Apolipoprotein A-I (apoA-I) activates the plasma enzyme lecithin:cholesterol acyltransferase (LCAT), catalyzing the rapid conversion of lipoprotein cholesterol to cholesterol ester. Structural mutants of apoA-I have been used to study the details of apoA-I-LCAT-catalyzed cholesterol ester formation. Several studies have shown that the α-helical segments corresponding to amino acids 143–164 and 165–186 (repeats 6 and 7) are essential for LCAT activation. In the present studies, we examined how the orientation of the hydrophobic face, independent of an increase in overall hydrophobicity, affects LCAT activation. We designed, expressed, and characterized a mutant, reverse of 6 apoA-I (RO6 apoA-I), in which the primary amino acid sequence of repeat 6 was reversed from its normal orientation. This mutation rotates the hydrophobic face of repeat 6 approximately 80°. Lipid-free RO6 apoA-I showed a marked stabilization when denatured by guanidine hydrochloride, but showed significant destabilization to guanidine hydrochloride denaturation in the lipid-bound state compared with wild-type apoA-I. Recombinant high density lipoprotein discs (rHDL) formed from RO6 apoA-I, sn-1-palmitoyl-sn-2-oleoyl phosphatidylcholine, and cholesterol were approximately 12 Å smaller than wild-type apoA-I rHDL. The reduced size suggests that one of the repeats did not effectively participate in phospholipid binding and organization. The sn-1-palmitoyl-sn-2-oleoyl phosphatidylcholine RO6 rHDL were a less effective substrate for LCAT. Mapping the entire lipid-free and lipid-bound RO6 apoA-I with a series of monoclonal antibodies revealed that both the lipid-free and lipid-bound RO6 apoA-I displayed altered or absent epitopes in domains within and adjacent to repeat 6. Together, these results suggest that the proper alignment and orientation of the hydrophobic face of repeat 6 is an important determinant for maintaining and stabilizing helix-bilayer and helix-helix interactions.

Apolipoprotein A-I (apoA-I)3 organizes phospholipid and cholesterol for use in the conversion of cholesterol to cholesterol ester by the plasma enzyme, lecithin:cholesterol acyltransferase (LCAT) (1–4). A number of investigations have focused on identifying the precise molecular mechanism by which apolipoprotein A-I activates LCAT. Studies of apoA-I deletion mutants have shown that the α-helical amino acid domains, 143–164 and 165–186 (repeats 6 and 7), were essential for LCAT activation and when removed from the primary sequence, severely disable (>95%) the ability of the protein to activate LCAT (5–8). Other investigations using monoclonal antibodies to inhibit cholesterol esterification (9–12) or mimic peptides to delineate key structural domains have suggested that other apoA-I regions, e.g. amino acids 99–120, may participate in the co-activation process (13). It still, however, remains unclear which domain(s) are most important in LCAT activation and the mechanism(s) by which they activate the enzyme.

Arranging all 10 apoA-I 11- or 22-mer repeats on Edmunson helical wheel diagrams clearly demonstrates the partitioning of the hydrophilic and hydrophobic amino acids onto opposite faces of the helical wheel, thus illustrating their amphipathic character (14–16). Based on this property, it has been suggested that each apoA-I repeat cooperatively participates in organizing phospholipid into a discoidal complex (rHDL) through the alignment of their hydrophobic faces parallel to the phospholipid acyl chains. It has been proposed that the penetration depth of each 22-mer amphipathic domain correlates to its total hydrophobicity, i.e. to its overall hydrophobic amino acid composition (16). However, only recently has it become apparent that an interrelationship exists between the hydrophobic amino acid distribution and the ability of an amphipathic α-helix repeat to activate LCAT (17).

By examining the role of the hydrophobic residues within repeat 6 we have been able to refine our understanding of how apoA-I activates LCAT. Previous studies showed that substitution of apoA-I’s repeat 10 for repeat 6 (10F6 apoA-I), significantly increased apoA-I rHDL’s helix-bilayer stability to denaturation without altering global secondary conformation (17). The increase in apoprotein-phospholipid bilayer stability was accompanied by a substantial reduction in the ability of 10F6 apoA-I rHDL to activate LCAT. We proposed that the increase in helix-bilayer and helix-helix interactions that result from the repeat 10 for 6 substitution cause the 121–165 domain (repeats 5 and 6) to penetrate more deeply into the lipoprotein disc (17).

This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Printed in U.S.A.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 273, No. 19, Issue of May 8, pp. 11776–11782, 1998

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rHDL phospholipid bilayer, thereby restricting LCAT's access to the boundary phospholipid acyl chains and reducing the overall catalytic efficiency (17).

In the present study we constructed a mutant apoA-I (RO6 apoA-I) having the hydrophobic face of repeat 6 rotated 80° with respect to wild-type apoA-I, and then characterized the ability of this mutant to bind lipid and activate LCAT. Discoidal rHDL containing RO6 apoA-I were significantly smaller in diameter, and exhibited a 5-fold lower ability to activate LCAT than wild-type apoA-I rHDL. Monoclonal antibody epitope mapping was used to analyze conformational changes. This technique demonstrated altered or absent epitopes spanning the domains adjacent to repeat 6 in both the lipid-free and lipid-bound RO6 apoA-I. These studies confirm the idea that the optimal alignment and orientation of repeat 6's hydrophobic face are important determinants in maintaining and stabilizing apoA-I helix-phospholipid bilayer interactions and that these interactions are key structural features regulating LCAT activation.

**EXPERIMENTAL PROCEDURES**

High performance liquid chromatography-grade organic solvents were from Fisher Scientific. All other chemical reagents were purchased from Sigma unless otherwise noted. [3H]-Cholesterol was from NEN Life Science Products Inc. Tissue culture reagents, restriction endonucleases, and other DNA modifying enzymes were from Life Technologies, Inc. Design and Expression of Wild-type and Mutant pBlueBac III:ApoA-I Constructs—Wild-type, ∆6 apoA-I (lacking residues 143–164), ∆6/6 apoA-I (lacking residues 121–164), and 10F6 apoA-I (replacement of repeat 6 for repeat 6) cDNA:pBlueBac III constructs were used in conjuction with the Autographa californica nuclear polyhedrosis virus linear viral DNA for co-transfection into Sf9 cells, as described previously (17, 18). The apoA-I mutant, called RO6 apoA-I, contains the reverse or inverted primary sequence of repeat 6 at residues 143–164 and was cloned into the Life Technologies, Inc. pFastBac1 expression vector. The RO6 apoA-I bacmid vector was prepared using the wild-type apoA-I pBlue BaciIII construct as template, our standard 3’ most apoA-I cDNA primer (17, 18) and the RO6 mutagenic primer which reverses the orientation of residues 143–164 or repeat 6. The RO6 mutagenic primer sequence: 5’-CTCGTCGTCGCTGTAGGGCAGGCCCTCCTCC-ATGCCGGTCGCCGCGCCGATCAGCTGCAGCCGCGCCTGATG- CAGGGTGGGCTGACCTGCTCT-3’. Following the first round of polymerase chain reaction the fragment which contained the desired mutation was used to generate full-length RO6 apoA-I using our standard 3’ apoA-I cDNA primer (17, 18). Transformation and selection were carried out according to standard protocols (19). The cDNAs were then subcloned into a vector containing the eukaryotic expression vector. Constructs were then transfected into Sf9 cells and plaque assays performed to determine viral titer, as described previously (17, 18). To prevent degradation of the expressed protein, pepstatin A and leupeptin (at final concentrations of 700 and 500 μg/liter, respectively) were added to the culture medium 36 h post-infection (17, 18).

Preparation, Purification, and Characterization of Recombinant Wild-type and Mutant Forms of ApoA-I from Baculovirus-infected Sf9 Cells—Preparation and purification of recombinant wild-type and 10F6 apoA-I protein were carried out as previously reported (17, 18). The final purified protein was dissolved in 10 mM ammonium bicarbonate, pH 7.4, and its concentration determined by the method of Lowry (20). The molecular weights of the proteins were confirmed by electrospray mass spectrometry on a Quattro II mass spectrometer. Preparation of rHDL Containing Wild-type and Mutant Forms of ApoA-I—A molar ratio of 80:4:1, POPC:cholesterol:apoA-I was used for making rHDL essentially as described for the preparation of l-a-dimyristoylphosphatidylcholine (DMPC) wild-type and 10F6 apoA-I rHDL at a starting molar ratio of 100:5:1 (17, 18). Briefly, 1.9 mg of POPC in chloroform (30 mg/ml) was added to 48 μg of cholesterol dissolved in ethanol (10 mg/ml) and 10 μl (10 μCi) of radiolabeled [1,2-3H]cholesterol (50 Ci/mmol) in ethanol. Organic solvents were removed under a stream of argon and the tubes were placed under vacuum for 30 min. Sodium cholate, 2.1 mg, was added, the solution vortexed and then incubated for 30 min at 27 °C. The mixture was briefly vortexed 3 times during the 10-min incubation. To this mixture, 0.9 mg of wild-type or mutant apoA-I was added and the incubation continued for an additional 20 min at 4 °C. Sodium cholate was removed by dialysis against 10 mM Tris, pH 7.4, 140 mM NaCl, 0.25 mM EDTA, and 0.15 mM sodium azide at 27 °C. The rHDL were then purified by passage through a Superose 12 (Pharmacia) column (55 × 1.8 cm) at a flow rate of 1 ml/min. Phospholipid, cholesterol, and protein contents were determined by the methods of the rHDL (17, 18). A final concentration of 1% SDS was used in the Lowry method for protein determination of rHDL complexes to prevent solution turbidity. The number of molecules of mutant apoA-I per substrate particle was determined by apoprotein cross-linking with dimethyl suberimidate as described by Swanne (21) followed by SDS–4–30% polyacrylamide gels for electrophoresis.

Non-denaturing Gradient Gel Electrophoresis—DMPC and POPC rHDL sizes were determined using 4–30% non-denaturing gradient gel electrophoresis (22), as described previously (17, 18). rHDL particle size was determined by comparison to protein standards of known Stokes’ diameter (23).

Circular Dichroism Spectroscopy and Guanidine Hydrochloride Denaturation Studies—Circular dichroism spectra were recorded with a Jasco J720 spectropolarimeter at 25 °C using a 0.1-cm path length cell. Ellipticity was measured at 222 nm. Five scans were recorded, averaged, and the background subtracted. Mean molar residue ellipticity (q) is reported as degrees cm²dmol⁻¹, and calculated from the equation, $q_m = \frac{1150}{l_c} - c$, where $q_m$ is the observed ellipticity at 222 nm in deg cm²dmol⁻¹, $l_c$ is the mean residue molecular weight of the protein, $c$ is the optical path length in centimeters, and $c$ the protein concentration in g/ml. The percent α-helix content was calculated from the formula of Chen et al. (24) $\% a-helix = \left(\frac{3000f_{\alpha}}{2.340}\right) - 2.340$. Stabilities of lipid-free and lipid-bound POPC rHDL were determined by plotting the mean residue ellipticity versus guanidine hydrochloride concentration and expressed as the concentration of GdnHCl that reduced the ellipticity by 50% (D₀).

Epitope Mapping of Lipid-free and Lipid-bound RO6 ApoA-I—Competitive solid-phase immunoassays were used to assess the binding of monoclonal antibodies to lipid-free apoA-I and POPC rHDL containing apoA-I. With two exceptions (antibodies AI-17 and AI-141.7) each antibody used in this study has been described and their epitopes on wild-type apoA-I documented (25). The epitopes of antibodies AI-17 and AI-147.7 were localized to residues 143–165 and 220–242, respectively, on the basis of their binding to apoA-I mutant proteins. The immunoassays were performed as described (17). Briefly, isolated human plasma apoA-I or plasma HDL (0.05 ml of 5 μg/ml) were immobilized onto 96-well Falcon 3911 Microtest III flexible assay plates. After post-coating the plates, increasing amounts of a purified apoA-I or recombinant antibody was added to wells containing 0.025 ml of phosphate-buffered saline (PBS) containing 3% normal goat serum were added to wells in the presence of 0.025 ml of aspartic acid containing a limiting amount of monoclonal antibody (typically dilutions of 1 × 10⁻⁴ to 1 × 10⁻⁴). Competitive concentrations listed in the figures represent the final concentrations (μg/ml) in the 0.05-ml reaction mixture. The plates were incubated overnight at 4 °C. After washing the wells, mouse antibody binding to the immobilized antigen was detected by a second 1-h incubation at 37 °C with 125I-labeled goat anti-mouse IgG. All data were expressed as B/B₀, where B₀ is the counts/min bound in the presence of competitor, and B is the counts/min bound in the absence of competitor. To compare the affinity of the antibodies for POPC rHDL containing wild-type or mutant apoA-I as competitor, the slopes of logit-transformed B/B₀ ratios were determined by linear regression and subjected to tests of equality.

**LCAT Reaction Kinetics of POPC rHDL Containing Wild-type and Mutant ApoA-I**—The LCAT reaction was monitored by following the cholesterol to cholesterol ester conversion using POPC rHDL containing either wild-type or mutant apoA-I. The complexes were assayed in duplicate using 0–1.0 μg of substrate cholesterol in a final concentration of 10 mM Tris, pH 7.4, 140 mM NaCl, 0.25 mM EDTA, and 0.15 mM sodium azide, 0.6% fatty acid-free bovine serum albumin, 2 mM β-mercaptoethanol, and 25 ng of purified human plasma LCAT (26). The reactions were carried out for 15–60 min at 37 °C, and the conversion of [3H]cholesterol to [3H]cholesterol ester was determined by lipid extraction of the incubation mixture followed by thin layer chromatography. The extent of cholesterol esterification was kept below 15% to maintain fidelity of LCAT and to prevent solution turbidity. The number of molecules of mutant apoA-I per substrate particle was determined by apoprotein cross-linking with dimethyl suberimidate as described by Swanne (21) followed by SDS–4–30% polyacrylamide gel electrophoresis.
RESULTS

Edmunson helical wheel diagrams of repeat 6, shown in Fig. 1, for wild-type and RO6 apoA-I show that the hydrophobic and hydrophilic face orientations differ by approximately 80°. We hypothesized that the rotation of the hydrophobic face relative to the adjacent helical domains would disrupt the protein-lipid stabilization normally present in wild-type apoA-I. Potential stabilizing apoprotein helix-phospholipid bilayer interactions affected by the rotation include hydrophobic interactions between the hydrophobic face of repeat 6 residues and the phospholipid bilayer as well as hydrophobic and hydrophilic interactions between repeat 6 and adjacent repeats 5 and 7.

To characterize the properties of RO6 apoA-I we prepared rHDL containing either DMPC or POPC at a 100:5:1 or 80:4:1, phospholipid:cholesterol:apoprotein molar ratio, respectively. As shown in Fig. 2, nondenaturating gradient gel electrophoresis showed that the column purified DMPC or POPC RO6 apoA-I rHDL were approximately 8–11 Å smaller in diameter than DMPC or POPC wild-type apoA-I rHDL. The final molar compositions of both DMPC and POPC RO6 apoA-I rHDL are listed along with their approximate particle diameters in Table I. Recombinant HDL composed of Δ6 apoA-I, a mutant apoA-I having the complete deletion of repeat 6 (amino acids 143–164), formed complexes of the same size as the RO6 apoA-I, Table I. The DMPC or POPC rHDL made with Δ5/6 (complete deletion of repeats 5 and 6, amino acids 121–164) were approximately 14–20 Å smaller than those made with wild-type apoA-I.

The denaturation of lipid-free and lipid-bound RO6 apoA-I by GdnHCl showed that lipid-free RO6 apoA-I had a significantly higher $D_{1/2} = 1.39 ± 0.05$ Å, than lipid-free wild-type apoA-I, $D_{1/2} = 1.02 ± 0.05$ Å ($n = 3$, $p < 0.05$), suggesting that repeat 6 of RO6 apoA-I is more stabilized by inter-helical interactions than lipid-free wild-type apoA-I. Mutant lipid-free Δ6 and 10F6 apoA-I had $D_{1/2}$ values of 1.30 ± 0.10 and 1.46 ± 0.09 Å, respectively, similar to RO6 apoA-I, whereas, lipid-free Δ5/6 apoA-I had a $D_{1/2} = 1.00 ± 0.06$ Å, that was similar to wild-type apoA-I (Table II). These results suggest that the greater degree of apoprotein inter-helical association in 10F6, RO6, and Δ6 apoA-I results from a conformation adopted to minimize hydrophobic residue exposure to the polar aqueous environment. In agreement with other reports (27, 28), no significant difference in α-helix content was seen among the various lipid-free mutant apoproteins compared with wild-type apoA-I, since most of the α-helical structure has been shown to reside in the N-terminal half of the molecule.

In contrast to the $D_{1/2}$ observed for lipid-free apoA-I, POPC RO6 apoA-I rHDL had a significantly lower $D_{1/2} (2.30 ± 0.08$ Å, $p < 0.05$), compared with POPC wild-type apoA-I rHDL. $D_{1/2}$ was also found to be significantly different from POPC wild-type apoA-I rHDL with $D_{1/2} = 2.35 ± 0.07$. In contrast, 10F6 apoA-I rHDL had a significantly higher $D_{1/2} (3.05 ± 0.14$ Å, $p < 0.05$), compared with wild-type apoA-I rHDL, indicating a more stable association between apoprotein and the lipid bilayer. The 10F6 apoA-I substitution mutation was also shown to have a significantly higher $D_{1/2}$ when compared with wild-type apoA-I complexed to DMPC (17). As noted for the lipid-free apoprotein state, there were no significant differences in the α-helix content of POPC mutant apoA-I containing rHDL and POPC wild-type apoA-I rHDL.

The ability of the POPC RO6 apoA-I rHDL to activate LCAT was compared with POPC wild-type apoA-I rHDL. As shown in Fig. 3 and Table III, POPC RO6 apoA-I rHDL and POPC Δ5/6 apoA-I rHDL had similar rates of cholesterol esterification and showed a 4–5-fold lower rate (nmol/h ml of LCAT) than POPC wild-type apoA-I rHDL. In contrast, POPC Δ6 apoA-I rHDL and POPC 10F6 apoA-I had even lower rates of cholesterol esterification compared with wild-type (approximately 7-fold reduction). Table III shows the apparent kinetic constants of the LCAT reaction for all of the POPC mutant apoA-I containing rHDL complexes. Interestingly, no differences were seen
The conformational changes caused by the association of RO6 apoA-I with lipid were also studied using the same 13 antibodies that identify epitopes between residues 137 to 147 and 178 to 187 that were found to bind very poorly. As shown in Fig. 4, the binding ratios that indicate that these epitopes were expressed in both apoproteins in a similar manner and suggest that there were fewer minimal differences in the extent of epitope expression between the two apoproteins. In contrast, two antibodies that identify epitopes between residues 137 to 147 and 178 to 200 on wild-type apoA-I either did not bind to RO6 apoA-I or bound very poorly, as shown in Fig. 4, panels A and C, respectively.

To probe the conformational changes caused by the RO6 apoA-I mutation, we carried out epitope mapping studies of lipid-free and lipid-bound RO6 apoA-I. First, the binding capacity of each antibody for lipid-free and lipid-bound RO6 and wild-type apoA-I were compared using competitive immunooassays (17, 25). Of the 13 antibodies tested, eight N-terminal epitopes between residues 1 and 137, one within the 143 to 165 residue region, and two antibodies that identify C-terminal epitopes between residues 187 and 243 were found to bind well to both apoproteins (data not shown). These antibodies had binding ratios that indicate that these epitopes were expressed in both apoproteins in a similar manner and suggest that there were only minimal differences in the extent of epitope expression between the two apoproteins.

The starting molar ratio of POPC:cholesterol:apoA-I was 80:4:1. Each rHDL preparation was found to contain 2 molecules of protein per particle, determined by cross-linking with bis(sulfosuccinimidyl) suberate and then used in the LCAT assays. Each assay contained 20 ng of purified human LCAT. Wild-type apoA-I rHDL (contains substitution of repeat 10 or residues 220–241 for repeat 6 or residues 143–164) (17); RO6 apoA-I rHDL (contains inversion of residues 143–164) (25); 5/6 apoA-I rHDL (contains deletion of residues 121–164) (25); 6 apoA-I rHDL (contains deletion of residues 143–164) (17); 10F6 apoA-I (contains substitution of repeat 10 or residues 220–241 for repeat 6 or residues 143–164) (17). All points represent the average of three individual experiments of duplicate determinations for each cholesterol concentration.

The apparent kinetic parameters were determined from Hanes-Woolf plots of reaction velocity and cholesterol concentration as described under “Experimental Procedures.” All values represent the mean ± S.D. of three independent measurements.
Fig. 4. Binding of monoclonal antibodies to lipid-free wild-type and RO6 apoA-I or POPC rHDL containing these apoproteins. Antibodies are indicated on top of each panel with their epitope assignment in parentheses. Panel A corresponds to binding curves obtained with the antibody A1–137.1 (amino acids 137–147), panel B with A1–17 (amino acids 143–165), and panel C with A1–178.1 (amino acids 178–200). Microtiter plates were coated with either pure plasma derived apoA-I or plasma HDL at 5 μg/ml and incubated with a predetermined limiting amount of each antibody in the presence of increasing amounts of either wild-type (○) or RO6 (●) apoA-I pure protein (left panel) or POPC rHDL (right panel) as described under “Experimental Procedures.” To compare the affinities of each antibody for the two complexes, the $B/Bo$ binding ratios were subjected to logit transformation where logit ($y$) = $\ln(y/(1-y))$ and $y = B/Bo$. The slopes were calculated by linear regression analysis of the logit-transformed data and were subjected to tests of equality. Similar slopes indicated similar affinities of the antibodies for wild-type and RO6 apoA-I. The displacement of the RO6 curve to the right of the wild-type curve observed with the 178.1 antibody indicates that the antibody had comparable affinity but reduced binding capacity for RO6 apoA-I.

and lipid-bound RO6 apoA-I (Fig. 4, panel A, right), showed a complete absence of the epitope corresponding to residues 137 to 147 (junction of repeats 5 and 6). Lipid-bound RO6 apoA-I showed a reduced binding capacity to the epitope corresponding to residues 178 to 200 (repeats 7 and 8) compared with wild-type apoA-I. However, antibody A1–17 corresponding to residues 143–165 (repeat 6) bound equally well to lipid-bound RO6 and wild-type apoA-I, suggesting that although the repeat 6 sequence of RO6 apoA-I had been inverted, its wild-type epitope conformation had been maintained.

DISCUSSION

In this study we describe the biophysical and biochemical properties of a mutant form of apoA-I, RO6, in which the hydrophobic and hydrophilic faces of repeat 6 (amino acids 143–164) have been rotated approximately 80° from their native position in wild-type apoA-I repeat 6. These studies support an earlier hypothesis which suggested the role of apoA-I in LCAT activation was to disrupt the water-bilayer interface and expose the buried substrate for LCAT (29, 30). The present study extends this idea and demonstrates that both the orientation and amino acid composition of the hydrophobic face of repeat 6 and thus, its interaction with the phospholipid bilayer are the major structural features regulating interfacial LCAT activation and catalysis (16, 17).

Previous studies have clearly demonstrated that the region corresponding to residues 121–186 was involved in LCAT activation (5–8, 17, 31). Investigations designed to pinpoint structural features of this region that activate LCAT have demonstrated the importance of the hydrophobic face (17). Those studies strongly suggest that the overall hydrophobicity of the repeat 6 22-mer can modulate LCAT activation without altering the overall substrate size and composition (17). POPC 10F6 apoA-I rHDL showed a 17-fold reduction in catalytic efficiency as compared with POPC wild-type apoA-I rHDL. In either DMPC (17) or POPC rHDL 10F6 apoA-I had a significantly higher $D_{1/2}$ than the respective wild-type apoA-I rHDL (Table II). Combined, these results strongly suggest that the greater fractional hydrophobic area occupying the helix face coupled with a higher hydrophobicity per residue content for the substituted repeat 6 (repeat 10) resulted in a tighter association between the hydrophobic face and the phospholipid bilayer, thus potentially restricting interaction between LCAT and bilayer acyl chains. Additional support for the apoA-I hydrophobic face-bilayer stabilization comes from NMR studies of a peptide activator of LCAT in which the hydrophobic interactions between nonpolar amino acid side chains and lipid ap-
peared to be the most important factor in stabilizing the apoprotein-lipid complex (32). Furthermore, NMR analysis of a 166–185 residue apoA-I fragment bound to micelles suggested that helix-helix interactions were not common and may not be important stabilization factors. This is consistent with the suggestion that cationic side chains located between the polar and nonpolar faces of adjacent helices would likely cause repulsive interactions if tightly spaced (33).

The RO6 apoA-I studies support the hydrophobic face-LCAT activation hypothesis since displacement of the hydrophobic and hydrophilic faces of repeat 6 functionally prevent this domain from independently interacting with the substrate phospholipid bilayer without rotation of the helix. The diameters and final molar compositions of either DMPC or POPC RO6 apoA-I rHDL were shown to be similar to the respective Δ6 apoA-I rHDL, which lacks repeat 6 entirely (Table I), suggesting that one α-helix per molecule of apoA-I has been removed from the periphery of the rHDL. In addition, GdnHCl denaturation $D_{50}$ values were found to be similar for both RO6 and Δ6 apoA-I in both lipid-free and lipid-bound states (Table II). Our interpretation of these data are supported by the work of Jonas and colleagues (34–37) which demonstrated that rHDL containing 2 molecules of apoA-I per particle may vary in diameter as a function of the number of α-helical segments per apoA-I molecule in contact with lipid. Using the area taken up by an α-helix (i.e. approximately 12 Å) rHDL with diameters of 78 Å, 86 Å, or 96 Å would contain a total of 6, 7, or 8 apoA-I α-helical segments, respectively, of wild-type apoA-I per particle in contact with lipid. Based on this hypothesis rHDL containing RO6 or Δ6 apoA-I with diameters of 86 Å would have one less α-helical segment per molecule in contact with phospholipid compared with rHDL containing wild-type apoA-I. In the case of Δ6 apoA-I this change resulted from the deletion of repeat 6, whereas, for RO6 apoA-I this change resulted from a shift in the orientation of the hydrophobic and hydrophilic faces within repeat 6. Thus, we interpret these data as indicating that RO6 apoA-I repeat 6 is functionally absent, i.e. not in direct contact with the phospholipid bilayer and possibly more tightly associated with adjacent helical domains.

Alterations in apoA-I secondary structure are not necessarily associated with changes in rHDL size or composition, e.g. 10F6 apoA-I rHDL (17). 10F6 apoA-I rHDL were dramatically deficient (17-fold) in their LCAT catalytic efficiency as compared with wild-type apoA-I rHDL (Table III), but this apparent reduction in LCAT catalytic efficiency did not result from global alterations in rHDL size or composition. In the 10F6 apoA-I mutation the epitope corresponding to residues 143–164 was absent in 10F6 apoA-I, as would be expected (17). While, adjacent domains corresponding to repeats 4 and 5 (monoclonal epitopes mapped to residues 119–144, and 137–147) were altered compared with wild-type apoA-I (17). Overall, these results suggest that the activation of LCAT is sensitive to the secondary structure of apoA-I in the 121–164 residue domain and its activation may depend on the precise alignment and interaction among adjacent helices (6, 17, 38–40). We have proposed that helix-helix interactions may provide optimal molecular spacing for LCAT’s active site to access interfacial phospholipids, however, it is also possible that these interactions form a region which acts as a recognition site for direct interaction with LCAT or its active site (3).

Secondary structure mapping of RO6 apoA-I using monoclonal antibodies (Fig. 4B) showed that the epitopes corresponding to amino acids 143–165 in RO6 apoA-I (repeat 6) had a similar binding affinity and binding capacity to repeat 6 of wild-type apoA-I in either the lipid-free or lipid-bound form (Fig. 4). In contrast, the epitope spanning the junction of repeats 5 and 6 (amino acids 137–147) in lipid-free or lipid-bound RO6 apoA-I was completely absent, compared with wild-type apoA-I (Fig. 4A). These results can be explained if the hydrophilic face of RO6 repeat 6 is exposed and conformationally similar to wild-type apoA-I repeat 6, while the hydrophobic face, interacts more tightly with repeat 5. Presumably these interactions between apoA-I and the phospholipid bilayer reduce the size of the RO6 rHDL.

LCAT activation studies by Jonas and colleagues (34–37) have shown that full-length wild-type apoA-I can form heterogeneous rHDL and that LCAT activation varied with rHDL size and composition. We have shown that Δ6 and RO6 apoA-I form rHDL that were smaller than wild-type apoA-I (Table I and Fig. 2). Therefore, a direct quantitative comparison of wild-type apoA-I rHDL LCAT catalytic efficiency with that of Δ6 or RO6 apoA-I rHDL is complex. In this study, we have demonstrated that rotation of apoA-I repeat 6 does not alter the rHDL size, composition, α-helix content, or $D_{50}$ compared with Δ6 apoA-I (Tables I and II). Consistent with Jonas and colleagues (34–37) Δ6 and RO6 apoA-I rHDL LCAT activation properties are similar (Table III and Fig. 3). However, the Δ6 apoA-I rHDL showed a consistently lower LCAT catalytic efficiency and may result from an entire 22-mer repeat deletion which could alter the hydrophobic face orientation of all downstream repeats. Consistent with this idea, results from monoclonal epitope mapping of lipid-free and lipid-bound Δ6 apoA-I demonstrate alterations in secondary conformation within repeats 6, 7, 8, and 9. In addition, the relative LCAT activation properties of RO6, Δ6, and wild-type apoA-I were measured after being incorporated onto small unilamellar vesicles of uniform size and composition in order to control for differences in substrate particle size. Unilamellar vesicles of approximately 100 Å in diameter were used as substrates for LCAT assays after preincubation with RO6 apoA-I, Δ6, or wild-type apoA-I. These studies showed that Δ6 and RO6 apoA-I had only 5 and 15% of wild-type apoA-I activation, respectively. Thus, the rotation of the hydrophobic face of a single 22-mer repeat has dramatic consequences on LCAT activation independent of substrate size.

Lipid-free apoE (fragment Δ191–299) has been shown by x-ray crystallography to exist as a four-helix bundle (41). The substrate of hydrophobic and hydrophilic amino acids in these bundled helices was regularly repeated approximately every seven residues following a heptad repeat pattern. Leucine residues were found mostly at positions 1 and 4, thereby placing them on a common helical face, giving one helical turn corresponds to approximately 3.6 residues. Recent x-ray crystallography of a lipid-free fragment of apoA-I (Δ1–43 amino acids) has suggested a functional role for heptad repeats within apoA-I helices 2–7 (42). On the basis of sequence homology, the 11-residue amphipathic α-helical motif of apoA-I can be segregated into two helix types, A and B (38, 43). While the type B helix follows the classical 1 and 4 heptad repeat pattern, the type A helix exhibits a modified heptad repeat in which the hydrophobic residues are found at positions 1 and 5 (42). These authors conclude that the modified heptad repeats (type A) serve to rotate the orientation of the hydrophobic face relative to the unmodified heptad repeats (type B). This frequent reorientation of the hydrophobic face may be necessary to allow the apoprotein to wrap around the surface of a curved lipid-containing particles while keeping the hydrophobic faces associated with the edge of the rHDL (42, 44, 45). In wild-type apoA-I the type A and B repeat pattern between

3 L. Curtiss, M. Sorci-Thomas, unpublished results.
4 M. Sorci-Thomas, M. W. Kearns, and M. Landrum, unpublished observations.
residues 141 and 168 (including repeats 5/6 junction, repeat 6, and repeats 6/7 junction) is B-A-B. For 10F6 apoA-I the pattern is the same (B-A-B). It should be noted that 10F6 apoA-I has additional highly hydrophobic residues (2 leucines and 1 valine) at positions other than 1–4 or 1–5 within the substituted repeat. The greater number and hydrophobicity of residues increases the hydrophobic face hydrophobicity, causing the substituted repeat to penetrate deeper in the phospholipid bilayer and causing a dramatic reduction in LCAT activation. In RO6 apoA-I the type A and B repeat pattern is inverted, and reads A-B-A. The inversion of the repeat 6 hydrophobic face may result in its inability to interact optimally with the phospholipid bilayer, in addition to restricting its hydrophobic face alignment relative to adjacent helices 5 and 7. Given the limited flexibility of the repeat 6 helix around the proline residues at positions 143 and 165 (44, 45), this limited flexibility may cause repeat 6 of RO6 apoA-I to interact more strongly with adjacent helices. In summary, we have demonstrated that the proper alignment and orientation of the hydrophobic face of apoA-I repeat 6 is an important determinant in maintaining and stabilizing helix-bilayer and helix-helix interactions and that these interactions are a key structural feature regulating LCAT activation.

Acknowledgments—We gratefully acknowledge the technical assistance of Carol Banka, Kathi Richards, Abraham Gebre, and the editorial assistance of Nancy H. Nelson.

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The Hydrophobic Face Orientation of Apolipoprotein A-I Amphipathic Helix Domain 143–164 Regulates Lecithin:Cholesterol Acyltransferase Activation
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J. Biol. Chem. 1998, 273:11776-11782.
doi: 10.1074/jbc.273.19.11776

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