Conservation of Apolipoprotein A-I's Central Domain Structural Elements upon Lipid Association on Different High-Density Lipoprotein Subclasses

Michael N. Oda,[†] Madhu S. Budamagunta,[‡] Ethan G. Geier,[†] Sajiv H. Chandradas,[†] Baohai Shao,[§] Jay W. Heinecke,[§] John C. Voss,[‡] and Giorgio Cavigiolio*[,†]

[†]Children's Hospital Oakland Research Institute, Oakland, California 94609, United States
[‡]Department of Biochemistry and Molecular Medicine, University of California, Davis, California 95616, United States
[§]Department of Medicine, University of Washington, Seattle, Washington 98195, United States

S *Supporting Information*

ABSTRACT: The antiatherogenic properties of apolipoprotein A-I (apoA-I) are derived, in part, from lipidation-state-dependent structural elements that manifest at different stages of apoA-I's progression from lipid-free protein to spherical high-density lipoprotein (HDL). Previously, we reported the structure of apoA-I's N-terminus on reconstituted HDLs (rHDLs) of different sizes. We have now investigated at the single-residue level the conformational adaptations of three regions in the central domain of apoA-I (residues 119−124, 139−144, and 164−170) upon apoA-I lipid binding and HDL formation. An important function associated with these residues of apoA-I is the activation of lecithin:cholesterol acyltransferase (LCAT), the enzyme responsible for catalyzing HDL maturation. Structural examination was performed by site-directed tryptophan fluorescence and spin-label electron paramagnetic resonance spectroscopies for both the lipid-free protein and rHDL particles 7.8, 8.4, and 9.6 nm in diameter. The two methods provide complementary information about residue side chain mobility and molecular accessibility, as well as the polarity of the local environment at the targeted positions. The modulation of these biophysical parameters yielded new insight into the importance of structural elements in the central domain of apoA-I. In particular, we determined that the loosely lipid-associated structure of residues 134−145 is conserved in all rHDL particles. Truncation of this region completely abolished LCAT activation but did not significantly affect rHDL size, reaffirming the important role of this structural element in HDL function.
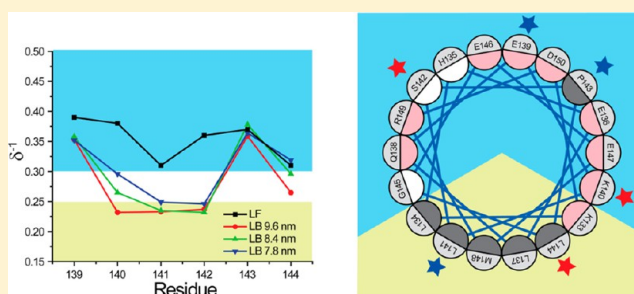
Human high-density lipoprotein (HDL) is a lipid−protein complex composed of approximately 50 proteins and a heterogeneous neutral lipid core.[1,2] Multiple functions such as cellular cholesterol homeostasis, plasma cholesterol transport, and anti-inflammatory, antioxidant, and antithrombotic activities contribute to HDL's antiatherogenic effect.[3−8] Although these activities may be catalyzed by an array of HDL resident proteins, HDL's function is influenced by the structure of the main protein scaffold, primarily composed of apolipoprotein A-I (apoA-I).[9]

According to the double-belt model for nascent HDL, the initial state of HDL during biogenesis, apoA-I assumes a curved amphipathic α-helical ring structure in which two apoA-I molecules are associated in an antiparallel fashion on the perimeter of a phospholipid bilayer. The model was originally derived from the crystal structure of an N-terminally truncated lipid-free apoA-I[10] and has been confirmed by several laboratories using *a priori* modeling[11] and an array of biophysical methods.[12−27] Wu et al. proposed an alternative model for nascent 9.6 nm diameter HDL, termed the double-

superhelix model, in which apoA-I assumes an asymmetrical helical shape that wraps around a central micellar lipid phase.[28] However, the double-superhelix model has been the subject of considerable debate.[29,30] Recently, Jones et al.[30] used molecular dynamics, fluorescence spectroscopy, and electron microscopy to test this model. The preponderance of evidence refuted the double-superhelix model and further substantiated the double-belt model.

Within the various models based on the double-belt scaffold, specific structural details have also been the object of scrutiny and debate. Several computational and experimental studies detected discontinuities in the α-helical secondary structure of apoA-I on nascent HDL; however, there is little agreement on the position, size, and structure of these regions.[19,22,24,31−34] The loose lipid association of these nonhelical segments in the central domain is postulated to serve a functional role relevant
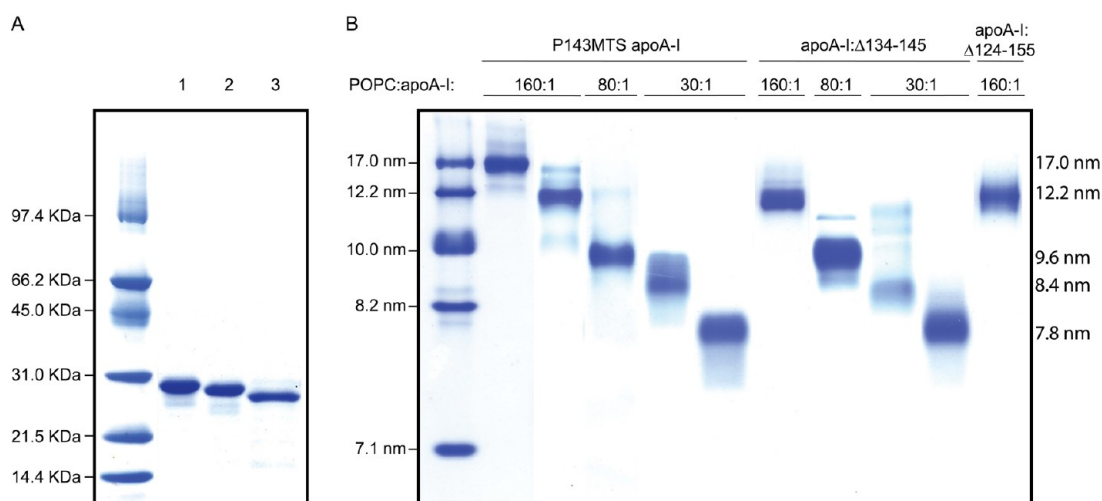
Figure 1. (A) SDS−PAGE analysis of a full-length apoA-I variant (lane 1, spin-labeled P143C apoA-I is representative of the 40 full-length protein variants analyzed) and truncation variants apoA-I:Δ134−145 (lane 2) and apoA-I:Δ124−155 (lane 3). (B) Nondenaturing gradient gel electrophoresis analysis of purified rHDL subclasses obtained by spin-labeled P143C apoA-I (representative of the 40 full-length apoA-I variants) and truncation variants apoA-I:Δ134−145 and apoA-I:Δ124−155. Wild-type apoA-I (see ref 45) and all full-length apoA-I variants were capable of forming five rHDL subclasses. Four rHDL subclasses were generated by apoA-I:Δ134−145; no rHDL larger than 12.2 nm was reconstituted. Lipidation of apoA-I:Δ124−155 resulted only in the production of ∼12.2 nm rHDL.

to lecithin:cholesterol acyltransferase (LCAT) activation.[22,24,33] In 2006, we identified a non-α-helical region (residues 134−145) in the central domain of apoA-I in 9.6 nm diameter HDL by site-directed spin-label electron paramagnetic resonance (SDSL-EPR) and fluorescence analysis and proposed the "looped-belt" model.[22] We speculated that this structural element is a "portal" facilitating access of LCAT to the acyl chain substrate during the cholesterol−acyl transfer reaction. This hypothesis has been corroborated recently by molecular dynamics experiments.[33] In contrast, Wu et al.[24] proposed the "solar-flares" model, in which a non-lipid-associated loop region spans residues 159 and 180 and no α-helical discontinuity was observed for region 134−145. The veracity of the solar-flares model was evaluated by molecular dynamics experimentation,[35] and the loop conformation of residues 159−180 was found to be unstable. The recent hydrogen−deuterium exchange mass spectrometry study by Chetty et al. also provides evidence that residues 158−180 on 9.6 nm rHDL are α-helical and do not adopt a loop conformation.[34]

While the first conformational analysis of plasma-isolated HDL subclasses was recently published,[9] a majority of HDL structural information is based on studies of reconstituted HDL (rHDL), which have largely been focused on 9.6 nm diameter discoidal particles. However, plasma nascent HDL is a heterogeneous population of particles of 7.5−17.0 nm in diameter.[2,27,36,37] To gain a more functional understanding of the role of specific apoA-I structural features in the biogenesis and metabolism of HDL, knowledge of their representation on different sized rHDL is essential. Recently, Chetty et al. investigated the secondary structure of apoA-I on rHDL and identified the position of α-helical regions within apoA-I on 9.6 and 7.8 nm rHDL particles.[34] Although exhaustive, these results are derived from the overall thermodynamic stability of peptide segments and do not define the structure of apoA-I at the single-residue level. Examination of specific structural elements at the single-residue level is needed to understand the structure−function relationship of apoA-I on different HDL subclasses. Such knowledge would provide insight into the functional consequences of specific molecular modifications of

apoA-I and their contribution to HDL dysfunction-associated pathologies like cardiovascular disease.

We recently investigated the conformation of apoA-I's N-terminus on different sized rHDL by SDSL-EPR analysis at the single-residue level and found that specific structural elements are present in a particle size-dependent fashion. Here, we extended our analysis to three regions of apoA-I's central domain through a combination of SDSL-EPR and fluorescence spectroscopies. Residues 139−144 (loop segment in the looped-belt model), 164−170 (loop segment in the solar-flares model), and 119−124 (amphipathic α-helical lipid-associated residues in both models) were analyzed by tryptophan and spin-label scanning in the lipid-free protein and HDL subclasses 7.8, 8.4, and 9.6 nm in diameter. This array of structural approaches allowed us to differentiate between structural features of apoA-I and chemical features of the protein. Because the solar-flares model was proposed on the basis of hydrogen−deuterium exchange analysis, we hypothesize that this data more likely represented the local chemistry and pK_a of the protein rather than a direct structural aspect of apoA-I. Our structural analysis, in combination with the functional characterization of truncated apoA-I variants, provides new insight into the role of specific central domain structural elements in nascent HDL particle biogenesis.

EXPERIMENTAL PROCEDURES

Materials. Thio-specific nitroxide spin-label (MTS) [(1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl) methanethiosulfonate] was a generous donation from K. Hideg (University of Pecs, Pecs, Hungary). 1-Palmitoyl-2-oleoyl-*sn*-glycerophosphocholine (POPC) was from Avanti Polar Lipids, Inc. (Alabaster, AL).

Production of Recombinant Proteins and Spin-Labeling. Twenty-one single-Cys, 19 single-Trp, and two central region truncation apoA-I cDNA variants were produced using either primer-directed polymerase chain reaction (PCR) mutagenesis or the megaprimer PCR method.[38] In the single-Trp variants, endogenous Trp fluorescence was eliminated by substituting the four native Trp residues (8, 50, 72, and 108)

with Phe as described previously.[22,39] The mutations were verified by dideoxy automated fluorescent sequencing. The proteins were expressed in BL21 *Escherichia coli* (InVitrogen) and purified as previously reported.[40] MTS spin-labeling of cysteines was performed as described previously.[41,42] Protein samples were stored in Tris-buffered saline (TBS) [8.2 mM Tris, 150 mM NaCl, and 1 mM EDTA (pH 7.4)]. Protein purity (>95%) was confirmed by SDS−PAGE analysis (Figure 1A).

Preparation of apoA-I−POPC Complexes. Spin-labeled apoA-I, single-Trp apoA-I, and truncated apoA-I-containing rHDLs were prepared by the deoxycholate method.[43−45] Different rHDL subclasses with 7.8, 8.4, 9.6, 12.2, and 17.0 nm diameters were isolated as described previously.[45] Briefly, POPC, unesterified cholesterol, and proteins were combined in a molar ratio of 160:8:1 to generate 17.0 and 12.2 nm rHDL, 80:4:1 to yield preferentially 9.6 nm rHDL, and 30:2:1 to produce 8.4 and 7.8 nm rHDL. Final rHDL protein−lipid composition was as described previously.[45] The rHDL subclasses were isolated by size exclusion chromatography on a Superdex 200 prep grade XK 16/100 column (GE Biosciences Inc.). The size and purity of the rHDL subclasses were confirmed by nondenaturing gradient gel electrophoresis (NDGGE) (Figure 1B). rHDL samples were stored at 4 °C in TBS (pH 8.0) and used for spectroscopic analysis within 1 week of preparation. For apoA-I:Δ124−155, the lipidation yields were low and the product mixtures very heterogeneous at lipidation molar ratios of 30:1 and 80:1.

Fluorescence Spectroscopy. To investigate the local environment of residues in the central domain of apoA-I, we produced apoA-I variants in which all four native Trp residues were substituted with Phe and a single Trp was introduced at strategic positions (Figure S1 of the Supporting Information). Steady-state fluorescence spectra of lipid-free proteins and corresponding rHDL particles were recorded at 20 °C on a Horiba Jobin-Yvon Fluoromax-4 spectrofluorometer, using a 1.5 nm slit width for both the excitation and emission monochronomators. Samples in TBS were normalized at a 0.5 mg/mL protein concentration and excited at 295 nm. Fluorescence spectra were recorded over the 300−420 nm range. The emission λ_{max} of Trp reports on the polarity of the local environment. According to Burstein et al., Trp residues can be classified as class I (330−332 nm), in apolar protein regions; class II (340−342 nm), on the protein surface but with limited solvent exposure; or class III (350−352 nm), solvent-exposed.[46] Furthermore, λ_{max} has been largely employed as a means of determining Trp "lipid exposure" in lipoproteins.[47,48] It has been empirically established that λ_{max} in the 324−335 nm range indicates residues in contact with lipids or buried in hydrophobic protein pockets, whereas a λ_{max} larger than 345 nm is suggestive of Trp facing the solvent-exposed side of amphipathic α-helices.[19,49] See Figure S2 of the Supporting Information for a representative example of fluorescence emission spectra of a single-Trp protein variant (L144W) in the lipid-free state and on rHDL.

Fluorescence Quenching Analysis. Potassium iodide quenching was employed as a means of establishing positional solvent accessibility, as described previously.[49,50] Sodium thiosulfate (1 mM) was added to a freshly prepared stock solution of KI (4 M) as an antioxidant to prevent formation of triiodide ion (I_3^-).[51] To lipid-free protein or rHDL samples (0.5 mg/mL in apoA-I) was added the KI stock solution in small increments to final KI concentrations from 0 to 1.75 mM. An

emission fluorescence spectrum was collected after each increase in KI concentration, and the emission intensity was corrected for the dilution caused by the corresponding volume increase. The Trp fluorescence collisional quenching by KI was analyzed by Stern−Volmer regression: $F_0/F = 1 + K_{sv}[Q]$. F_0 and F are the maximal emission intensities at an appropriate emission wavelength (in our case λ_{max}) in the absence and presence of KI, respectively. [Q] is the molar concentration of the quencher.[52] The Stern−Volmer quenching constant (K_{sv}) is derived as slope of the F_0/F versus [Q] plot. K_{sv} is proportional to the solvent accessibility of the Trp residue. For totally exposed Trp residues, in the absence of electrostatic or viscosity effects, $K_{sv} = 12$ M^{-1}; on the other hand, for totally protected Trp residues, $K_{sv} = 0$ M^{-1}.[53] For apolipoproteins, K_{sv} values of <2.5 have been empirically assigned to Trp residues in a nonpolar environment and K_{sv} values of >3.5 M^{-1} to solvent-exposed Trp residues.[49]

Electron Paramagnetic Resonance (EPR) Spectroscopy. The 100 G width EPR spectra were collected on a JEOL X-band EPR spectrometer fitted with a loop-gap resonator.[54,55] Aliquots (5−6 μL) of protein or lipoprotein samples (60 μM spin-labeled protein) were loaded into quartz microcapillaries sealed at one end and placed in the resonator for EPR measurements. Spectra were acquired at room temperature (20−22 °C) from a single 2 min scan over a field of 100 G at a microwave power of 4 mW with a modulation amplitude optimized to the natural line width of the individual spectrum (1.0−1.6 G). Normalization was calculated from the sample alleviated from broadening by integrating the spectrum obtained in the presence of sodium dodecyl sulfate [2% (w/v)].

The motional freedom of the spin-label determines the spectral features of the X-band EPR spectrum; thus, analysis of the spectra of a series of consecutive spin-labeled side chains provides information about the level of local structural order. We measured the mobility parameter, δ^{-1}, of single spin-labeled apoA-I variants as the inverse of the central line width of the EPR spectra and analyzed the results in the context of an amphipathic α-helix model. Restriction in mobility may arise from intra or intermolecular protein−protein contacts and, in lipoproteins, by protein−lipid contacts.

The molecular accessibility to the spin-label was probed by measuring the EPR signal quenching by two diffusible relaxation agents, the polar chromium oxalate (CrOx) and the nonpolar oxygen (O_2). The accessibility parameters ($\Pi_{1/2}$) of the two quenchers were calculated using software provided by C. Altenbach as described previously.[41,42,56] The polarity index (or contrast function, Φ) was calculated as the logarithmic ratio of the $\Pi_{1/2}$ values of the polar and nonpolar quenchers. For lipid-associated proteins, the polarity index provides a measure of the membrane penetration depth of the spin-label.[57,58] Residues with a Φ of greater than −0.5 were scored as lipid-buried, whereas residues with a Φ of less than −1.5 were considered largely solvent-exposed. Intermediate Φ values were assigned to residues in the water−lipid interface.[59,60]

LCAT Activation Assay. LCAT activation by rHDL was monitored by following the conversion of cholesterol to cholesteryl ester as previously reported.[45,61] Briefly, five rHDL subclasses were generated as described above but in the absence of unesterified cholesterol and were characterized by NDGGE and chemical composition (POPC:apoA-I ratio). [1,2-³H]-Cholesterol was introduced exogenously into the rHDL complexes. rHDL samples (2 μg in apoA-I) were diluted in

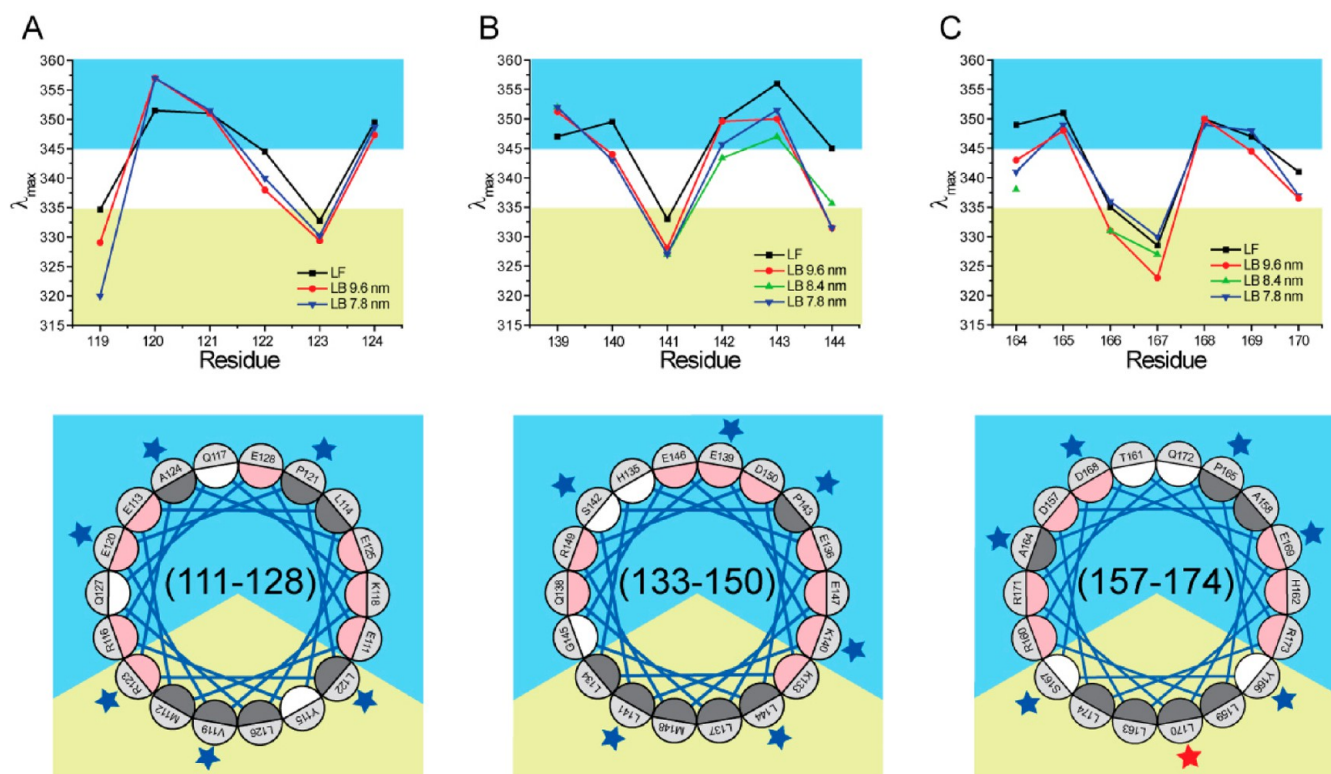**Figure 2.** Maximal fluorescence emission wavelengths (λ_{max}) of residues 119−124 (A), 139−144 (B), and 164−170 (C). Bottom quadrants are α18/5 pitch Schiffer−Edmunson helical wheel diagrams of regions 111−128, 133−150, and 157−174, respectively. Residues are colored according to side chain polarity: dark gray for hydrophobic, white for polar and uncharged, and pink for charged residues. The orientation of the amphipathic wheels was solely based on clustering of hydrophobic residues on a sector of the wheel. The apolar and polar solvation space are colored yellow and blue, respectively. Residues 119−124, 139−144, and 164−170 are marked by blue or red stars if their λ_{max} values are or are not consistent with a lipid-associated amphipathic α-helical conformation, respectively.

TBS and equilibrated with radiolabeled cholesterol by incubation at 37 °C for 30 min with 1.39 pmol of [1,2-^{3}H]cholesterol (57.6 Ci/mmol; PerkinElmer Life and Analytical Sciences Inc., Boston, MA). The mixtures were incubated at 37 °C for 1 h in the presence of 5 mg/mL (final) BSA and 4 mM (final) β-mercaptoethanol. LCAT (50 ng) was added, and the reaction mixture was incubated for 1 h at 37 °C; the reactions were terminated by the addition of 1 mL of absolute ethanol. Lipids were extracted with hexane, and the cholesterol and cholesteryl ester were separated by thin layer chromatography using a hexane/ethyl ether/acetic acid mixture [108:30:1.2 (v:v:v)] as the solvent phase. The lipid spots were visualized under iodine vapor and recovered for scintillation analysis (LS 6500 multipurpose scintillation counter, Beckman Coulter, Fullerton, CA). The LCAT reaction conditions were chosen on the basis of the linear response range of LCAT.

Statistical Analysis. Results are the average of at least three independent experiments, in which the process of protein expression, labeling, HDL reconstitution, rHDL purification, and spectroscopic acquisition was repeated. Because there was a small and consistent ($\leq$10%) standard deviation for a majority of the data, error bars are not reported in Figures 2−5 for the sake of clarity. A Student's two-tailed, unpaired t test was used for statistical analyses, and a P of <0.05 was considered significant.

Assessment of ApoA-I Conformation. To survey the structural adaptation of apoA-I from lipid-free to rHDL particles of different sizes, 19 residues in three regions of the central domain of apoA-I were mutated to a single Trp (residues 119−124, 139−144, and 164−170) and 21 residues were mutated to a single Cys (residues 119−124, 133, 139−144, 146, and 164−170). Single-cysteine-bearing apoA-Is were spin-labeled with a nitroxide spin-label for EPR analysis. Single-Trp or single spin-label-containing protein variants were reconstituted into HDL particles 7.8, 8.4 (for positions 139−143 and 164−170), and 9.6 nm in diameter. Lipid-free and rHDL samples were analyzed using two physical methods, fluorescence spectroscopy and EPR. Each apoA-I residue examined was evaluated on the basis of four metrics of protein conformation. (1) Local polarity was assessed from the maximal fluorescence emission wavelength (λ_{max}) of single-Trp-bearing apoA-I mutants. (2) Solvent accessibility was measured by the susceptibility of single-Trp-bearing apoA-Is to fluorescence quenching by potassium iodide. (3) Residue polarity was evaluated by measuring the molecular accessibility ($\Pi_{1/2}$) of two diffusible relaxation agents by EPR and the derived polarity index (Φ). For lipoproteins, Φ is dependent upon the depth of penetration of the spin-label into the phospholipid bilayer. (4) Residue mobility was determined from the line width (δ^{-1}) of the EPR spectrum. These data were then modeled in the context of an ideal amphipathic α-helix of α11/3 (11 residues) or α18/5 (18 residues) pitch (Figure S3 of the Supporting Information). The identification of solvent-exposed surface and the periodicity of the backbone structure were determined from the modulation of these parameters as a function of sequence position.
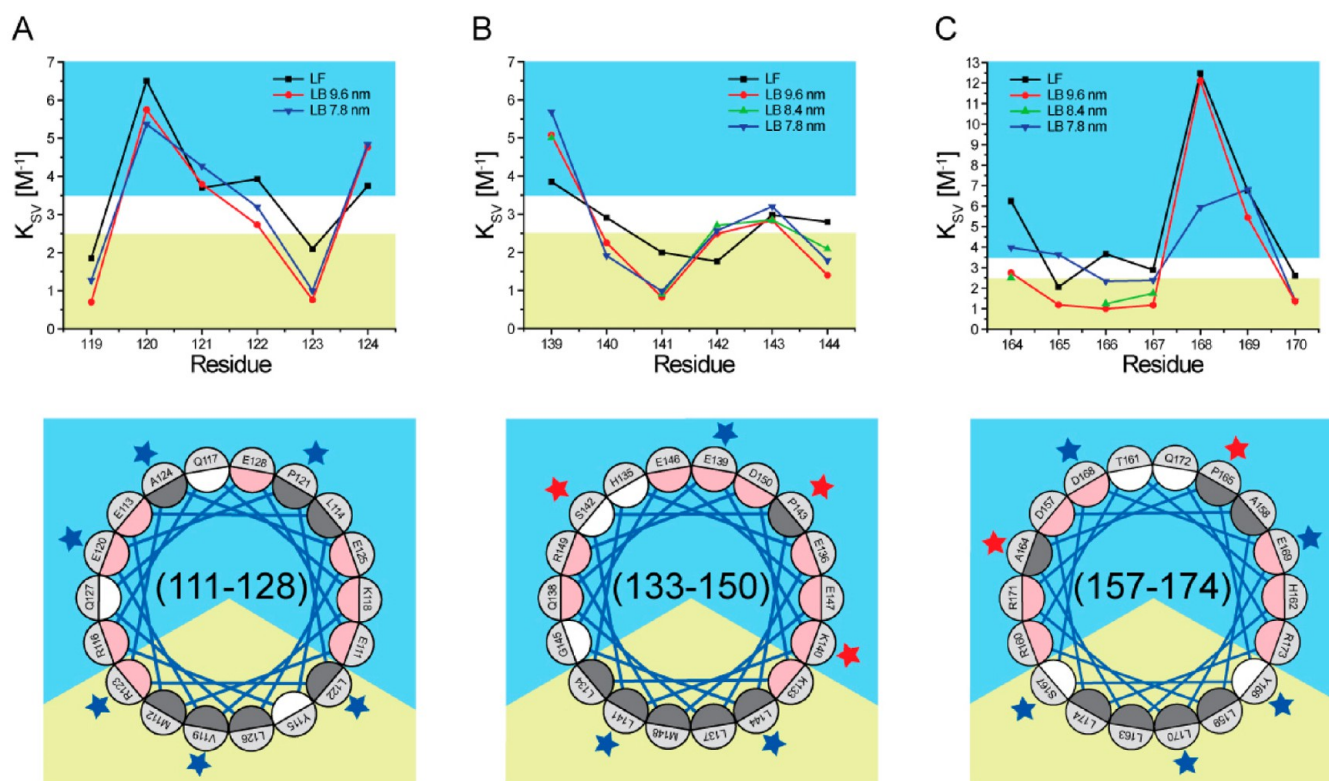
Figure 3. Stern−Volmer constants (K_{sv}) of residues 119−124 (A), 139−144 (B), and 164−170 (C). Bottom quadrants are Schiffer−Edmunson helical wheel diagrams of residues 111−128, 133−150, and 157−174, respectively, oriented and colored as described in the legend of Figure 2. Residues 119−124, 139−144, and 164−170 are marked by blue or red stars if their K_{sv} values are or are not consistent with a lipid-associated amphipathic α-helical conformation, respectively.

■ RESULTS

Selection of Scanned Positions within apoA-I. The residues selected for analysis represent three unique portions of apoA-I on HDL. Amino acids 139−144 are within a loop region in the looped-belt model (residues 134−145).[22] Residues 164−170 are in the center of a loop region in the solar-flares model (residues 159−180).[24] In contrast, residues 119−124 have been reported to form a lipid-associated amphipathic α-helical structure on two-apoA-I-containing rHDL[20−25,34] and were evaluated as a "control" set of residues. All three regions contain a single Pro and span an amphipathic α-helical junction, traditionally termed H4−H5, H5−H6, and H6−H7 for residues 119−124, 139−144, and 164−170, respectively (Figure S1 of the Supporting Information).[62,63]

Trp Fluorescence Scanning. To assess the secondary structure of residues 119−124, 139−143, and 164−170 of apoA-I, we determined the polarity of the local environment surrounding these residues by Trp scanning fluorescence analysis. To permit this survey, we substituted all four Trp residues of wild-type apoA-I with Phe and produced 19 single-Trp variants. The protein variants were used to reconstitute 7.8 and 9.6 nm rHDL. For residues 139−144 and 164−170, 8.4 nm rHDL particles were also generated. The emission λ_{max} values of lipid-free and rHDL-associated proteins were evaluated. When apoA-I was associated to rHDL, the λ_{max} for the resident Trp was blue-shifted up to 14 nm, compared to the lipid-free protein (Figure S2 of the Supporting Information). This blue shift is the hallmark of changes in local polarity experienced by the tryptophan.[52]

The λ_{max} values of the residues examined (Figure 2) exhibit consistent periodic patterns, yielding three primary observations. First, for a majority of the 19 residues, there were no significant differences in λ_{max} between lipid-free and HDL-associated apoA-I. Thus, the environments surrounding the examined residues have similar polarity regardless of whether protein−protein or protein−lipid interactions are predominant.

Second, local polarity was not significantly different among the three rHDL subclasses analyzed. Only residues 119, 142, 143, and 167 showed small differences among the various rHDL subclasses, which however did not affect the general score for the four residue environments (i.e., solvent-exposed for residues 142 and 143 and apolar for residues 119 and 167).

Third, the general pattern of λ_{max} periodicity is consistent with a lipid-associated amphipathic α-helical model for all three regions in the three rHDL subclasses analyzed (Figure 2). Only the λ_{max} of residue 170 is inconsistent with a residue within a lipid-associated amphipathic α-helix, in which it would be deeply buried in the lipid bilayer. However, although in rHDL the λ_{max} of Trp 170 (336 and 337 nm for 7.8 and 9.6 nm rHDL, respectively) approaches the value empirically determined as being indicative of a residue undergoing the transition from polar to nonpolar environments (335 nm), this blue shift is too small to reclassify the residue as residing in a nonpolar environment.

Solvent Accessibility by Trp Fluorescence Quenching Scanning. The relative solvent exposure of the central domain residues was measured by potassium iodide quenching of Trp fluorescence for the 19 single-Trp apoA-I variants in the lipid-free and HDL-associated forms.
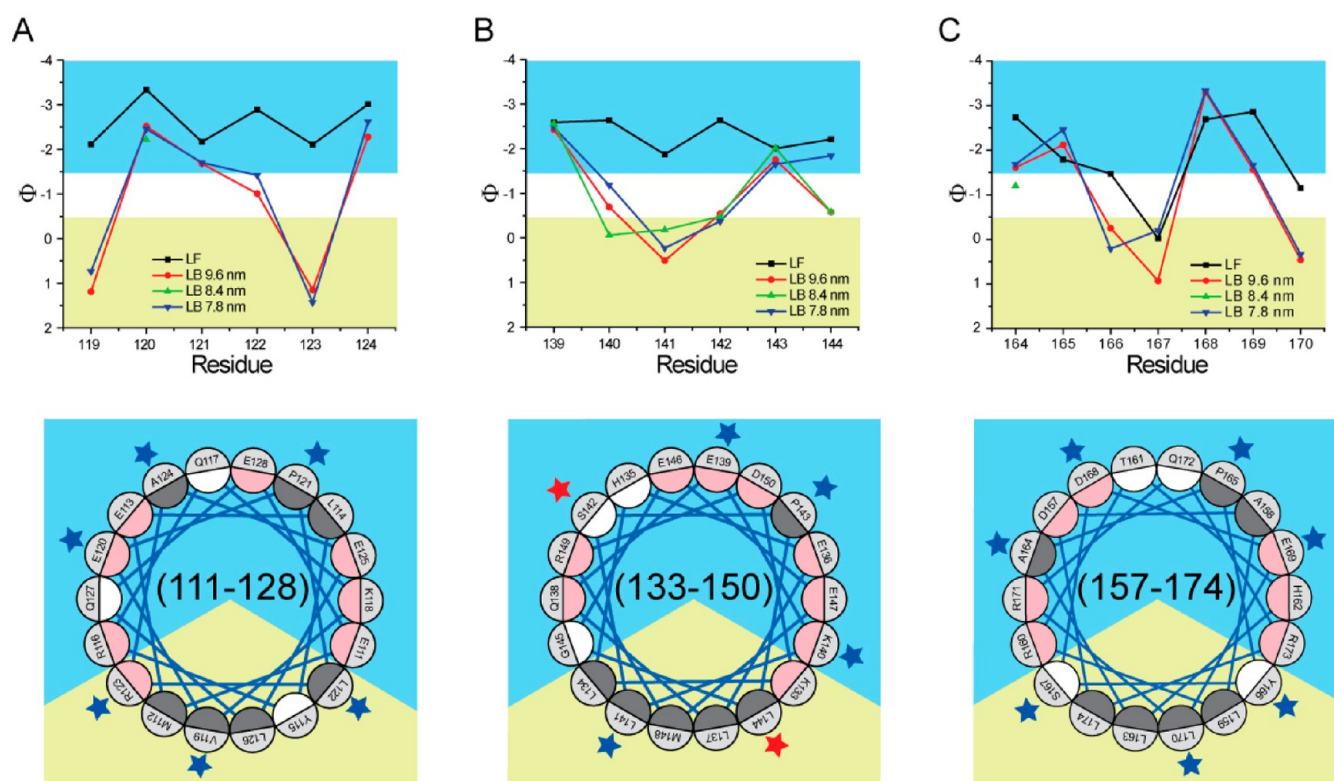
Figure 4. Polarity indexes (Φ) of residues 119−124 (A), 139−144 (B), and 164−170 (C). Bottom quadrants are Schiffer−Edmunson helical wheel diagrams of residues 111−128, 133−150, and 157−174, respectively, oriented and colored as described in the legend of Figure 2. Residues 119−124, 139−144, and 164−170 are marked by blue or red stars if their polarity index values are or are not consistent with a lipid-associated amphipathic α-helical conformation, respectively.

Consistent with the local polarity results obtained by Trp λ_{max} scanning, solvent accessibility was not significantly different between lipid-free and HDL-associated apoA-I. Thus, the native folding of lipid-free apoA-I determines the local polarity and solvent accessibility of the protein segments under analysis that are similar to the local environment occurring upon binding of the protein to the edge of a phospholipid bilayer. It is important to notice that protein−protein interactions in lipid-free apoA-I can be intra or intermolecular. Under the experimental conditions described here (~18 μM), lipid-free apoA-I is self-associated and intermolecular contacts may contribute to the folding of the protein. This is particularly true for Trp to Phe apoA-I variants that exhibit higher levels of self-association compared to that of wild-type apoA-I[64] or the single-Cys mutants used for EPR analysis.

In the rHDL particles analyzed, the periodicity of Stern−Volmer constants (K_{sv}) (Figure 3) for residues 119−124 was compatible with the lipid-associated amphipathic α-helical model and similar to the trend observed for Trp fluorescence λ_{max} (Figure 2). In contrast, K_{sv} values for three residues (140, 142, and 143) of the six in region 139−144 were too low to correspond to an orientation toward a solvent-exposed environment and inconsistent with a lipid-associated amphipathic α-helical conformation. Thus, the K_{sv} results do not support an ideal lipid-associated amphipathic α-helical structure for residues 139−144. No significant differences in K_{sv} were observed for residues 119−124 and 139−143 for different rHDL subclasses, which is in agreement with the fluorescence λ_{max} analysis results.

The K_{sv} results for residues 164−170 were unanticipated. First, the K_{sv} constants of residue 168 in the lipid-free protein

and 9.6 nm rHDL (12.48 and 12.12 M^{-1}, respectively) were much higher than those of any other residues analyzed and similar to that of totally exposed Trp.[53] Second, unique in the whole series, K_{sv} values of five (164−168) of seven residues in region 164−170 were significantly different between 7.8 and 9.6 nm rHDL. Third, although K_{sv} values of all residues in 7.8 nm rHDL were compatible with the lipid-associated amphipathic α-helical model, K_{sv} values of two residues ($K_{sv} = 2.75$ M^{-1} for residue 164, and $K_{sv} = 1.19$ M^{-1} for residue 165) in 9.6 nm rHDL did not match the solvent accessible prediction of the model.

Combined, the fluorescence results (λ_{max} and K_{sv}) indicate that residues 119−124 form a lipid-associated amphipathic α-helix in all rHDL particles analyzed. In contrast, while fluorescence λ_{max} results for residues 139−144 are compatible with an amphipathic α-helical structure, K_{sv} analysis does not support the same conclusion. The fluorescence results for residues 164−170 are consistent with a lipid-associated amphipathic α-helix in 7.8 nm rHDL, but some K_{sv} results are outliers both in absolute value (residue 168) and in trend (residues 164 and 165) for an amphipathic α-helical structure in 9.6 nm rHDL. The apparently contradictory K_{sv} results for residues 164−170 may have been produced by the unique charge properties of this region.

Side Chain Polarity Determined by EPR. The polarity of residue side chains labeled with a nitroxide spin-label can be quantified from the relative collision frequency of the nitroxide with polar (CrOx) or nonpolar (O$_2$) relaxation agents. The polarity index (Φ) was calculated for all residues analyzed and used to generate regional topological maps of apoA-I (see Experimental Procedures). Specifically, the secondary structure
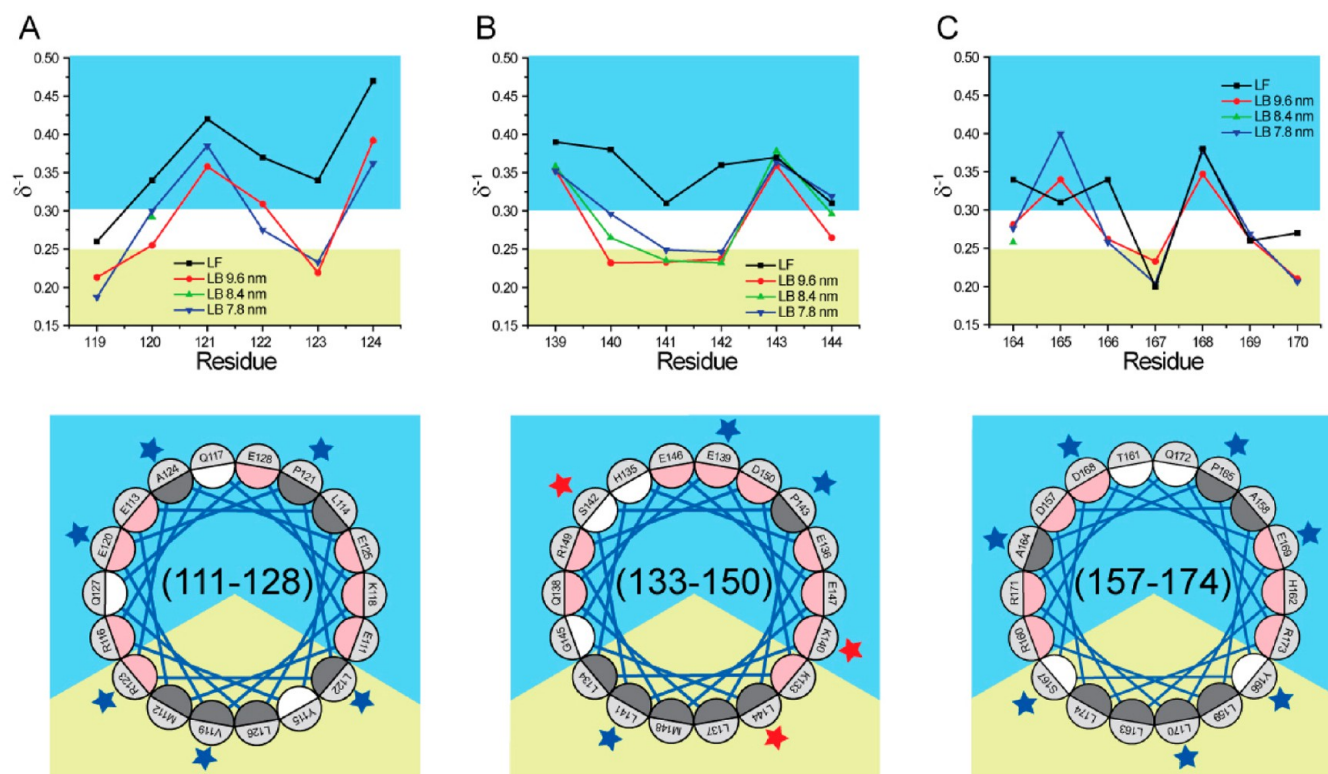
Figure 5. Mobility parameters (δ^{-1}) of residues 119−124 (A), 139−144 (B), and 164−170 (C). Bottom quadrants are Schiffer−Edmunson helical wheel diagrams of regions 111−128, 133−150, and 157−174, respectively, oriented and colored as described in the legend of Figure 2. Residues 119−124, 139−144, and 164−170 are marked by blue or red stars if their mobility scores are or are not consistent with a lipid-associated amphipathic α-helical conformation, respectively.

was inferred from the periodicity of Φ as a function of residue number; amphipathic α-helical structures typically display a periodicity of 3.6, whereas β-strand structures exhibit an alternating periodicity.

Interestingly, all residues in regions 119−124 and 139−144 of the lipid-free protein exhibited a very negative ($\Phi \leq -1.87$) polarity index (very polar environment) (Figure 4). In contrast, the range of the polarity index for residues 164−170 was much higher (less polar) (Φ, −2.86 to −0.02) compared to those of residues 119−124 (Φ, −3.34 to −2.11) and 139−144 (Φ, −2.64 to −1.87). In particular, residue 167 was the only residue of 19 with a polarity index greater than −0.5 (−0.02).

Once apoA-I had bound lipids, the polarity index values of 10 of 12 residues in regions 119−124 and 139−144 increased. Only the polarity index of residues 139 and 143 did not change significantly (Figure 4). In contrast, although the polarity index of five of seven residues within region 164−170 (164, 166, 167, 169, and 170) increased, the polarity index of residues 165 and 168 (lipid-free, Φ values of −1.69 and −2.69, respectively) significantly decreased when the protein was HDL-associated (for 7.8 nm, Φ values of −2.46 and −3.33, respectively; for 9.6 nm, Φ values of −2.12 and −3.29, respectively).

The polarity index pattern of the residues in regions 119−124 and 164−170 is consistent with these residues assuming a lipid-associated amphipathic α-helical conformation for all rHDL sizes analyzed. In contrast, within region 139−144, two (residues 142 and 144) of the six positions examined bore polarity values inconsistent with an interpretation indicating that these residues assume an amphipathic α-helical structure, as position 142 is too hydrophobic for an aqueous exposed residue and position 144 is too hydrophilic for a lipid-

embedded position. This is true for 7.8, 8.4, and 9.6 nm rHDL, with sites 142 and 144 having Φ values of −0.37, −0.48, and −0.54 and −1.84, −0.58, and −0.58 for the three rHDL sizes, respectively. In contrast, the polarity indexes of analogous residues in region 119−124 (for positions 119 and 124, Φ = 0.74 and 1.19 for 7.8 nm rHDL and Φ = −2.62 and −2.28 for 9.6 nm rHDL, respectively) are consistent with a lipid-associated amphipathic α-helical conformation.

Steric Environment of Residue Side Chains. Local steric constraints imposed upon a spin-labeled side chain can be quantified from the inverse of the central peak-to-peak width of the spin-label EPR spectrum, which defines the mobility parameter (δ^{-1}). The patterns of side chain mobility (δ^{-1}) (Figure 5) and polarity index (Φ) (Figure 4) for all residues examined are strikingly similar. This observation is even more remarkable considering that the two data sets are based on different physical properties and derived from independent experiments.

In the absence of lipids, a majority of the 19 residues examined were highly mobile ($\delta^{-1} \geq 0.27$). Although residues 119, 167, 169, and 170 were partially restricted in terms of mobility, only δ^{-1} of residue 167 was below 0.25, the value empirically selected for marking the transition between mobile and immobilized residues (Figure 5).

The conformational adaptation that occurs upon apoA-I lipid binding produced significant changes in residue mobility. With the exception of position 143, all residues in regions 119−124 and 139−144 exhibited reduced side chain mobility on all rHDL particles analyzed (Figure 5). In rHDL, residues 121 and 124 retained a high level of mobility ($\delta^{-1} \geq 0.36$), as expected for residues that are solvent-oriented within a lipid-associated
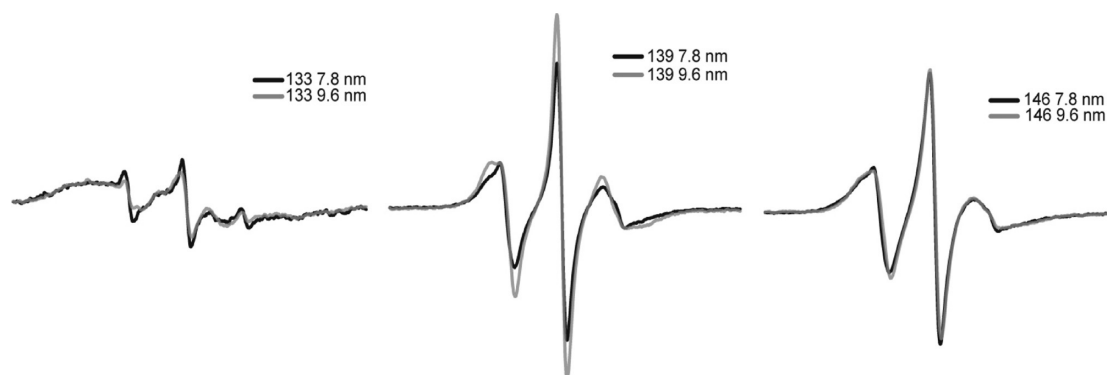
Figure 6. EPR spectra of spin-labeled K133C, E139C, and E146C apoA-I variants, in 7.8 nm (black) and 9.6 nm (gray) rHDL.

amphipathic α-helical structure. In contrast, the mobility of residues 119 and 123 was very low on all rHDL particles ($\delta^{-1} \leq 0.23$), consistent with the orientation of these residues toward a lipid environment. The mobility of residues 120 and 122 was also significantly lower in all lipid-bound forms of the protein ($\delta^{-1} \leq 0.30$ and $\delta^{-1} \leq 0.31$, respectively). Whereas a decrease in the mobility of residue 122 can be ascribed to the proximity of this residue to the water−lipid interface, the mobility of residue 120 in 9.6 nm rHDL ($\delta^{-1} = 0.26$) is unexpectedly low for a residue oriented toward solvent in a lipid-associated amphipathic α-helix.

Notably, the side chain mobility for residues 139−144 of lipid-free apoA-I was smaller (δ^{-1} ranging between 0.31 and 0.39, average of 0.35) than for residues 119−124 (δ^{-1} ranging between 0.26 and 0.47, average of 0.37). Although lipid binding produced a drastic decrease in side chain mobility ($\Delta\delta^{-1}$ ranging from −0.04 to −0.15), the interpretation of this change is not straightforward. If forming an amphipathic α-helix, mobilities of residues 141 and 144 are anticipated to be the most affected by lipid binding. Interestingly, the decrease in the mobility of residues 141 and 144 ($\Delta\delta^{-1}$, −0.08 and −0.04, respectively) was not as large as for analogous residues 122 and 123 ($\Delta\delta^{-1}$, −0.09 and −0.11, respectively) in region 119−124. Furthermore, δ^{-1} of residue 144 was significantly reduced on 9.6 nm rHDL ($\Delta\delta^{-1}$, −0.04) but not on 7.8 nm rHDL ($\Delta\delta^{-1}$, 0.01) or 8.4 nm rHDL ($\Delta\delta^{-1}$, −0.01), compared to that of the lipid-free protein. Residues 140 and 142, although predicted by the lipid-associated amphipathic α-helical model to be marginally affected by lipid interaction, experienced the largest mobility reduction of all residues examined ($\Delta\delta^{-1}$, −0.15 and −0.13, respectively). Furthermore, the mobility of residue 143, predicted to reside on the apolar face of an amphipathic α-helix, was unaffected by lipid binding, whereas the mobility of the highly unrestricted residue 139 was significantly reduced in all lipid-bound forms, which is inconsistent with the prediction of an amphipathic α-helical model.

The overall side chain mobility of residues 164−170 in the lipid-free protein was considerably lower than that of residues 119−124 and 139−144. Notably, the motion of residue 167 was highly restricted in the lipid-free state ($\delta^{-1} = 0.20$), suggesting strong immobilization due to protein contacts of a buried site.[65]

Because of the lower mobility of residues 164−170 in the lipid-free protein, compared to that of residues 119−124 or 139−144, lipid binding yielded an assortment of mobility changes, whereas for residues 119−124 and 139−144, lipid binding led to wholesale reductions in side chain mobility.

Specifically, three of seven residues from position 164 to 170 exhibited a significant increase in mobility (residues 165 and 168) or no change at all (residue 169). Residue mobilities on rHDL particles of all sizes were consistent with a lipid-associated amphipathic α-helical conformation for this region (164−170). Residues 166, 167, and 170, predicted to be in contact with lipids on the basis of their positions within an amphipathic α-helix, were indeed the least mobile ($\delta^{-1} \leq 0.26$). Also consistent with an amphipathic α-helical conformation, residues 165 and 168 were highly mobile ($\delta^{-1} \geq 0.34$), indicating solvent exposure. Finally, residues 164 and 169 exhibited intermediate mobility values (0.26 and 0.28, respectively), consistent with a position at the oil−water interface, as predicted by the amphipathic α-helical model.

Combined, the EPR results (side chain mobility and polarity index) support three primary conclusions. (1) In the lipid-free protein, residues in regions 119−124 and 139−144 are not restricted in motion or polar solvent accessibility, whereas two residues in region 164−170 exhibit restricted mobility and polar relaxation agent accessibility ($\Pi_{CrOx} = 0.042$ and 0.194 for residues 167 and 170, respectively). (2) In the lipid-bound states, regions 119−124 and 164−170 fit a lipid-associated amphipathic α-helical model, whereas residues 139−144 do not exhibit an ideal amphipathic α-helical pattern. (3) Notably, the side chain mobility and polarity index of a majority of the 19 residues analyzed do not significantly vary for different HDL subclasses. The spectra of spin-labels at the other two positions (133 and 146) of the three key positions identified by the looped-belt model (residues 133, 139, and 146) were also virtually identical in 7.8 and 9.6 nm rHDL (Figure 6).

rHDL Particle Formation and LCAT Activation by Central Loop Truncation Variants. To test the hypothesis that residues 134−145 are essential for LCAT activation but contribute minimally to the overall structure of two-apoA-I-containing rHDL particles (7.8, 8.4, and 9.6 nm rHDL[45]), we produced two apoA-I truncation variants (apoA-I:Δ134−145 and apoA-I:Δ124−155) in which 12 and 22 residues, respectively, in the central domain were deleted. Removal of a non-lipid-associated portion of apoA-I would have a minimal effect on the size of the rHDL particles. In contrast, elimination of residues that form a portion of the "belt" circumscribing the HDL particle perimeter would have a noticeable effect on the size of the reconstituted particles.

Wild-type apoA-I was combined in various ratios with POPC to reconstitute HDL particles of five sizes, 7.8, 8.4, 9.6, 12.2, and 17.0 nm (see Experimental Procedures and Figure 1B). When reconstituted under the same conditions, apoA-I:Δ134−

145 retained the ability to form four of five particle sizes, with a particle equivalent to the 12.2 nm wild type being the largest achievable rHDL (Figure 1B). Interestingly, the largest truncation (apoA-I:Δ124−155), yielding the shortest apoA-I variant tested, was able to generate a particle equivalent to 12.2 nm wild-type rHDL, but none of the two-apoA-I-containing rHDL particles (Figure 1B).

Formation of cholesteryl esters by LCAT in the presence of rHDL particles of different sizes and protein compositions was measured. Consistent with our hypothesis that the region encompassing residues 134−145 predominantly facilitates LCAT activation, the LCAT activation ability of rHDL particles reconstituted with apoA-I:Δ134−145 was dramatically reduced (Figure 7), although the apparent size of the particles was not
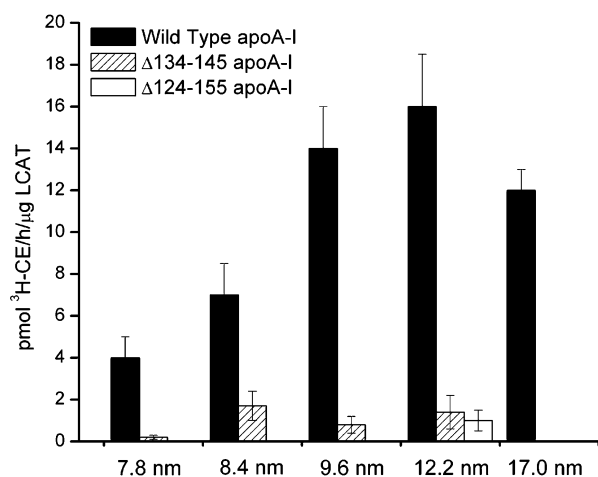

Figure 7. LCAT activation by wild-type rHDL (five particle sizes), Δ134−145 apoA-I rHDL (four particle sizes), and Δ124−155 apoA-I rHDL (12.2 nm rHDL only). Means and standard deviations (error bars) are from three independent experiments.

significantly altered (Figure 1B). In contrast, when residues essential for rHDL formation (124−133 and 146−155) and the structure essential for LCAT activation (residues 134−145) were deleted, both functional [LCAT activation (Figure 7)] and structural [HDL forming ability (Figure 1B)] aspects of HDL were compromised. Notably, with a change in the stoichiometry of apoA-I per particle to greater than two apoA-I molecules, the ability of apoA-I:Δ124−155 to form rHDL was rescued (Figure 1B; 12.2 nm rHDL), but its LCAT activation function was not (Figure 7).

■ DISCUSSION

Despite the prominent antiatherogenic role of apoA-I, the detailed structures of the lipid-free and HDL-associated forms of this protein are largely unknown. Because structural determinants that reside in the central domain of apoA-I are hypothesized to mediate HDL function, a detailed comparative analysis at the single-residue level of the structural adaptation of the central domain of apoA-I upon the transition from lipid-free to different subclasses of rHDL may provide insight into how HDL function is modulated in an HDL particle size-dependent fashion. For example, the binding and activation of LCAT are critical elements in cholesterol metabolism and involve residues in helices 4−6 (residues 99−164) of HDL-associated apoA-I.[63,66−80] Specific structural features in this domain may influence apoA-I's susceptibility to oxidative reactions that

weaken HDL's ability to mediate LCAT activation. These structural details have recently been the subject of much debate.[22,24,30,35]

Previously, we examined the conformation of residues within the central domain of apoA-I (61−221) on 9.6 nm rHDL by SDSL-EPR.[22] Residues 133 and 146 displayed a high degree of spin coupling, suggesting that the single spin-label at these positions is proximal to the analogous spin-label of the paired apoA-I molecule on 9.6 nm rHDL. Furthermore, position 139 showed a low level of spin coupling and an unusually high degree of mobility, suggesting that this residue may be part of a very flexible segment. A model compatible with these observations (the looped-belt model) assigned positions 133 and 146 at the "hinges" of a loop segment, with position 139 at the apex of the loop. According to the looped-belt model, residues 134−145 are likely poorly lipid-associated and therefore differ from the rest of the residues examined, which form a lipid-associated amphipathic α-helical belt circumscribing the rHDL particle perimeter. These hypotheses were supported by fluorescence resonance energy transfer experiments,[22] which confirmed that apoA-I on 9.6 nm rHDL forms an antiparallel association aligned at residue 133.

Subsequent hydrogen−deuterium exchange studies performed by Wu et al.[24] resulted in the solar-flares model, in which residues 159−180 compose a large loop region that extends off the surface of 9.6 nm rHDL. In addition to a unique proton exchange profile, this loop structure, which resides within the LCAT activation domain, should also have dynamic and polarity properties distinctive from those of the remainder of apoA-I (rHDL-associated).

To gather more detailed structural information about the loop regions postulated by the solar-flares and looped-belt models, we undertook direct measurements of solvent accessibility, local polarity, and backbone dynamics for three protein segments (residues 119−124, 139−144, and 164−170) by using site-directed fluorescence and EPR spectroscopies. While both EPR (of site-directed spin-labels) and fluorescence (of Trp-substituted residues) measurements have been extensively used to explore lipid−protein interactions independently, a direct comparison of their results for a successively scanned region of protein sequence has not been performed. Therefore, we evaluated the complementarity of these methods in determining the local charge, backbone structure, and orientation of protein segments associated with lipid particles. These methodologies yielded distinct and complementary information about the local chemistry and dynamics of the targeted positions in apoA-I.

This multivariable approach provides a robust means of cross-validation and data interpretation. If a residue exhibits biophysical properties that are inconsistent with the prediction of a secondary or tertiary structural model, the apparent outlier value can be reconciled by quantifying other biophysical parameters at the same location.

Taken together, the results reported here support the conclusion that residues 119−124 form a stable lipid-associated amphipathic α-helix that is not significantly affected by changes in rHDL particle size. This latter observation is in contrast with the results of the hydrogen exchange mass spectrometry study of Chetty et al., which suggest that residues 115−158 do not form a lipid-associated α-helix but a disordered loop in 7.8 nm rHDL.[34] However, sample preparation was slightly different in the two studies. Whereas in our rHDL preparations we used a small amount of unesterified cholesterol (∼4 cholesterol

molecules per 7.8 nm rHDL particle), Chetty et al. analyzed cholesterol-free rHDL. The presence of a small amount of cholesterol in rHDL (<6 molecules per particle) facilitates deeper penetration of apolipoproteins into the lipid bilayer with a small increase in helical content.[81,82] Furthermore, residues in the "central domain" of apoA-I are particularly sensitive to the presence of cholesterol.[83] We speculatively propose that the presence of a low molar ratio of cholesterol in our rHDL preparations may be sufficient to stabilize the amphipathic α-helical structure of residues 119−124, explaining the discrepancy between our results and those of Chetty et al.[34] Further investigation is necessary to validate this hypothesis.

In the looped-belt model,[22] residues 134−145 form a loop dissociated from the lipid bilayer. The current data recapitulate the high degree of spin coupling for residues 133 and 146 and the high mobility of residue 139 on 9.6 and 7.8 nm rHDL (Figure 6), suggesting that the relative alignment of apoA-I on rHDL is not affected by particle size and that the conformation of residues 134−145 is a consistent feature of rHDL-associated apoA-I. Interestingly, residues 134−145 comprise a loop region in the lipid-free form of the protein.[84]

For residues 139−144 within the postulated loop, the periodicity of three of four biophysical parameters suggests that these amino acids do not form an ideal amphipathic α-helical structure in any of the three rHDL subclasses analyzed. Specifically, the side chain mobility and KI quenching of Trp fluorescence of three positions and the EPR polarity index of two of six residues did not have the periodicity (3.6 per turn) expected for an ideal amphipathic α-helical structure. These results suggest that residues 139−144 are non-α-helical or reside on a flexible α-helical segment in rapid association−dissociation dynamics with the edge of the phospholipid bilayer. Importantly, the biophysical properties of residues 139−144 are not dependent on the rHDL subclass.

A series of computational studies support the hypothesis that apoA-I helix 5 (residues 122−142) in discoidal HDL is loosely associated with the lipid bilayer to form an amphipathic presentation tunnel that assists the transfer of phospholipids and unesterified cholesterol to the active site of LCAT.[32,33] The results presented here provide experimental evidence in favor of this hypothesis. The presence of a non-lipid-associated structural motif on rHDL particles of different sizes (7.8, 8.4, and 9.6 nm rHDL) and the ability of these particles to activate LCAT[45] led to the hypothesis that this conserved structural feature facilitates LCAT activation. Results from apoA-I truncation variants suggest that the computationally postulated amphipathic presentation tunnel is the structure (residues 134−145) that we identified experimentally. Notably, deletion of residues 134−145 completely abolished apoA-I's ability to activate LCAT (Figure 7), without compromising rHDL particle size (Figure 1), supporting the hypothesis that positions 134−145 reside on a non-lipid-associated segment that does not contribute to particle size.

To compare the results obtained for residues 139−144 to those of another postulated loop region, we extended our analysis to residues 164−170, which in the solar-flares model for 9.6 nm rHDL form an extended loop structure.[24] Our biophysical analysis indicates that this region bears unique structural and chemical features. In the lipid-free form of the protein, side chain mobilities of residues 167 and 170 were the lowest and their polarity indexes the highest of the 19 residues investigated here, suggesting that this region forms a hydrophobic pocket in which protein−protein contacts significantly restrict side chain mobility. In contrast, however, residue 168 had an unusually high K_{sv} (12.48 M^{-1}; extremely solvent accessible), which is almost 2 times greater than the next highest value within the range of residues examined (for residue 120, K_{sv} = 6.51 M^{-1}). While typically this result would lead to the conclusion that residue 168 is highly exposed to solvent, the three other biophysical parameters examined (side chain mobility, polarity index, and Trp λ_{max}) do not substantiate this interpretation. Thus, we conclude that the high K_{sv} value for this residue reflects a unique local chemistry rather than an unexpected protein structure.

Although region 164−170 may experience unique tertiary−quaternary structural interactions in the lipid-free form of the protein, our data are consistent with these residues assuming a standard lipid-associated amphipathic α-helical structure when the protein is HDL-associated. This is substantiated by the periodicity of the side chain mobility, polarity index, and Trp λ_{max} for all rHDL subclasses analyzed and by the periodicity of K_{sv} for 7.8 nm rHDL. In contrast, KI quenching results for residues 164 and 165 in 9.6 nm rHDL are inconsistent with an amphipathic α-helical structure. However, KI as a fluorescence quenching probe is strongly influenced by local charge, and the microenvironment surrounding positions 164 and 165 is rich in charged residues (R160, R171, D157, D168, and E169). Thus, we propose that the K_{sv} values for residues 164 and 165 may be a reflection of the unusual local pK_a in the region.

In conclusion, examination of three unique segments of apoA-I's central domain (residues 119−124, 139−144, and 164−170) using four distinct biophysical techniques (Trp λ_{max}, Trp fluorescence KI quenching, polarity index, and side chain mobility) revealed that while these residues undergo a large structural rearrangement upon lipid association, their lipid-bound conformation is maintained in rHDL particles of different sizes (7.8−9.6 nm). Thus, while discoidal HDL particles may alter their general morphology to accommodate changes in lipid composition,[27,33,45,85−87] from our measurements of either backbone−side chain dynamics or differential accessibility, we can conclude that the three regions analyzed retain their secondary structure within the rHDL subclasses examined.

The conservation of the central domain structure on rHDL particles of different sizes is consistent with the ability of all native HDL particles to activate the LCAT cholesterol esterification reaction. LCAT-mediated lipid polarity changes drive global structural rearrangements and formation of spherical HDL, which likely requires alteration of apoA-I structure and of protein−lipid interactions. Thus, it is plausible that the structure of residues 134−145 is conserved in discoidal HDL particles and essential for LCAT activation but is lost upon formation of spherical HDLs, which are poor LCAT activators.[88−90] The structure of the central domain in spherical HDL is currently under investigation using a similar multi-technique approach.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Map of protein segments analyzed in this study within the sequence of human wild-type apoA-I, representative fluorescence emission spectra, and Schiffer−Edmunson helical wheel projections of α18/5 and α11/3 pitch of regions 111−128, 133−150, and 157−174. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Children's Hospital Oakland Research Institute, 5700 Martin Luther King Jr. Way, Oakland, CA 94609. E-mail: gcavigiolio@chori.org. Telephone: (510) 450-7630. Fax: (510) 450-7910.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

HDL, high-density lipoprotein; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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