ApoA-I Structure on Discs and Spheres

VARIABLE HELIX REGISTRY AND CONFORMATIONAL STATES*

Received for publication, July 8, 2002, and in revised form, July 31, 2002
Published, JBC Papers in Press, August 6, 2002, DOI 10.1074/jbc.M206770200

Hui-hua Li, Douglas S. Lyles, Wei Pan, Eric Alexander, Michael J. Thomas, and Mary G. Sorci-Thomas

From the Departments of Pathology, Microbiology and Immunology, and Biochemistry, The Wake Forest University Baptist Medical Center, Winston-Salem, North Carolina 27157

Apolipoprotein A-I (apoA-I) readily forms discoidal high density lipoprotein (HDL) particles with phospholipids serving as an ideal transporter of plasma cholesterol. In the lipid-bound conformation, apoA-I activates the enzyme lecithin:cholesterol acyltransferase stimulating the formation of cholesterol esters from free cholesterol. As esterification proceeds cholesterol esters accumulate within the hydrophobic core of the discoidal phospholipid bilayer transforming it into a spherical HDL particle. To investigate the change in apoA-I conformation as it adapts to a spherical surface, fluorescence resonance energy transfer studies were performed. Discoidal rHDL particles containing two lipid-bound apoA-I molecules were prepared with acceptor and donor fluorescent probes attached to cysteine residues located at specific positions. Fluorescence quenching was measured for probe combinations located within repeats 5 and 5 (residue 132), repeats 5 and 6 (residues 132 and 154), and repeats 6 and 6 (residue 154). Results from these experiments indicated that each of the 2 molecules of discoidal bound apoA-I exists in multiple conformations and support the concept of a “variable registry” rather than a “fixed helix-helix registry.” Additionally, discoidal rHDL were transformed in vitro to core-containing particles by incubation with lecithin:cholesterol acyltransferase. Compositional analysis showed that core-containing particles contained 11% less phospholipid and 633% more cholesterol ester and a total of 3 apoA-I molecules per particle. Spherical particles showed a lowering of acceptor to donor probe quenching when compared with starting rHDL. Therefore, we conclude that as lipid-bound apoA-I adjusts from a discoidal to a spherical surface its intermolecular interactions are significantly reduced presumably to cover the increased surface area of the particle.

Decades of epidemiological evidence solidly support the association between plasma apoA-I concentration and its use as a negative prognostic factor of coronary heart disease in human populations. Despite the extensive associative data, the mechanisms explaining how HDL apoA-I accomplishes its antiatherogenic role remain debatable. One of the most popular hypotheses is that apoA-I HDL directs “reverse cholesterol transport” involving the organization of phospholipid, and cholesterol, the activation of LCAT and its conversion of cholesterol to cholesterol ester, which is then delivered to SR-B1 for elimination by the liver (1, 2). Another hypothesis suggests that HDL provides a variety of antioxidant and anti-inflammatory factors to the artery wall, thus, aiding in the stabilization of the “susceptible plaque” to potential rupture and thrombosis (3, 4). Both mechanisms likely provide the basis for the potent anti-atherogenic role for HDL, whereas they also emphasize the common feature, apoA-I, as the unique molecule driving both mechanisms.

Since the publication of the x-ray crystal structure of lipoprotein A-I (5), numerous studies have verified that apoA-I folds in a “belt-like” conformation when bound to a phospholipid bilayer (6–13). This structure contrasts with the “picket fence” conformation which had been favored for over 20 years. The term belt was coined to specify the orientation of the apoprotein helices lying perpendicular to the phospholipid acyl chains, as distinguished from the picket fence where the acyl chains lie parallel to the protein helices (9, 11). Although the majority of studies now support the belt conformation describing lipid-bound apoA-I, it is not clear whether disc bound apoA-I favors intramolecular interactions such as in a “hairpin” configuration (13, 14), or intermolecular interactions that would predominant in an “extended” belt conformation (6). Although these two types of belt conformation are not easily distinguished by most techniques, they have very different consequences regarding the helix registry of lipid-bound molecules of apoA-I.

Recently, two studies using quantitative fluorescence resonance energy transfer (FRET), measured the molecular distance between fluorescent probes attached to lipid-bound apoA-I (6, 13). The earlier study from our laboratory, Li et al. (6), measured the distance at one site between two apoA-I molecules, whereas a later study by Tricerri et al. (13) measured the distance between acceptor and donor probes placed at three different sites. Remarkably, both studies reported a similar 20–36% quenching from steady state measurements for all probe combinations, whereas a 50 or 75% predicted quenching was expected based on the ratio of acceptor to donor probe. Therefore, both studies concluded that the data demonstrating fluorescence quenching were consistent with the belt conformation and excluded the picket fence conformation.

* This work was supported by National Institutes Health Grants HL48373, HL64163, HL64963 (to M. S. T.), AI15892 (to D. S. L.), and HL-60079 (to M. J. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: LCAT, lecithin:cholesterol acyltransferase; HDL, high density lipoprotein; FRET, fluorescence resonance energy transfer; DPPC, 1-o-phosphatidylcholine dipalmityl; DSP, di-thiodia/succinimidyl propionate; 5-IAF, 5-(iodoacetamido)fluorescein; 5-TMRIA, tetramethylrhodamine-5-iodoacetamide; LDL, low density lipoprotein.

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However, each study interpreted the "missing" quenching differently. Tricerri et al. (13) hypothesized that the only model consistent with all the data including the missing fluorescence quenching was the alternative belt conformation, termed a hairpin that was hypothesized to exist in an equal ratio of head to head and head to tail. Li et al. (6) were not able to distinguish between an extended versus hairpin belt arrangement, and hypothesized that the missing fluorescence was because of nonrandom distribution of acceptor-labeled apoA-I molecules. Therefore, neither approach conclusively established the exact conformation of lipid-bound apoA-I nor do they explain the cause of the missing fluorescence quenching.

Therefore, to more clearly distinguish between alternative conformations of lipid-bound apoA-I and to address the issue of helix registry we have used FRET analysis to measure the distance between fluorescent probe pairs within repeats 5 and 6, 5 and 7, 5 and 8, and 6 and 7. We have also initiated studies to determine what conformational changes apoA-I undergoes as it adapts from a discoidal to a spherical surface.

**EXPERIMENTAL PROCEDURES**

Chemicals used were reagent grade and the highest purity commercially available. Dithiothreitol, aprotinin, leupeptin, t.-α-phosphatidylcholine dipalmitoyl (DPPC), and phenylmethylsulfonyl fluoride were purchased from Sigma. The mini-EDTA-free protease inhibitor tablets were purchased from Roche Molecular Biochemicals. Oligonucleotides were synthesized by International DNA Technologies. Plasmid DNA was purified using the Wizard purification systems from Promega Inc. Deoxyribonuclease I (DNase I) was purchased from Worthington. The expression vector pTBY12, Escherichia coli strain ER2566, and chitin strain ER2566, and chitin were purchased from New England Biolabs. Restriction enzymes, T4 DNA ligase, DH5α competent cells, and isopropyl-β-d-thiogalactopyranoside were purchased from Invitrogen. The cross-linker dithiobis(succinimidyl propionate) (DSP) was obtained from Pierce. The fluorescent probes, 5-(iodoacetamido)fluorescein (5-IAF; donor probe) and tetramethylrhodamine-5-iodoacetate (5-TMRIA; acceptor probe), were purchased from Molecular Probes (Eugene, OR). Q-Sepharose Fast Flow and Nap-25 columns were obtained from Amersham Biosciences. Cholesterol (> 99%) was from Nu-Chek Prep. Ultraferr-15 centrifugal filter units (BioMax – 10K membrane) were from Millipore Corp. Sodium cholate was from Calbiochem and all other chemical reagents were purchased from Sigma unless otherwise noted.

**Purification and Preparation of Cysteine Mutant ApoA-I**—The mutants Q132C and A154C apoA-I were cloned and expressed using the IMPACT system with the pTYB12 vector (New England Biolabs)./H9262

**Preparation and Purification of Discoidal DPPC rHDL**—Discoidal DPPC rHDL was incubated with LDL and rHDL to generate spherical core-containing particles following previously published methods (18–22). Briefly, a final volume of 1 ml contained the following components: 0.1 mg of LDL protein, 0.1 mg of LDL protein, 6 mg/ml bovine serum albumin, and 200 μl of human His-LCAT (equivalent to 40 μg of protein) and a final concentration of 2 mM β-mercaptoethanol, 1 mM Tris-HCl, 0.02% sodium azide, 50 mM imidazole, and 10% glycerol at 4 °C. LCAT-containing fractions were identified by their A<sub>280</sub> and by the measurement of their enzymatic activity. Fractions containing the highest activity were pooled and stored at −70 °C.

**Preparation of Core-containing HDL Particles**—Discoidal DPPC rHDL was incubated with LDL and rHDL to generate spherical core-containing particles following previously published methods (18–22). Briefly, a final volume of 1 ml contained the following components: 0.1 mg of LDL protein, 0.1 mg of LDL protein, 6 mg/ml bovine serum albumin, and 200 μl of human His-LCAT (equivalent to 40 μg of protein) and a final concentration of 2 mM β-mercaptoethanol, 1 mM Tris-HCl, 0.02% sodium azide, 50 mM imidazole, and 10% glycerol at 4 °C. LCAT-containing fractions were identified by their A<sub>280</sub> and by the measurement of their enzymatic activity. Fractions containing the highest activity were pooled and stored at −70 °C.

**Preparation and Purification of Fluorescent Probe-labeled ApoA-I**—Two fluorescent acceptor-labeled apoA-I rHDL were produced with either 5-IAF (donor) or 5-TMRIA (acceptor) probes based on the method described previously (6, 15) with slight modifications. Briefly, mutant apoA-I was reacted in 10 mM sodium phosphate, pH 7.4, 50 μM dithiothreitol with a 5-fold molar excess of 5-IAF or 5-TMRIA over total thiols and incubated for 1 h at room temperature. Probe-labeled apoA-I was separated from free probe using a Sephadex G-25 column (Amersham Biosciences) pre-equilibrated in 10 mM sodium phosphate, pH 7.4. The fractions containing 5-IAF-labeled or 5-TMRIA-labeled apoA-I were pooled and dialyzed against 10 mM sodium bicarbonate, pH 7.4, 3 μM EDTA, and 15 μM sodium azide. The protein purity and molecular weight were determined using a Quattro II mass spectrometer as previously reported (6, 15).

**Cross-linking of DPPC rHDL ApoA-I with DSP**—Approximately 16 μg of DPPC rHDL protein and 16 μg of lipid-free apoA-I were adjusted to 10 mM sodium phosphate, 140 mM NaCl, pH 7.4, with a final protein concentration of 0.05 and 1.0 μg/μl, respectively. Immediately before an experiment 1 mg of DSP was dissolved in 1000 μl of MeSO for a final concentration of 1 μg/μl. The dissolved DSP was added to the reaction mixture for a 30 DSP to 1 apoA-I molar ratio. After the addition, the tube was capped, inverted several times to mix, and immediately placed at 37 °C for 5 min. Following the reaction, tubes were iced, and 1 mM of bovine serum albumin, pH 7.4, was added to a 0.03 μl final concentration to stop the reaction. Aliquots containing 6 μg of protein were analyzed on nonreducing SDS-PAGE.

**Isolation and Purification of LDL and LCAT**—Low density lipoproteins (d = 1.019–1.063 g/ml) used in these studies were from African green monkey plasma that had been spun at d < 1.225 then run over 2 Superose-6 columns as described (16). The fractions containing the LDL fractions were pooled and used directly.

Recombinant human His-tagged LCAT (His-LCAT) was isolated from stably transfected CHO cell medium as previously described (17). The medium was passed over a Talon™ (Clontech) cobalt metal affinity column and the His-tagged LCAT was eluted with 10 mM Tris-HCl, pH 7.4, 140 mM NaCl, 0.02% sodium azide, 50 mM imidazole, and 10% glycerol at 4 °C. LCAT-containing fractions were identified by their A<sub>280</sub> and by the measurement of their enzymatic activity. Fractions containing the highest activity were pooled and stored at −70 °C.

**Fluorescence Measurements**—Steady-state fluorescence measurements were performed at 25°C using a SLM Aminco model 5000 spectrofluorometer. The emission wavelength was set at 520 nm, and the excitation wavelength was set at 310 nm. The number of apoA-I molecules in DPPC rHDL was determined by photo-oxidation of 5-IAF-labeled apoA-I using a SLM Aminco Model 5000 spectrofluorometer (d = 1.225 ± 0.005 g/ml) and a 1-ml glass cuvette filled with 0.5 ml of a 20 mM dithiothreitol, 50 mM Tris-HCl, pH 7.4, containing 30% non-denaturing gradient gel electrophoresis. After separation the gels were stained with Coomassie Brilliant Blue G and then scanned using a ChemiImager™5500 (Alpha Innotech Corp.). Particle size was determined by comparison to proteins of known Stokes diameter as described previously (6, 15).
technique using an ISS K2 multifrequency fluorometer as described previously (6), except the data were fit to a Gaussian distribution of lifetimes rather than to discrete exponentials. In this analysis, the data were fit to a distribution of intensities, \( I(\tau) \), by fitting two parameters, a peak lifetime, \( \tau_0 \), and a distribution width, \( \omega \). The distribution of intensities is described as the following.

\[
I(\tau) = \exp \left( - (\tau - \tau_0)^2 / 2 \omega^2 \right) 
\]

(Eq. 1)

If each donor molecule fluoresces independently of other donor molecules, the contribution of each component to the total intensity is inversely proportional to its lifetime. Therefore, \( I(\tau) \) is more heavily weighted toward the longer lifetime components. To correct for this weighting, the distribution of donor molecules, \( G(\tau) \), was approximated by Equation 2 and was converted to a fractional distribution, \( g(\tau) \), by Equation 3.

\[
G(\tau) = I(\tau) / \tau 
\]

(Eq. 2)

\[
g(\tau) = G(\tau) / \int G(\tau) d\tau 
\]

(Eq. 3)

These equations are an approximation because \( G(\tau) \gg 1 \) for \( \tau < 1 \). Because \( \tau_0 \) was \(-4\) ns and \( \omega = 0.5 - 1.5\) ns, the distributions were well described by calculating \( G(\tau) \) for 0.2 ns < \( \tau < 10\) ns. The integrated distribution \( \int G(\tau) d\tau \) was estimated by numerical integration as \( 2G(\tau) \Delta \tau \) for 0.2 ns < \( \tau < 10\) ns and \( \Delta \tau = 0.2\) ns. The average lifetime, \( \tau_{AV} \), was calculated as the following.

\[
\tau_{AV} = \int \tau^2 G(\tau) d\tau / \int G(\tau) d\tau = \int I(\tau)^2 d\tau / \int I(\tau) d\tau
\]

(Eq. 4)

Quenching of donor probes by acceptor probes was calculated as \( I_{AV}(\text{donor + acceptor}) / I_{AV}(\text{donor alone}) \times 100\% \).

**Results**

In these studies the lipid-bound conformation of apoA-I was studied using FRET, allowing the distance to be measured between acceptor and donor fluorescent probes. Fluorescent probes were attached to cysteine residues substituted at specific locations within apoA-I repeat 5(Q132C) and repeat 6(A154C) (15). Substitution sites were selected based on Edmanhelical wheel diagrams to identify noncharged residues located on the negatively charged or polar face of the \( \alpha \)-helical 22-mer repeat under examination. Fluorescein (donor) or rhodamine (acceptor) probes were attached to apoA-I and then used in the preparation of DPPC rHDL discoidal complexes using a 3:1 molar ratio of acceptor to donor-labeled apoA-I, as previously described (6, 15).

DPPC rHDL containing probe combinations in repeats 5 and 5 (residue 132), repeats 5 and 6 (residues 132 and 154), and repeats 6 and 6 (residue 154) were prepared and then purified by fast protein liquid chromatography. Some of the probe-labeled DPPC rHDL were used for the in vitro conversion to core containing spherical particles by incubation with LDL and LCAT. Following incubation for 2 h at 37°C with LDL and LCAT, all HDL particles were reisolated by sequential ultracentrifugation and then characterized by size and composition. Fig. 1 shows the particle size of rHDL, rHDL incubated with LDL, and rHDL incubated with LDL and LCAT. The Coomassie Blue-stained nondenaturing 4–30% gradient gel electrophoresis (NDGGE) of rHDL before and after incubation with LDL or LDL + LCAT; Panel A: lane 1, untreated DPPC rHDL containing acceptor and donor probe-labeled A154C apoA-I prepared as described under “Experimental Procedures”; lane 2, DPPC rHDL incubated with an equal protein mass of LDL for 2 h at 37°C and then isolated by sequential ultracentrifugation; lane 3, DPPC rHDL incubated with an equal protein mass of LDL and 4 \( \mu \)g of human His-LCAT for 2 h at 37°C and then isolated by sequential ultracentrifugation. All lanes represent the equivalent of 5 \( \mu \)g of rHDL apoA-I separated on a 4–30% NDGGE stained with Coomassie Blue. After destaining gels were scanned and analyzed using a Alpha Innotech ChemiImager \(^{TM} \)5500 and particle size was determined by comparison to standards of known Stokes diameter (nm): thyroglobulin, 17.2; ferritin, 12.4; catalase, 9.8; lactate dehydrogenase, 8.6; and albumin, 7.0; Panel B, various preparations of DPPC rHDL containing probe-labeled apoA-I were incubated with an equal protein mass of LDL and \( \pm 40 \) \( \mu \)g of human His-LCAT (lane 3). Incubation of rHDL with LDL alone caused a slight shift in the rHDL size to a smaller particle, which was reversed when the rHDL was incubated with LDL and LCAT.

To determine whether rHDL containing different fluorescent probe combinations affected the size of the starting rHDL or the core-containing particles generated, additional rHDL preparations were examined. Shown in Fig. 1B, various prepara-
The starting discoidal DPPC rHDL was prepared, purified, and characterized as described under “Experimental Procedures.” Generation of core containing rHDL was carried out by combining 0.1 mg of rHDL with an equal mass of LDL protein, 40 μg of human His-LCAT and incubating for 2 h at 37 °C. Following incubation the core-containing HDL was purified by sequential ultracentrifugation, dialyzed, and then analyzed as described under “Experimental Procedures.” Particle size was determined by comparison to protein standards of known Stokes diameter using a ChemiImager™ 5500. Cross-linking analysis indicated that discoidal rHDL contained 2 molecules of apoA-I, whereas the rHDL incubated with LDL and LCAT contained 3 molecules of apoA-I per particle. All values represent the mean ± S.E. of four to six independent measurements. Statistically significant differences are indicated by lowercase letters at p < 0.003. No significant differences were seen as a function of the probe attached within labeled repeats 5:5 rHDL, labeled repeats 5:6 rHDL, or labeled repeats 6:6 rHDL in any of the three treatment groups.

| Probe-labeled apoA-I repeats in rHDL | Original rHDL | rHDL + LCAT | rHDL + LDL + LCAT |
|-------------------------------------|---------------|-------------|-------------------|
| Repeat 5: repeat 5                 | 9.57 ± 0.04   | 9.25 ± 0.02 | 9.62 ± 0.01       |
| Repeat 5: repeat 6                 | 9.52 ± 0.06   | 9.40 ± 0.05 | 9.68 ± 0.03       |
| Mean ± S.E.                        | 9.55 ± 0.01   | 9.33 ± 0.03 | 9.64 ± 0.01       |

To determine the number of apoA-I molecules per particle, chemical cross-linking with DSP was carried out on rHDL before and after incubation with LDL and LCAT. Following incubation with LDL and LCAT, samples were purified by sequential ultracentrifugation and then treated with DSP. Fig. 3 shows a Coomassie-stained 4–30% SDS-PAGE analysis of...
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FIG. 3. Coomassie-stained 4–30% SDS electrophoresis of rHDL and rHDL incubated with LDL and 40 μg of human His-LCAT for 2 h at 37 °C, purified by sequential ultracentrifugation, and then treated with the cross-linker DSP as described under “Experimental Procedures.” Lane 1, purified lipid-free wild-type apoA-I treated with DSP; lane 2, purified lipid-free wild-type apoA-I, not treated with DSP; lane 3, DPPC rHDL containing probe-labeled A154C apoA-I, treated with DSP; lane 4, DPPC rHDL containing probe-labeled A154C apoA-I incubated with an equal protein mass of LDL and 40 μg of human His-LCAT, purified by sequential ultracentrifugation and then treated with DSP. All lanes show the equivalent of 6 μg of protein run under nonreducing conditions.

rHDL and core-containing HDL. Lane 1 shows purified lipid-free wild-type apoA-I treated with DSP that exists in various oligomeric states from monomer to pentamer, and serves as a molecular weight marker. Lane 2 shows purified lipid-free wild-type apoA-I, not treated with DSP and lane 3 shows DPPC rHDL containing probe-labeled A154C apoA-I, treated with DSP, which is primarily shown as a dimer. Lane 4 shows DPPC rHDL containing probe-labeled A154C apoA-I incubated for 2 h at 37 °C with an equal protein mass of LDL and 40 μg of human His-LCAT. Following the incubation, HDL was re-isolated by ultracentrifugation and then treated as described with DSP. The results of this study indicate that the starting rHDL contained 2 molecules of apoA-I per particle, which increased to 3 molecules per particle once the rHDL had been converted to a core-containing spherical particle by incubation with LDL and LCAT.

Using the data derived from compositional analysis and cross-linking studies, the diameter of a sphere was calculated based on the final molar stoichiometries listed in Table II. Combined with the reported volumes of each of the particles components (cholesterol ester, 1200 Å³/molecule; cholesterol, 675 Å³/molecule; phospholipid, 1320 Å³/molecule; apoA-I, 55,400 Å³/molecule) (18) the diameter of a sphere was calculated to be 95.8 Å, which agrees well with the experimental value of 96.4 Å (Table I).

To assess the apoA-I conformational changes that accompany the conversion from a disc to sphere, the energy transfer between donor and acceptor probe-labeled lipid-bound apoA-I was measured. The energy transfer was measured by the extent of quenching of donor probe fluorescence in the presence of acceptor probe. Fluorescence quenching was determined both by steady state fluorescence intensities as well as by the decrease in fluorescence lifetime of the donor probe. These data are shown in Table III.

The efficiency of energy transfer from repeat 5 in one apoA-I molecule to repeat 5 in the second apoA-I molecule in DPPC rHDL (Table III, 5:5 Disc) was 32% based on the quenching of steady state fluorescence. Quenching measured by the decrease in the average donor lifetime was only 9% of the total quenching. The quenching measured in this study is similar to previously published data (6). We and others have attributed this difference between the steady state and lifetime measurements to the existence of a population of rHDL in which the two repeats 5 are so close together that quenching is nearly complete, so that this population is not detected in the lifetime experiment (6, 13). From the difference between the steady state and lifetime measurements, this population would be 23% of donor-labeled apoA-I. Assuming that quenching in this population is >90%, the distance between probes in repeat 5 can be calculated to be <30 Å. Because amino acids at position 132 in repeat 5 are separated by ~16 Å, as reported from the x-ray structure, this finding is consistent with the belt conformation. However, if all of the apoA-I in rHDL were in the belt conformation, the steady state quenching would be expected to be 75%. Thus, we and others have concluded that apoA-I probably resides in multiple environments in rHDL.

Conversion of rHDL discs into spheres containing apoA-I labeled with donor and acceptor probes in repeat 5 reduced the quenching of steady state donor fluorescence from 32 to 25% (Table III, 5:5 Sphere). There was no decrease in the quenching measured in the lifetime experiment, indicating that the decrease in steady state quenching was because of a change in the population with repeats 5 too close together to detect in the lifetime experiment, which was reduced from 23 to 13%. This result is fully consistent with conversion of discs containing two apoA-Is to spheres containing three apoA-Is, in which two molecules of the apoA-I move apart, but maintain some repeat 5 to 5 contact, whereas the third apoA-I adapts an alternate conformation. Because the starting molar ratio of acceptor to donor-labeled apoA-I was 3:1, it is more likely that the third apoA-I is an acceptor-labeled apoA-I. If the sphere has two acceptors and one donor-labeled apoA-I, the energy transfer would be expected to be higher, not lower, as we have observed in our studies. Thus, because of the addition of the third molecule of apoA-I it is likely that the observed reduction in steady state quenching represents an underestimation of the distance that the apoA-I molecules have moved away from each other on the surface of the sphere.

The efficiency of energy transfer between repeats 5 and 6 was measured using apoA-I labeled with donor probe in repeat 6 (position 154) and with acceptor probe in repeat 5 (position 132). These data provided further support for multiple apoA-I conformations in rHDL. In DPPC rHDL (Table III, 5:6 Disc), the quenching measured in the steady state experiments was 25%, of which 15% could be accounted for by the decrease in the measurable fluorescence lifetime. Thus, 10% of the observed steady state quenching was because of probes too close together to be detected in the lifetime experiment and are, therefore, <30 Å apart. The proximity of repeats 5 and 6 in this population of apoA-I is inconsistent with the belt conformation, as understood by x-ray crystal data, in which the labeled positions in these helices are rigidly fixed with a separation of 40 Å. Instead, these results support the idea of a “variable registry” model in which a population of rHDL contain molecules of apoA-I in which repeat 5 has significant interaction with repeat 6.

The 90% of apoA-I labeled in repeats 5 and 6 whose lifetimes could be detected (Table III) consisted of at least two subpopulations. As in the case of energy transfer between two labels in repeat 5, at least 25% of apoA-I labeled in repeat 6 must be paired with another donor-labeled molecule, and therefore would not be quenched. The 15% quenching observed in the lifetime experiment was because of a second subpopulation with an average separation between labeled repeats of ~56 Å. Whereas this is not fully consistent with the 40-Å separation predicted from the x-ray structure, it could result from an unresolved mixture of molecules, in some of which repeats 5 and 6 are 40 Å apart and in others the separation is greater. Such a mixture would also be consistent with the variable registry model.

Conversion of rHDL disc into spheres containing apoA-I labeled with probes in repeats 5 and 6 resulted in a reduction in...
quenching of steady state donor fluorescence from 25 to 17% (Table III, 5:6 Sphere). This resulted from a small reduction in the % of repeats too close together to detect in the lifetime measurement (from 10 to 5%) as well as a small increase in the average separation in repeats whose lifetimes could be measured (from 56 to 60 Å). These results are similar to those with probes in two repeats 5, in which the extent of energy transfer was reduced upon conversion of discs to spheres and may represent an underestimation of the absolute distance these probes have moved apart.

There was a small amount of energy transfer (~4%) from repeat 6 in one lipid-bound apoA-I molecule to repeat 6 in the other apoA-I in DPPC rHDL (Table III, 6:6 Disc). Although this quenching was observed reproducibly, it is near the limit of detection for these experiments. This level of quenching corresponds to a separation of ~70 Å, which is consistent with the distance between the labeled positions in repeat 6 in the x-ray structure. There was little if any change in this result upon conversion of rHDL discs into spherical particles (Table III, 6:6 Sphere).

The discrepancies between the steady state quenching and the quenching observed in the lifetime experiments in Table III indicate that donor-labeled apoA-I must exist in multiple environments when bound to rHDL. As shown in Table IV, we postulate that there must be at least three different populations of donor-labeled apoA-I to account for the fluorescence properties observed. One is the population in which the repeats are too close together to be detected in the lifetime experiment, as described above. The second is the population in which the repeats are far enough apart to detect an intermediate level of energy transfer in the lifetime experiment. There must also be a third population in which donor-labeled apoA-Is are paired with another donor rather than acceptor-labeled apoA-I, and, therefore, would not be quenched. This is because of the fact that the initial molar ratio of acceptor:donor-labeled apoA-I was 3:1. Thus, 75% of donors would be paired with acceptors in discs, and 25% would be paired with another donor, if the distribution of apoA-I molecules was completely random.

Table IV shows the calculated distribution of apoA-I among these three different populations. Because the lifetimes of the second and third populations were so close, they could not be resolved as separate fluorescence components. However, their percentages can be calculated by assuming a random distribution of donor- and acceptor-labeled apoA-I. The energy transfer between discs with probes in repeats 5 (Table IV, 5:5 Disc) can best be described as a mixture containing 23% of molecules with repeats 5 very close together (<30 Å), which is consistent with the x-ray structure, and 52% of molecules with repeats 5 ~60 Å apart. The remaining 25% of donors would be paired with other donors, and thus would not participate in energy transfer. The 52% of molecules with repeats ~60 Å apart would be consistent with a variable registry model (discussed below). However, this distance would not be consistent with the head-to-tail hairpin model, because the two repeats 5 would be ~85–96 Å apart. Additionally, the major change in energy transfer between repeats 5 upon conversion of discs to spheres was because of the increase in this population from 52 to 81%.

Further evidence that apoA-I exists in multiple environments on rHDL was obtained from detailed analysis of fluorescence lifetimes. In our previously published work and that of others (6, 13), it was clear that the fluorescence decay of donor probe-labeled apoA-I in rHDL could not be described by a single lifetime component, even in the absence of acceptor. This result provides evidence for heterogeneity within the probe environment. Our earlier data (6) were fit to a two-component decay model in which most of the donor probes had a lifetime of about 4 ns and a small amount of the steady state fluorescence (1–4%) was because of a shorter lifetime component (0.4–0.8 ns). However, in the present series of experiments, we found that a two-component fit of the data could often be described just as well by a major component with a slightly shorter lifetime (3.5 ns) and a minor component with a longer lifetime (10 ns). This suggested to us that two-component fits might not be appropriate for these data. Instead these data may be more appropriately described as a single lifetime component with a distribution of lifetimes. In fact all of the new data, as well as our previous data, can be fit very well by the single lifetime distribution model. The quality of the fits was at least as good as the two-component model with discrete lifetimes, as judged by the X2 statistic. This quality of the fits was obtained by fitting fewer independent variables, because the lifetime distribution model uses two variables (a single peak lifetime and the width of the distribution), whereas the two-component model uses three (two lifetimes and a fractional intensity for one of the components).

Table V shows the peak lifetimes and widths of the lifetime distributions resulting from fitting the data to the lifetime distribution model. These data are fully consistent with a variable registry model in which donor probes would experience a distribution of slightly different environments dictated by
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### Table IV

|                      | 5.5 Disc | 5.5 Sphere | 5.6 Disc | 5.6 Sphere | 6.6 Disc | 6.6 Sphere |
|----------------------|----------|------------|----------|------------|----------|------------|
| % of Total donor-labeled apoA-I |
| Repeats too close to detect lifetime<sup>a</sup>       | 23       | 13         | 10       | 5          | <1       | <1         |
| Repeats far enough apart to detect lifetime<sup>b</sup>  | 52       | 81         | 65       | 89         | 75       | 94         |
| Donors not paired with acceptors<sup>c</sup>            | 25       | 6.25       | 25       | 6.25       | 25       | 6.25       |
| Total               | 100      | 100        | 100      | 100        | 100      | 100        |
| Distance between repeats |
| Repeats too close to detect lifetime<sup>d</sup>       | <30 Å    | <30 Å      | <30 Å    | <30 Å      | <30 Å    | <30 Å      |
| Repeats far enough apart to detect lifetime             | 61 Å     | 62 Å       | 56 Å     | 60 Å       | 74 Å     | 74 Å       |
| Donors not paired with acceptors                        | 6.25 Å   | 6.25 Å     | 6.25 Å   | 6.25 Å     | 6.25 Å   | 6.25 Å     |

<sup>a</sup> Calculated as the quenching measured in the steady state experiment versus the quenching measured in the lifetime experiment from Table III.

<sup>b</sup> Calculated as 100% – (% too close together) – (% donors not paired with acceptors).

<sup>c</sup> Calculated from the 3:1 ratio of acceptor-donor-labeled apoA-I used in making rHDL. For discs, it was assumed that 25% of donor-labeled monomers are not paired with acceptors, and for spheres, it was assumed that 6.25% of donor-labeled monomers are not paired with acceptors since there are three molecules of apoA-I per spherical particle. The probability that a donor-labeled apoA-I will not be paired with an acceptor-labeled apoA-I is 25% for each of the other two molecules thus 25 x 25 = 6.25% probability that neither apoprotein will be acceptor labeled.

<sup>d</sup> Calculated from a Förster distance ($R_0$) of 45 Å for the fluorescein-rhodamine donor-acceptor pair and assuming that quenching is >90% in this population of molecules.

### Table V

| Donor repeat | Acceptor repeat | Peak Tau | Distribution width | Average Tau<sup>a</sup> |
|--------------|----------------|----------|--------------------|-------------------------|
| Discs        |                |          |                    |                         |
| 5            | None           | 4.17 ± 0.10 | 0.62 ± 0.51        | 4.00 ± 0.03             |
| 5            | 5              | 4.18 ± 0.07 | 1.27 ± 0.23        | 3.83 ± 0.18             |
| 6            | None           | 4.20 ± 0.12 | 0.98 ± 0.17        | 3.92 ± 0.25             |
| 6            | 5              | 4.01 ± 0.34 | 1.36 ± 0.01        | 3.34 ± 0.42             |
| 6            | None           | 4.17 ± 0.07 | 0.83 ± 0.31        | 3.95 ± 0.19             |
| 6            | 6              | 4.07 ± 0.06 | 0.94 ± 0.14        | 3.81 ± 0.16             |
| Spheres      |                |          |                    |                         |
| 5            | None           | 4.20 ± 0.04 | 0.83 ± 0.07        | 4.01 ± 0.01             |
| 5            | 5              | 4.08 ± 0.05 | 1.26 ± 0.16        | 3.54 ± 0.12             |
| 6            | None           | 4.23 ± 0.12 | 1.05 ± 0.10        | 3.92 ± 0.21             |
| 6            | 5              | 4.03 ± 0.10 | 1.38 ± 0.11        | 3.34 ± 0.25             |
| 6            | None           | 4.15 ± 0.06 | 0.89 ± 0.23        | 3.91 ± 0.20             |
| 6            | 6              | 4.10 ± 0.05 | 1.08 ± 0.06        | 3.74 ± 0.11             |

<sup>a</sup> Calculated as the quenching measured in the steady state experiment minus the quenching measured in the lifetime experiment from Table III.

<sup>b</sup> Calculated as 100% – (% too close together) – (% donors not paired with acceptors).

<sup>c</sup> Calculated from the 3:1 ratio of acceptor-donor-labeled apoA-I used in making rHDL. For discs, it was assumed that 25% of donor-labeled monomers are not paired with acceptors, and for spheres, it was assumed that 6.25% of donor-labeled monomers are not paired with acceptors since there are three molecules of apoA-I per spherical particle. The probability that a donor-labeled apoA-I will not be paired with an acceptor-labeled apoA-I is 25% for each of the other two molecules thus 25 x 25 = 6.25% probability that neither apoprotein will be acceptor labeled.

<sup>d</sup> Calculated from a Förster distance ($R_0$) of 45 Å for the fluorescein-rhodamine donor-acceptor pair and assuming that quenching is >90% in this population of molecules.

The main focus of this study was to obtain information regarding the structure of lipid-bound apoA-I on discs and spheres. FRET between acceptor and donor probes placed at specific residues within apoA-I permitted the determination of the intermolecular distance between helix 5 and 5, helix 5 and 6, and helix 6 and 6. Several recent studies (6–13) support the conclusion that lipid-bound apoA-I adopts a “belt-like” conformation, first described by the crystal structure of lipid-free A43 apoA-I (5). However, the striking results from studies using FRET, including those results described here, is that no single apoA-I conformation on 96-Å particles explain all of the data. Rather, the present studies suggest that two apoA-I molecules can rotate or assume a number of interhelix registries with respect to each other when bound to phospholipid discs. Therefore, we propose that the two lipid-bound apoA-I molecules do not conform to a tightly associated or rigid dimer, i.e. “fixed helix registry” model, but instead apoA-I can assume a more flexible interaction between molecules called a “variable helix registry.”

Tricerri et al. (13) first hypothesized the existence of two populations of lipid-bound apoA-I molecules from FRET data. In this model, they hypothesized an equal proportion of discs containing apoA-I dimers arranged in a head to head and head to tail hairpin orientation. Hairpins resemble extended belts, bent in the center and folded back on themselves forming intramolecular interactions. Our present data do not allow us to uniquely distinguish between an extended belt versus the hairpin conformation. However, the lifetime data are more consistent with the hypothesis that two apoA-I molecules adopt a belt conformation in which they assume multiple registries that can rotate or “ratchet” with respect to one another over intermediate distances when bound to phospholipid discs. The concept of multiple interhelical interactions does not imply that molecules of lipid-bound apoA-I freely rotate around the periphery of the disc or sphere. If this were true, the steady state quenching would be reduced to ~9% (Table III).

Given our variable registry hypothesis, it is highly likely that the conformation(s) of two apoA-I molecules assumed on 96-Å
arginine at 173 is replaced with cysteine and forms dimers with or presence (solid line) of acceptor-labeled apoA-I. Phase and modulation data from a representative experiment were fit to a single lifetime distribution characterized by a lifetime peak and width. Fitted lifetime distributions were corrected for the heavier weighting of longer lifetime components and are shown as a fraction of total donor molecules. Figure 4. Lifetime distributions for donor-labeled apoA-I with probes in repeats 5 in the absence (solid line) or presence (dashed line) of acceptor-labeled apoA-I. Phase and modulation data from a representative experiment were fit to a single lifetime distribution characterized by a lifetime peak and width. Fitted lifetime distributions were corrected for the heavier weighting of longer lifetime components and are shown as a fraction of total donor molecules.

Fig. 4. Lifetime distributions for donor-labeled apoA-I with probes in repeats 5 in the absence (solid line) or presence (dashed line) of acceptor-labeled apoA-I. Phase and modulation data from a representative experiment were fit to a single lifetime distribution characterized by a lifetime peak and width. Fitted lifetime distributions were corrected for the heavier weighting of longer lifetime components and are shown as a fraction of total donor molecules. discs may not be the same as that found on discs containing 3 or 4 molecules of apoA-I. Consider the results from studies conducted on a mutant form of apoA-I in which the helix registry is fixed by the formation of a cysteine dimer. ApoA-IMilano is a naturally occurring mutant form of apoA-I in which arginine at 173 is replaced with cysteine and forms dimers with itself. In a study by Calabresi et al. (23) the apoA-IMilano dimer was used to form discoidal particles and compared with discs containing wild-type apoA-I. The size, composition, and LCAT reactivity of the mutant apoA-I discs were carefully examined and the authors found that only two sizes of discs were formed with the apoA-IMilano dimer, corresponding to a diameter of 78 and 125 Å (23). The 78-Å particle contained 1 dimer or 2 molecules of the mutant apoA-I, whereas the 125-Å particle contained 2 dimers or 4 molecules of mutant apoA-I, while discs made with wild-type apoA-I contained 2 molecules of apoA-I and 3 molecules of apoA-I, respectively. The apoA-IMilano cysteine dimer forces repeats 7 and 7 to exist in register, which is thoroughly inconsistent with the published crystal structure. Thus, it appears that under these constraints the apoA-IMilano dimer cannot adapt to the conformation necessary to form a 96-Å disc, although, a wide range of protein to phospholipid ratios were tested (23). The idea of discrete populations of apoA-I conformations on different sized discs reinforces the concept of a highly flexible apoprotein as it was first described by studies in which lipid-free apoA-I was shown to conform to a “molten globular” state (24).

In the present studies, we provide a number of experimental observations that are consistent with the crystal structure of lipid-free apoA-I. First, a population of apoA-I molecules was identified with a highly efficient energy transfer between probes in repeat 5 and 5. These results show that repeat 5 in two molecules of apoA-I are in close contact (<30 Å). Second, most of the energy transfer between repeats 5 and 6 of apoA-I resulted from probes separated by an intermediate distance, ~30–60 Å, which is consistent with the crystal structure. Finally, a small amount of energy transfer was measured between probes in apoA-I repeats 6 and 6. This observation is consistent with repeat 6 helices that fold in an antiparallel belt orientation separated by a distance of ~70 Å, consistent with the x-ray crystal structure.

Whereas several observations reported here are consistent with the x-ray crystal structure of Δ43 apoA-I and earlier FRET data, other results are not. For instance, a large proportion of lipid-bound apoA-I molecules labeled in repeats 5 and 5 had an intermediate level of energy transfer consistent with a 60-Å separation. This distance is not consistent with the x-ray crystal structure that predicts a 16-Å separation between repeats 5 in two molecules of apoA-I. In addition, a small population of labeled apoA-I in repeats 5 and 6 displayed highly efficient energy transfer, indicating close proximity (<30 Å). This observation is also not consistent with the x-ray crystal structure that predicts a separation of 42 Å between these repeats.

To reconcile all of the data, we propose a simple model that represents a variation of the x-ray crystal structure or fixed helix registry, termed the variable helix registry. The x-ray crystal structure suggest that probes in repeat 5 and 5 of two molecules of lipid-bound apoA-I should be in a fixed register within ~16 Å of each other. Instead, we find a population of apoA-I molecules corresponding to this orientation and another population of repeat 5 probes at an intermediate distance from each other. Thus, to explain all of the data we suggest that a distribution of discrete intermolecular helix pairs stabilize the lipid-protein structure, and separate the donor-acceptor molecules by distances ranging between 30 and 70 Å. Our findings are also consistent with FRET studies of apolipophorin-III (25), in which a 15–20 Å increase in probe separation was seen upon the unfolding of the lipid-free protein when bound to phospholipid.

Another important conclusion derived from our studies is that the intermolecular distance between 2 lipid-bound apoA-I molecules increases once apoA-I conforms to a spherical surface. Cross-linking studies indicate that the core-containing spheres contain 3 molecules of apoA-I per particle. Once apoA-I conforms to the surface of a sphere an increase in distance separating these apoA-I molecules occurs, presumably to cover the increased surface area of the spherical particle. A reduction in the steady state quenching was observed as the probe-labeled apoA-I on the disc conformed to the surface of a sphere. Because the starting molar ratio of acceptor to donor-labeled apoA-I was 3 to 1, it is more likely that the third apoA-I is an acceptor-labeled apoA-I. If the sphere has two acceptors and one donor-labeled apoA-I, the energy transfer would be expected to be greater, not lower, as we have observed in our
studies. Thus, because of the addition of the third molecule of apoA-I it is likely that the observed reduction in steady state quenching represents an underestimation of the distance that the apoA-I molecules have moved away from each other on the surface of the sphere.

In summary, the information presented in this report provides important information regarding the flexibility of apoA-I and the nature of its interaction with phospholipids on discoidal and spherical surfaces. Additional studies will be needed to distinguish between extended belts and hairpins and the frequency of these conformations on particles of different sizes.

Acknowledgments—We thank Dr. John Parks for providing the purified human His-LCAT. We also thank Dr. Martha Wilson and Kathryn Kelley for the purified monkey LDL.

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