatically, while apoA-I or apoA-II binding to A-I–POPCrHDL inhibits their reaction with LCAT.

TABLE 1. Size rearrangements of POPCrHDL that occurred upon incubation for 24 h at 37°C with lipid-free apoA-I or apoA-II added in a 1/1 molar ratio, rHDL/lipid-free apolipoprotein

Sample	Initial rHDL Size[a]	Added Apo[b]	Remodeled rHDL Size[a]
	Å		Å
A-II–POPCrHDL	103	apoA-I	98
		or	90
		apoA-II	78
A-I–POPCrHDL	98	apoA-I	105
		or	98
		apoA-II	88
			78

[a] The standard deviation in the calculated size of the rHDL is less than 2 Å.

[b] While the same sizes of remodeled rHDL were observed, whether apoA-I or apoA-II was added, the relative proportions of the remodeled rHDL varied.

Regarding the kinetics of apolipoprotein binding to rHDL particles, the observed rate of interaction is rapid, and equilibrium is reached within 5 min for both apoA-I and apoA-II. As it is widely known that apoA-II has a higher affinity for binding lipids than does apoA-I (32), and the on-rates appear to be very similar in the present experiments, then it follows that the desorption rate of apoA-II from the rHDL will be slower. A slower desorption rate of apoA-II is likely explained by the ability of its C-terminal helix to penetrate more deeply into the lipid bilayer (33) than the amphipathic helices of apoA-I. Thus, the higher affinity of apoA-II for lipids and its slower desorption rate underlie the ability of apoA-II to displace apoA-I from HDL and rHDL particles (33). The physiologic significance of our observation that both major apolipoproteins of HDL bind rapidly to rHDL particles is that apoA-I and apoA-II probably do not persist in free form in circulation, rather they adsorb rapidly to HDL lipid surfaces that are present in large excess. Initially the adsorption of apoA-I would be readily reversible, but subsequent particle remodeling would change the nature of the interactions of apoA-I with lipid and decrease the proportion of weakly bound apoA-I.

Our experiments on the remodeling of rHDL particles by added apoA-I or apoA-II demonstrate that HDL rearrangements can occur even without the action of the plasma proteins (CETP, PLTP) and enzymes (LCAT, HL) known to promote the remodeling of HDL (4–8). It suffices to change the proportion of total apolipoprotein to lipid in these systems, initiating particle remodeling and shifting the systems to a new equilibrium state. Some of the particle rearrangements can occur within 30 min, while other changes continue beyond 24 h. It appears that the particle remodeling rates are strongly dependent on the temperature and the nature of the rHDL phospholipid. Indeed, at 4°C particle remodeling is not detectable over 24 h (D. Durbin and A. Jonas, unpublished results), and A-I–DPPCrHDL particles exposed to apoA-II do not rearrange under the same conditions as do A-I–POPCrHDL particles (19). Clearly the nature of the added apolipoprotein also affects the remodeling kinetics and the properties of the resulting particles as observed in this study.

In general, the increase in the apolipoprotein content (30 and 41% increases) over the phospholipid present in these experimental systems should lead to the formation of smaller particles. This is the case for the experiment depicted in Figs. 2, 3, and 4 where the initial 103 Å rHDL particles give rise to 78 Å and 90 Å species. The 90 Å parti-

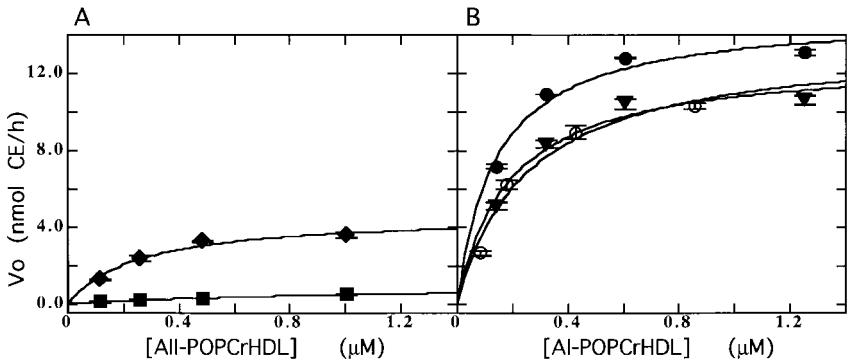

Fig. 8. Effect of lipid-free apoA-I or apoA-II on the reactivity of POPCrHDL with LCAT. Initial velocity (nmol CE formed per h) versus concentration of POPCrHDL substrate is shown for the LCAT reaction in the presence of added lipid-free apoA-I, apoA-II, or buffer. The data points represent the mean of triplicate samples; the error bars represent the standard deviation. Panel A: lipid-free apoA-I (◆) or buffer (■) was added to A-II–POPCrHDL to give a final molar ratio of 1:1, lipid-free apos:rHDL particles, and incubated at 37°C for 20 min before the addition of LCAT. The reaction was stopped after 30 min. The data obtained for the addition of lipid-free apoA-II to A-II–POPCrHDL was not significantly different from the addition of buffer to A-II–POPCrHDL and LCAT and is not shown. Panel B: lipid-free apoA-I (▼) or apoA-II (○) or buffer (●) was added to apoA-I–POPC–rHDL and reacted with LCAT as described for panel A.

cles contain only apoA-II and probably arise from the original particles by the removal of lipid by apoA-I or apoA-II and the subsequent loss of a molecule of lipid-bound apoA-II. The released components form the 78 Å and other minor complexes and the lipid-free fraction that contains both apolipoproteins. We have demonstrated by crosslinking experiments that the "lipid-free" apolipoproteins observed by GGE are in fact weakly bound to various complexes in solution, but are stripped during electrophoresis (D. Durbin and A. Jonas, unpublished results). In the experiment shown in Figs. 5, 6, and 7 the initial 98 Å A-I–POPCrHDL exposed to apoA-I or apoA-II form smaller particles, as expected, but also give rise to larger species. This can be explained by the rearrangement of 98 Å A-I–POPCrHDL into smaller and larger particles when phospholipid is depleted by various means (17). In fact, we showed in previous work (17) that particles similar to the 105 Å species contain three apoA-I molecules and a lower proportion of phospholipid. They result from the fusion and disproportionation of particles containing two apoA-I that are destabilized due to loss of phospholipid.

Whether or not HDL rearrangements induced by "free" or, rather, weakly lipid-bound apolipoproteins are significant in vivo will depend on the relative rates of other HDL remodeling processes. Nevertheless, the present experiments emphasize the fact that any changes in HDL component proportions and levels will be accompanied by HDL remodeling.

The LCAT kinetic experiments reported here show that binding of apoA-I or apoA-II to rHDL particles can have opposite effects on their reactions with LCAT: apoA-I stimulates the reaction of A-II–POPCrHDL 24-fold, while either apoA-I or apoA-II inhibit the reaction of A-I–POPCrHDL. These effects can be explained by the modulation of LCAT binding to the lipid surface of the rHDL and its activation by apoA-I. Binding of apoA-I to the essentially unreactive A-II–POPCrHDL particles partially activates LCAT. The activation does not reach the level of the A-I–POPCrHDL control because the binding of apoA-I to the lipid is quite different in both systems. The added apoA-I initially binds to the lipid surface of the rHDL particles and probably adopts a conformation distinct from apoA-I

TABLE 2. Apparent kinetic constants for the reaction of LCAT with POPCrHDL prepared with either apoA-I or apoA-II

Sample	V_{max}	K_m	V_{max}/K_m	Relative Reactivity
	nmol CE/h	*μM*	*nmol CE/h·μM*	*%*
A-I–POPCrHDL	15.1 ± 0.9	0.14 ± 0.03	108	100
A-I–POPCrHDL + apoA-I	12.8 ± 1.1	0.19 ± 0.05	67	62
A-I–POPCrHDL + apoA-II	13.7 ± 1.7	0.25 ± 0.08	55	51
A-II–POPCrHDL	1.16 ± 0.14	1.5 ± 0.3	0.77	0.7
A-II–POPCrHDL + apoA-I	4.65 ± 0.44	0.26 ± 0.07	18	17

The reactivity of the rHDL with LCAT was evaluated in the presence of additional lipid-free apoA-I, apoA-II, or buffer. The initial velocity (nmol of CE/h) versus rHDL concentration was plotted from data representing the mean of triplicate samples. The apparent kinetic constants were derived from a hyperbolic curve fit to the data ± the error in curve fitting, as described in Methods. For ease of comparison, the apparent kinetic constants were normalized to that of a sample of A-I–POPCrHDL with LCAT, which was representative of at least five different experiments.

tightly bound to the edge of the disc in A-I–POPCrHDL, the optimal conformation for LCAT activation. The fact that added lipid-free apoA-I inhibited the reactivity of A-I–POPCrHDL with LCAT supports this statement. The presence of apoA-I on A-II–POPCrHDL may increase its reactivity with LCAT by interacting with the enzyme, decreasing its rate of dissociation from the A-IIrHDL surface and allowing longer time for the reaction to proceed. We have recently observed that the dissociation rate of LCAT from A-II–rHDL is about 5-fold faster than from A-IrHDL, which is likely due to protein–protein interactions between apoA-I and LCAT (L. Jin, J. Shieh, E. Grabbe, S. Adimoolam, D. Durbin, and A. Jonas, unpublished results). These interactions do not occur between apoA-II and LCAT, which may explain why there was neither activation nor inhibition of A-II–POPCrHDL in the presence of added apoA-II. In any case, we have shown that the binding of apoA-I to A-IIrHDL can enhance its reactivity with LCAT. If apoA-II does recruit excess cellular phospholipid and cholesterol in vivo in the initial step of reverse cholesterol transport, the subsequent binding of apoA-I and LCAT could facilitate the maturation of the particle into spherical mature LpA-I/A-II HDL. The work of Ikewaki et al. (28) suggests that the plasma levels of apoA-I in LpA-I/A-II correlate with the production of apoA-II. It is reasonable that there could exist a regulatory mechanism in place for coordinating the production and maturation of this HDL particle.

Addition of apoA-II to A-I–POPCrHDL inhibits the reaction with LCAT about 2-fold. The effect is due to an increase in the apparent K_m of the reaction without significant change in apparent V_{max}. We have shown that the binding of apoA-II destabilizes, without displacing, one of the two molecules of apoA-I on DPPCrHDL (19). If a similar situation occurs with apoA-II binding to A-I–POPCrHDL, destabilization of apoA-I may prevent it from making optimal contact with LCAT. Activation of LCAT could still occur by the remaining lipid-bound apoA-I, but overall reactivity would be reduced by half, as seen in our experiments. In the case of the addition of apoA-I to A-I–POPCrHDL, the observed inhibition may be due to the lipid-free apoA-I competing with the enzyme for the rHDL surface. Alternatively, some rearrangement of the rHDL into 78 Å particles occurs during the course of the LCAT reaction and these particles are known to be very unreactive with LCAT compared to 98 Å controls (34). Both of these effects probably determine the level of activity in this system.

Thus, lipid-free apolipoproteins by their interaction with nascent and mature HDL particles can markedly influence their reaction with LCAT in one of the key steps in reverse cholesterol transport. Furthermore, lipid-free apolipoproteins promote the remodeling of HDL to smaller particles, some of which have preβ-1 mobility and are expected to be very effective acceptors of cholesterol in reverse cholesterol transport.

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