The Carboxyl-terminal Hydrophobic Residues of Apolipoprotein A-I Affect Its Rate of Phospholipid Binding and Its Association with High Density Lipoprotein*

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We performed a series of mutations in the human apolipoprotein A-I (apoA-I) gene designed to alter specific amino acid residues and domains implicated in lecithin:cholesterol acyltransferase (LCAT) activation or lipid binding. We used the mutant apoA-I forms to establish nine stable cell lines, and developed strategies for the large scale production and purification of the mutated apoA-I proteins from conditioned media.

HDL and dimyristoyl phosphatidylcholine binding assays using the variant apoA-I forms have shown that replacement of specific carboxyl-terminal hydrophobic residues Leu222, Phe225, and Phe229 with lysines, as well as replacement of Leu211, Leu214, Leu218, and Leu219 with valines, diminished the ability of apoA-I to bind to HDL and to lyse dimyristoyl phosphatidylcholine liposomes. The findings indicate that Leu222, and Phe225, Phe229 located in the putative random coil region, and Leu211, Leu214, Leu218, and Leu219 located in the putative helix 8, are important for lipid binding. In contrast, substitutions of alamines for specific charged residues in putative helices 7, 8, or 9 as well as various point mutations in other regions of apoA-I, did not affect the ability of the variant apoA-I forms to bind to HDL or to lyse dimyristoyl phosphatidylcholine liposomes. Cross-linking experiments confirmed that the carboxyl-terminal domain of apoA-I participates in the self-association of the protein, as demonstrated by the inability of the carboxyl-terminal deletion mutants Δ185–243 and Δ209–243 to form higher order aggregates in solution. Lecithin:cholesterol acyltransferase analysis, using reconstituted HDL particles prepared by the sodium cholate dialysis method, has shown that mutants (Pro165 → Ala,Gln173 → Glu) (Leu311 → Val,Leu314 → Val,Leu318 → Val,Leu319 → Val), Leu222 → Lys,Phe250 → Lys,Phe250 → Lys) and Δ209–243 reduced LCAT activation (38–68%). Mutant (Glu191 → Ala,His193 → Ala,Lys196 → Ala) enhanced LCAT activation (131%), and mutant (Ala162 → Leu,Leu183 → Trp) exhibited normal LCAT activation as compared with the wild type proapoA-I and plasma apoA-I forms. The apparent catalytic efficiency (Vmax/app/Km(app)) of the apoA-I mutants ranged from 17.8 to 107.2% of the control and was the result of variations in both the Km and the Vmax in the different mutants. These findings indicate that putative helices 6 and 7, and the carboxyl-terminal helices 8 and 9 contribute to the optimum activation of lecithin:cholesterol acyltransferase. In addition to their use in the present study, the variant apoA-I forms generated will serve as valuable reagents for the identification of the domains and residues of apoA-I involved in binding the scavenger receptor BI, and facilitating cholesterol efflux from cells as well as aid in the structural analysis of apoA-I.

Apolipoprotein A-I (apoA-I) is the major protein constituent of HDL and plays an important role in HDL stability, lipid transport, and metabolism (1). As a component of HDL, apoA-I is the principal physiological activator of lecithin:cholesterol acyltransferase (LCAT), particularly with physiological lecithins (2, 3). Reconstituted HDL (rHDL) particles, formed in vitro by mixing apoA-I with phospholipid-cholesterol vesicles, serve as substrates of LCAT and are converted into cholesteryl ester containing spheres upon incubation with LCAT (4). It was shown that rHDL particles have different sizes and that their LCAT activation ability correlates with the conformation of apoA-I on these particles (5–7). ApoA-I also promotes the efflux of cholesterol from peripheral cells, thus providing a substrate for the LCAT reaction (8). Finally, apoA-I plays a role in receptor-dependent or receptor-independent binding of HDL to cell surfaces (9–12). Such binding may contribute to either cholesterol efflux (8) or selective lipid uptake (13). As a result of these activities, apoA-I may play an important role in regulating the cholesterol content of peripheral tissues through the reverse cholesterol transport pathway (4, 8, 14).

Amino acid (15) and nucleotide (16, 17) sequence analyses of apoA-I have shown that both the protein and the gene contain repeated units. The protein contains 22- or 11-residue repeated units, which are organized into amphipathic α-helices (15–17). Models of secondary structure have been proposed that predict the presence of 8 or 9 helical regions in the apoA-I molecule (18, 19). The model proposed by Atkinson predicts the existence of 9 antiparallel helices and is consistent with the formation of an antiparallel α-helical bundle structure in solution (18).

Several studies have examined the ability of synthetic peptides and fragments of apoA-I to associate with phospholipid vesicles and to activate LCAT (20–22). Moderate reduction in LCAT activation in the range of 40–70% of normal was ob-

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§ The abbreviations used are: apoA-I, apolipoprotein A-I; HDL, high density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; DMPC, dimyristoyl phosphatidylcholine; POPC, palmitoyl oleyl phosphatidylcholine; HDL, reconstituted high density lipoprotein; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco’s modified Eagle’s medium; BS3, bis(sulfosuccinimidyl)suberate.
Generation and Functional Analysis of ApoA-I Variants

EXPERIMENTAL PROCEDURES

Materials

The Klenow fragment of DNA polymerase I, T4 ligase, polynucleotide kinase, and restriction enzymes were purchased from New England Biolabs. calf intestinal alkaline phosphatase was from Stratagene (La Jolla, CA). [35S]Methionine (>1000 Ci/mmol) and [14C]cholesterol (45–60 mCi/mmol) were from NEN Life Science Products. The sequencing kit was from U. S. Biochemical Corp. Materials for the polymerase chain reaction were from Perkin-Elmer. Bactotryptone and Bacto-yeast extract were from VWR (Pittsburgh, PA). Dulbecco’s modified Eagle’s medium (DMEM) and methionine-free DMEM were from Life Technologies, Inc. Materials for the two-dimensional polycrylamide gel electrophoresis were described previously (36). IgGorb was from the Enzyme Center (Boston, MA). BSA, POPC, DMPC, sodium cholate, aprotinin, and benzamidine were from Sigma. Materials for oligonucleotide synthesis were from Applied Biosystems.

Methods

Mutagenesis and Plasmid Construction—The oligonucleotides utilized as primers for the in vitro mutagenesis of the apoA-I gene were synthesized by the solid-phase phosphate triester method using an Automated Oligonucleotide Synthesizer (Applied Biosystems, model 380-B), according to the instructions provided by the manufacturer. The oligonucleotides were purified by electrophoresis on 20% polyacrylamide, 7 M urea gels.

The Pest-Pest fragment of the apoA-I gene was ligated to SalI linkers and inserted into the SalI polynucleotide site of the pUC19 vector. This new plasmid designated pUCA-I, was digested with the pBMT3X vector, thus placing the apoA-I gene under the control of the 3’ chain reaction to introduce two new restriction sites, in intron 3 and at the apoA-I, between residues 95 and 185 are important for the activation of LCAT. These domains contain the putative helices 6 and 7 and the hinged domain, which includes the putative helices 4 and 5. The carboxyl-terminal region of apoA-I (residues 190–243) has been shown to be important for lipid and HDL binding as well as for binding to cell membranes (12, 29–31, 34, 35).

Although the data obtained with the carboxyl-terminal apoA-I mutants are informative, mapping of the apoA-I domains that are functionally important requires more precise point mutagenesis that disturbs minimally the three-dimensional structure of apoA-I. In the present study we describe the generation and characterization of novel apoA-I variants. Functional analysis established that specific hydrophobic residues in the putative loop region (223–231) and within putative helix 8 (187–223) are important for binding to HDL and for the initial association of apoA-I with multimolecular phospholipid vesicles. A relatively small reduction in LCAT activation was observed for several point mutants as a result of variations in both the apparent $K_m$ and $V_{max}$.

Purification of Variant ApoA-I Forms—Medium (1 liter) collected from the roller bottles was concentrated down to 50 ml using an Amicon Ultrafiltration Cell and membranes with a 10,000 molecular weight cut-off. The concentrated medium was then dialyzed against 0.1 M Tris, pH 8, filtered, and passed through a DEAE HiTrapQ column (Pharmacia Biotech Inc.), which had been equilibrated with the same buffer. The protein was then eluted with a step gradient (5% → 20% → 50% → 100%) of 1 M NaHCO$_3$ in the Tris buffer. The fractions were analyzed by SDS-PAGE, and those containing the apoA-I protein were pooled and further concentrated down to 2 ml using a Centicon concentrator (Amicon) with a molecular weight cut-off of 10,000. The concentrated sample was applied to a gel filtration column, HiPrep Sephacryl S-200 (Pharmacia), at a rate of 0.1 ml/min, and eluted with one column volume (320 ml) of 0.15 M Na$_2$CO$_3$, 0.02% NaN$_3$, 10 μM aprotinin, and 10 μM benzamidine. Collection of media was repeated every 3–4 days.

The concentration of the apolipoprotein was determined by the absorbance at 280 nm and an extinction coefficient of 1.15 mg$^{-1}$ cm$^{-1}$.

Flotation Properties of the ApoA-I Variants—Cell cultures expressing the variant apoA-I proteins were labeled with [35S]methionine in methionine-free medium as described (41). A 2-ml aliquot of the culture medium was adjusted to a density of 1.21 g/ml with 0.65 g of potassium bromide, placed in a cellulosine nurse tube, mixed with 100 μg of human HDL, and overlaid sequentially with 1.75 ml of a potassium bromide solution of $d = 1.15$ g/ml, 3 ml of each of potassium bromide solutions of $d = 1.063$ g/ml and $d = 1.019$ g/ml, followed by normal saline.

The tubes were then centrifuged in a Beckman SW41 rotor at 35,000 rpm for 22 h. After centrifugation, 12-ml fractions were collected from the top of the tube using a Haake/Buchler fraction collector. The samples were then extensively dialyzed against a 1 mM solution of cold methanol, dried up in a vac, resuspended in one-dimensional SDS-PAGE sample buffer, and analyzed by one-dimensional SDS-PAGE and autoradiography. To quantitate the 35S-labeled apolipoproteins, the protein bands of the one-dimensional gels were excised and solubilized in 25 ml of 30% (w/v) $H_2O_2$, at 60 °C in scintillation vials. The solubilized acrylamide was then mixed with 15 ml of scintillation fluid and counted in an LKB scintillation counter.
TABLE I
List of the variant apoA-I forms and rationale for their selection

| Helix no. | Repeat | Mutations |
|----------|--------|-----------|
| 6        | 6      | Ala152 -> Leu,Leu159 -> Trp^<a> |
|          |        | Pro165 -> Ala,Gln172 -> Glu^<b> |
| 7        | 7      | Glu191 -> Ala,His193 -> Ala,Lys195 -> Ala^<a> |
| 8        | 8      | Glu212 -> Ala,Asp213 -> Ala,Arg215 -> Ala^<a> |
|          | 8 and 9| Leu211 -> Val,Leu214 -> Val,Leu216 -> Val,Leu219 -> Val^<a> |
| 9        | 10     | Leu222 -> Lys,Phc225 -> Lys,Phc229 -> Lys^<a> |
|          | 8      | Glu234 -> Ala,Glu235 -> Ala,Lys238 -> Ala,Lys239 -> Ala^<a> |
|          | 8 and 9| Gly165 -> Stop^<a> |
|          | 9      | Pro209 -> Stop^<a> |
| 10       |        | |

^<a> Alter the putative bundle structure of apoA-I in solution.
^<b> Alter the relative orientation of helices 6 and 7.
^<c> Investigate the role of charged residues in inter or intrahelical ionic interactions.
^<d> Investigate the role of specific domains and residues of apoA-I in lipid binding and LCAT activation.

RESULTS
Characterization of Secreted Wild Type and Variant Apo-A-I Forms—To characterize the mutant apoA-I forms, cell clones expressing the mutant apoA-I genes (Table I) were labeled with [35S]methionine, immunoprecipitated, and analyzed by two-dimensional PAGE and autoradiography, using the plasma wild type apoA-I as an internal marker. The Coomassie-stained gel obtained from this analysis shows the position of the plasma apoA-I isoforms that were included in the sample, and the autoradiogram shows the position of the newly synthesized apoA-I. Superimposition of the gel on the autoradiogram establishes the charge and size differences between the plasma apoA-I and the newly synthesized variant apo-A-I forms (37). Thus, using this analysis, mutants with charge or size differences from the wild type can be distinguished unequivocally.

Fig. 1 (A–I) shows the two-dimensional gel electrophoresis analysis for the following apoA-I variants: A, (Gly185 -> Stop); B, (Pro209 -> Stop); C, (Leu222 -> Lys,Phc225 -> Lys,Phc229 -> Lys); D, (Leu211 -> Val,Leu214 -> Val,Leu216 -> Val,Leu219 -> Val); E, (Glu191 -> Ala,His193 -> Ala,Lys195 -> Ala); F, (Pro165 -> Ala,Gln172 -> Glu); G, (Ala152 -> Leu,Leu159 -> Trp); H, (Glu212 -> Ala,Asp213 -> Ala,Arg215 -> Ala); and I, (Glu234 -> Ala,Glu235 -> Ala,Lys238 -> Ala,Lys239 -> Ala). In panel A, the secreted radiolabeled variant form is smaller in size, and is two charge units more negative than the wild type proapoA-I (isoform 2). This is consistent with the net loss of two positive charges; the deleted sequence contains 8 negatively charged and 10 positively charged residues. The change in size is consistent with the deletion of 58 amino acids. In panel B, the secreted radiolabeled variant form is smaller in size, and is one charge unit more positive than the wild type proapoA-I. This is consistent with the net loss of one negative charge; the deleted sequence contains five negatively charged and four positively charged residues. The change in size is consistent with the deletion of 35 amino acids. In panel C, the secreted radiolabeled variant form has three additional positive charges as compared with the corresponding wild type proapoA-I. This is consistent with the acquisition of three positive charges due to the replacement of three neutral residues with three positively charged lysines. The additional acidic forms with the same Mr present on the gel are the result of deamidation or carbamylation of the major form, as described previously (45). The more basic, higher Mr isoform observed in this panel and in panels E, F, G, H, and I has been observed previously and represents the pre proapoA-I form (37). The acidic higher Mr isoforms of the newly secreted apoA-I present in this panel have been observed previously and may represent post-translationally modified forms of unknown
C127 cell lines expressing the mutant proteins were labeled with [35S]methionine. The radiolabeled proteins were immunoprecipitated and analyzed by two-dimensional gel electrophoresis and autoradiography, as described under "Methods." The figure shows superimposition of the autoradiogram on the corresponding gels, stained for protein. Note that in panel A, the newly synthesized apoA-I is smaller in size, and is two charge units more negative than the wild type proapoA-I, consistent with the net loss of two positive charges. In panel B, the newly synthesized apoA-I is smaller in size, and is one charge unit more positive than the wild type proapoA-I, consistent with the net loss of one negative charge. In panel C, the newly synthesized apoA-I has three additional positive charges as compared with the corresponding wild type proapoA-I, confirming the replacement of three non-charged residues with three positively charged lysines. In panels D, G, and I, the newly synthesized apoA-I overlaps completely with the plasma proapoA-I, confirming the lack of change in the total charge, due to the mutations. In panel E, the newly synthesized apoA-I has one additional negative charge as compared with the wild type proapoA-I, confirming the net loss of one negative charge due to the mutation. In panel F, the newly synthesized apoA-I has one additional negative charge as compared with the corresponding wild type proapoA-I form, confirming the acquisition of one extra negative charge, due to the mutations.

**Isolation of the Wild Type and Variant ApoA-I Forms from Conditioned Medium**—To obtain large quantities of apoA-I protein, the permanent cell lines expressing the different apoA-I variants were pooled and concentrated to a small volume mostly in the lipoprotein-free fraction (41). The density distribution of the recombinant wild type apoA-I expressed in C127 cells is distributed mostly in the lipoprotein-free fraction (d > 1.21 g/ml) (47). The ability of the variant apoA-I forms to bind to HDL was analyzed by KBr density gradient ultracentrifugation (41). The distribution of the radiolabeled recombinant variant and wild type apoA-I in different lipoprotein fractions, relative to the plasma apoA-I, is shown in Fig. 3 (A–I). The location of the mutations, in the model proposed by Nolte and Atkinson (18), described in this and subsequent figures is shown in panel J. The density distribution of the recombinant wild type apoA-I was similar to that of plasma apoA-I, suggesting an equilibrium between the recombinant and the plasma wild type forms. The majority of this recombinant protein floats in the HDL region, between densities 1.06 and 1.21 g/ml.

Deletion of the putative helices 8 and 9 ([185–243], or part of helix 8 and helix 9 ([209–243]), affected dramatically the flotation properties of the mutant proteins. The majority of the protein was recovered in the non-lipoprotein fraction (d > 1.21 g/ml) (Fig. 3, A and B). The findings confirm previous findings (29) that the carboxyl-terminal region of apoA-I is necessary for its ability to bind to the lipoprotein surface.

Point mutations were used to identify the specific residues within the carboxyl terminus of apoA-I that are involved in HDL binding. Substitution of alanines for specific charged residues (Glu191 → Ala, His193 → Ala, Lys193 → Ala) or (Glu213 → Ala, Asp217 → Ala, Arg215 → Ala) in putative helix 8, or (Glu234 → Ala, Glu235 → Ala, Glu238 → Ala, Glu239 → Ala) in putative helix 9 did not affect the flotation properties of the mutant proteins, suggesting that these mutated residues are not cru-
for the ability of the protein to associate normally with HDL (Fig. 3, E, H, and I).

However, substitution of lysines for three specific hydrophobic residues (Leu\textsuperscript{222}, Phe\textsuperscript{225}, and Phe\textsuperscript{229}) located in the predicted random coil region between putative helices 8 and 9, altered dramatically the ability of the mutant protein to bind to HDL. As shown in Fig. 3C, the majority of the mutant protein is recovered in the lipoprotein-free fraction (d < 1.21 g/ml), indicating that these specific hydrophobic residues are critical for the ability of the protein to bind HDL.

Similarly, replacement of a series of leucine residues (Leu\textsuperscript{211}, Leu\textsuperscript{214}, Leu\textsuperscript{215}, and Leu\textsuperscript{219}) in putative helix 8 by valines, which have similar hydrophobicity but are less bulky, resulted in the same dramatic alteration in the flotation properties of the variant protein (Fig. 3D), indicating the importance of these residues for the binding of apoA-I to HDL.

Substitution of alanine for proline between putative helices 6 and 7 and the alteration of a neighboring glutamine to glutamate did not affect the binding of the variant apoA-I to HDL (Fig. 3F). These mutations were predicted to alter the orientation of these helices due to the elimination of the helix-breaking proline residue, and the change of the predicted A type half-repeat to a B type half-repeat (18).

Finally, a mutation in helix 6, which introduces amino acids at positions that are expected to disrupt the formation of the helical bundle in solution (Ala\textsuperscript{152} → Leu, Leu\textsuperscript{159} → Trp) (48, 49) does not affect the flotation properties of the mutant protein (Fig. 3G), suggesting that these substitutions do not affect the binding of apoA-I to HDL.

**Lipid Binding Properties of the Variant ApoA-I Forms—**

DMPC binding experiments were performed to assess the effect of the mutations on the kinetics of interaction of apoA-I with DMPC multilamellar vesicles. The rate of the interaction was monitored by the change in absorbance at 325 nm. The experiments were performed at 24 °C, the transition temperature of the lipid, where the gel and liquid-crystalline phases co-exist, and where defects in the lipid matrix make it easier for apoA-I to interact.

As illustrated in Fig. 4 (A–G), both plasma and recombinant wild type proapoA-I bind and solubilize DMPC rapidly, as indicated by the dramatic decrease in turbidity of the DMPC dispersions. In contrast, the apoA-I mutants in which the putative helices 8 and 9 (Δ209–243) were deleted, interacted extremely slowly with the phospholipid (Fig. 4, A and B). After incubation at 24 °C for 8 h, the reaction mixture was adjusted to a density of 1.21 g/ml and was fractionated by ultracentrifugation. Approximately 85% of the wild type apoA-I was recovered in the top fraction, bound to the DMPC, whereas only approximately 20% of the deletion mutant (Δ209–243) protein was found in the top fraction (data not shown).

The same slow kinetics of interaction were observed for the mutant in putative helix 8, where four valines were substituted for leucines (Leu\textsuperscript{211} → Val, Leu\textsuperscript{214} → Val, Leu\textsuperscript{219} → Val, Leu\textsuperscript{219} → Val) (Fig. 4D). It is worth noting that even when the ratio of protein to lipid was doubled (2.5:2, DMPC:protein) the reaction was still extremely slow (data not shown).

Similarly slow kinetics of interaction with DMPC were obtained for the mutant in which hydrophobic residues in the predicted random coil region between helices 8 and 9 were changed to charged residues (Leu\textsuperscript{222} → Lys, Phe\textsuperscript{225} → Lys, Phe\textsuperscript{229} → Lys) (Fig. 4C). This variant associates initially with the phospholipid at a rate that is slightly higher than the rate of association of the mutants of Fig. 4 (A, B, and D). However, the rate of association is still slow compared with that observed for the plasma and the recombinant wild type apoA-I.

In contrast to the slow rate of solubilization of multilamellar vesicles of DMPC observed in apoA-I mutants with substitutions of hydrophobic carboxyl-terminal amino acids, other point mutants in the central and carboxyl-terminal region of the molecule do not affect the ability of the protein to solubilize efficiently the multilamellar vesicles of DMPC.

Fig. 4 (E–G) shows the kinetics of interaction of the mutant apoA-I forms (Glu\textsuperscript{191} → Ala, His\textsuperscript{193} → Ala, Lys\textsuperscript{193} → Ala), (Pro\textsuperscript{165} → Ala, Glu\textsuperscript{72} → Glu), and (Ala\textsuperscript{152} → Leu, Leu\textsuperscript{159} → Trp) with DMPC. All of these mutants interact spontaneously and solubilize DMPC with a rate comparable to those of the recombinant wild type apoA-I.

The results obtained with the DMPC binding assay are in agreement with the results from the HDL binding assay and indicate that those mutants (both the deletion and the point mutations) that lost their ability to associate normally with the HDL, also displayed slow kinetics of association with the multilamellar vesicles of DMPC. The findings show that the carboxyl terminus of apoA-I is important for lipid binding, and indicate for the first time that specific hydrophobic residues in
the predicted random coil region between putative helices 8 and 9, as well as the leucine residues present in putative helix 8, are critical for the ability of the protein to bind to both the HDL surface as well as to bind and solubilize multilamellar vesicles of DMPC.

**Self-association Properties of the Variant ApoA-I Forms**—To study the self-association properties of the mutants in comparison with the plasma apoA-I protein, we performed cross-link-
FIG. 4. A–G, solubilization of multilamellar vesicles of DMPC by wild type and variant apoA-I forms, monitored by the turbidity change as a function of time, at 24 °C. Multilamellar vesicles of DMPC were combined with wild type or variant apoA-I forms at a ratio of DMPC:apoA-I of 2.5/1 (w/w). The change in turbidity was monitored by the change in absorbance at 325 nm, at 5-min intervals, and was plotted as a function of time. Panel A, (Δ185–243); panel B, (Δ209–243); panel C, (Leu$^{222}$ → Lys, Phe$^{226}$ → Lys, Phe$^{229}$ → Lys); panel D, (Leu$^{211}$ → Val, Leu$^{214}$ → Val, Leu$^{218}$ → Val, Leu$^{219}$ → Val); panel E, Glu$^{191}$ → Ala, His$^{193}$ → Ala, Lys$^{195}$ → Ala); panel F, (Pro$^{165}$ → Ala, Glu$^{172}$ → Glu); panel G, (Ala$^{152}$ → Leu, Leu$^{159}$ → Trp).
FIG. 5. Cross-linking of wild type and variant apoA-I proteins, as analyzed by SDS-PAGE. Lipid-free wild type or variant apoA-I forms were cross-linked with BS² and analyzed on an 8–25% Phast gel (Pharmacia). Lanes 1 and 6 contain plasma apoA-I, lane 2 contains the variant (Leu²⁰² → Lys,Pro²⁰⁵ → Lys,Pro²⁰⁵ → Lys), lanes 3, 4, and 7 contain the variant (Δ209–243), lane 5 contains the variant (Δ185–243), lane 8 contains the variant (Asp¹⁵⁵ → Leu,Leu¹⁵⁹ → Trp), lane 9 contains the variant (Pro¹⁶⁵ → Ala,Gln¹⁷² → Glu, Glu) rHDL, lane 10 contains the variant (Asp¹⁹¹ → Ala,His¹⁹³ → Ala,Lys¹⁹⁵ → Ala). Note that the deletion of the carboxyl-terminal residues 185–243 or 209–243 prevents oligomerization of the mutant apoA-I protein. However, the point mutation (Leu²²² → Lys,Pro²²⁵ → Lys,Pro²²⁵ → Lys), which affected lipid binding, did not affect oligomerization. 

Photographing experiments using the cross-linking reagent BS³. 

Plasma apoA-I formed dimers, trimers, and tetramers in addition to monomers, when it was present in solution at a concentration of 1.5 mg/ml (Fig. 5). In contrast, both of the carboxyl-terminal deletion mutants (Δ185–243) and (Δ209–243) formed predominantly monomers, and a few dimers. The formation of the dimer was concentration-dependent, but even at concentrations as high as 2 mg/ml, the dimer represented only a minor component. 

The mutants (Ala¹²² → Leu,Leu¹⁵⁹ → Trp) in putative helix 6, (Pro¹⁶⁵ → Ala,Gln¹⁷² → Glu) in putative helix 7 and the turn preceding it, as well as the mutant (Asp¹⁹¹ → Ala,His¹⁹³ → Ala,Lys¹⁹⁵ → Ala) in putative helix 8, all formed higher order oligomers similar to the ones observed for the plasma apoA-I. These results show that alterations in these regions do not affect the self-association of apoA-I. 

The variant (Leu²²² → Lys,Pro²²⁵ → Lys,Pro²²⁵ → Lys) in the random coil region between putative helices 8 and 9, which did not bind to HDL and which interacted slowly with multilamellar vesicles of DMPC, self-associated to form dimers, trimers, and tetramers. This suggests that either these residues are not involved in the self-association of apoA-I or that self-association requires more than one region of the apoA-I molecule. 

The observation that deletion of a small part of the carboxyl-terminal region of apoA-I prevents the protein from self-associating at high concentrations in solution is of great importance. In the future it may be possible, using these mutants or derivatives, to determine the structure of apoA-I by x-ray crystallography or NMR spectroscopy. 

Generation of rHDL Substrates for the LCAT Reaction—The mutant proteins were reconstituted in particles containing POPC, and cholesterol (cold and labeled) at a ratio of 100:10:1 of POPC:cholesterol:apoA-I using the sodium cholate dialysis method (43), and were used as substrates for the LCAT reaction. The sodium cholate dialysis method allowed the formation of HDL particles, even with the mutants which interacted very slowly with phospholipid. These particles were sized by native gradient gel electrophoresis (Fig. 6). This analysis identified two populations of particles at a ratio of approximately 3:1, with diameters of 96 Å and 109 Å. LCAT assays were performed with the mixed particle population. 

The rHDL particles were also negatively stained with potassium phosphotungstate, overlaid on carbon-coated grids, and photographed with a Philips CM12 electron microscope.

Fig. 7 (A–H) shows formation of the rHDL particles with all of the variant apoA-I protein samples tested for LCAT activation. Under the negative staining conditions used, these particles form the typical “rouleaux” indicating that they are discoidal in shape and that they have the thickness of a phospholipid bilayer. The number of rouleaux observed depends on the concentration of the sample on the carbon grid. In the samples that are less concentrated, a large number of round particles that lie flat on the grid are also observed. These particles do not seem to pack hexagonally on the grid during aggregation (a characteristic of spherical structures), providing further evidence that these particles are, in fact, discoidal in shape. 

Activation of LCAT by the Variant ApoA-I Forms—Even though other apolipoproteins like apoC-1, apoC-IV, and apoE can activate the LCAT reaction in vitro using rHDL particles as substrates, none is as effective as apoA-I, when physiological lipoproteins are used as substrates (3). To identify the domains and residues of apoA-I that are responsible for LCAT activation, the mutant proteins were reconstituted in rHDL particles. The LCAT activity was assayed as the rate of production of labeled cholesterol esters from the rHDL particles. The labeled cholesterol esters were separated from the free cholesterol by thin layer chromatography. All LCAT assays were standardized by adding fixed amounts of apoA-I reconstituted in the HDL particles, and LCAT enzyme.
The ability of the wild type proapoA-I secreted from C127 cells to activate LCAT is comparable with that of plasma apoA-I (Fig. 8). The mutant in helix 6, which contains amino acid substitutions designed to destabilize the bundle structure of apoA-I (Pro165→Leu,Leu159→Val), was also able to activate LCAT to levels comparable with those of plasma apoA-I. However, the mutant apoA-I form (Pro165→Leu,Leu159→Val) activates LCAT to approximately 55% as compared with the wild type apoA-I. The substitutions of alanine for proline and glutamate for glutamine are predicted to change the orientation of these helices. These results indicate that the putative helices 6 and 7 contribute to the efficient activation of LCAT.

DISCUSSION

Background—Epidemiological and genetic data have shown convincingly that low levels of HDL or apoA-I are associated with an increased risk of developing coronary heart disease (51). In a systematic effort to map the domains of apoA-I important for its functions, we have mutagenized the human apoA-I gene and we have created permanent cell lines expressing the variant apoA-I proteins. Large scale cultures of the permanent cell lines in roller bottles containing collagen-coated microspheres allowed us to obtain sufficient quantities of the apoA-I substrate and analyze its functions.

Lipid and Lipoprotein Binding Properties of the Variant ApoA-I Forms—Consistent with previous observations, deletion of residues 185–243 (Δ185–243) or 209–243 (Δ209–243) of apoA-I severely altered the ability of the mutant proteins to bind to HDL (29). To identify specific residues within the car-
boxy terminus of apoA-I involved in lipid and/or lipoprotein binding, we introduced a series of point mutations in this region. Analysis of the ability of these mutants to bind to HDL has shown that substitution of a series of charged amino acids between residues 195 and 236 did not affect the ability of the mutant proteins to bind to HDL, indicating that inter- or intrahelical ionic interactions may not be essential for the binding of apoA-I to HDL. This is consistent with the presence, in the general population, of several substitutions of charged for neutral amino acids that do not affect HDL levels (52). This finding indicates that apoA-I may have the ability to tolerate substitutions of charged amino acids without adverse physiological consequences.

In contrast, substitution of the positively charged lysine for specific hydrophobic residues (Leu211, Leu214, Leu218, and Leu219) by valines in helix 8 altered dramatically the ability of the mutant protein to bind to HDL. The presence of valines in these positions, which have similar hydrophobicity to but are less bulky than leucines, might prevent leucine zipper type hydrophobic interactions between juxtapositioned leucine residues (55). This may cause conformational changes in the random coil region that diminish the ability of this region to attach to the surface of HDL. The structural alterations associated with these mutants are the subject of ongoing research.

Preliminary analysis of several other point mutations, along the predicted helices 1–6 of apoA-I, has shown that these mutations did not have any effect on the ability of the protein to bind to HDL, thus reinforcing the notion that the carboxy-terminal region of apoA-I plays a unique role in the binding of apoA-I to HDL.3

The results obtained from the kinetic analysis of DMPC binding are consistent with the results obtained from the HDL binding assay. Mutants that failed to bind to HDL also lysed multilamellar vesicles of DMPC very slowly, pointing to the possibility that the mechanism of the interaction of apoA-I with the HDL surface and the multilamellar phospholipid vesicles may be similar.

The carboxy-terminal domain (putative helices 8 and 9 and the random coil region), however, is most likely only involved in the initial penetration of the protein into the phospholipid bilayer, since the mutant proteins that did not bind to HDL and interacted very slowly with the DMPC bilayers were able to form rHDL particles when the sodium cholate reconstitution method was used. These results are in agreement with previously published studies, where a proteolytic fragment of apoA-I (1–192) interacted slowly with DMPC, but did form rHDL particles when the sodium cholate method was used (34).

Activation of LCAT by Variant ApoA-I Forms—rHDL particles formed using the sodium cholate dialysis method have been shown before to be excellent substrates for the LCAT reaction (56). In the present study, these particles were visualized by electron microscopy and sized by native gradient gel electrophoresis. This latter analysis identified two populations of particles with diameters of 96 Å and 109 Å. LCAT assays were performed with the mixed particle population. The ratio of the 96-Å to the 109-Å particle present in the mixture is 3:1 and is the same for the plasma, the recombinant wild type, and the mutant pro-apoA-I proteins. Therefore these two different size particles contribute equally to the total LCAT activity in the various samples. Since the ability of the 96-Å particles to activate LCAT is 10-fold higher than that of the 109-Å particle, the contribution of the 109-Å particle is only 1/30th of the overall activation. Thus our results, expressed as percent activation relative to the wild type apoA-I, reflect the relative change in LCAT activation of the 96-Å substrate.

\[ \text{TABLE II} \]
Kinetische Parameter für LCAT-Aktivierung für Plasma, wild type, und variant apoA-I

| rHDL Substrate | Apparent $K_m$ | Apparent $V_{max}$ | Apparent $V_{max}/apparent K_m$ |
|----------------|---------------|-------------------|----------------------------------|
| Plasma A-I     | $9.79 \pm 0.47 \times 10^{-7}$ | $8.4 \pm 0.18$ | $8.58 \times 10^6$ |
| Ala$^{155}$ → Leu,Leu$^{159}$ → Trp | $15.7 \pm 1.98 \times 10^{-7}$ | $10.8 \pm 0.72$ | $6.88 \times 10^6$ |
| Pro$^{165}$ → Ala,Gln$^{172}$ → Gru | $9.71 \pm 2 \times 10^{-7}$ | $5.19 \pm 0.48$ | $5.34 \times 10^6$ |
| Glu$^{181}$ → Ala,Lys$^{183}$ → Ala | $21.5 \pm 4.2 \times 10^{-7}$ | $19.78 \pm 3.71$ | $9.2 \times 10^6$ |
| Leu$^{211}$ → Val,Leu$^{214}$ → Val,Leu$^{218}$ → Val | $11.7 \pm 4 \times 10^{-7}$ | $5.74 \pm 1.07$ | $4.9 \times 10^6$ |
| Leu$^{222}$ ↔ Lys,Phe$^{225}$ ↔ Lys | $43 \pm 1.57 \times 10^{-7}$ | $6.58 \pm 0.38$ | $1.53 \times 10^6$ |
| $\Delta 209–243$ | $8.65 \pm 0.4 \times 10^{-7}$ | $4.04 \pm 0.97$ | $4.67 \times 10^6$ |

2 M. Kokkinidis and V. I. Zannis, unpublished data.

3 M. Laccostrate and V. I. Zannis, unpublished observations.
Previous studies have pointed out the importance of putative helices 6 and 7 (residues 145–183) of apoA-I in the activation of LCAT (21, 29, 30). Specifically, deletion of residues 148–186 or residues 143–164 and 165–185 reduced the capacity of the mutant proteins to activate LCAT to background levels (29, 30). The possibility exists, however, that these results were due to dramatic alterations in the protein structure caused by the large deletions.

The point mutant (Pro\(^{165} \rightarrow\) Ala; Gln\(^{172} \rightarrow\) Glu) eliminated a helix-breaking proline residue, and also changed a glutamine to glutamate. According to the model proposed by Nolte and Atkinson (18), type A half-repeats have neutral residues at the eighth position, whereas type B have negatively charged residues. Thus, the glutamine to glutamate substitution converts the predicted type A repeat to a type B repeat. It has been suggested that \(\beta\) turns occur mostly between A and B, rather than AA or BB repeats (18). Thus, the 45% reduction in the LCAT activation ability of this mutant suggested that the two amino acid substitutions may have distorted the orientation of the putative helices 6 and 7, which were shown previously to be important for LCAT activation (29, 30, 33). Consistent with this finding, a naturally occurring apoA-I variant (Pro\(^{165} \rightarrow\) Arg) has a 45–55% LCAT activation ability as compared with wild type apoA-I (57). It is possible that one or both of the helices interact electrostatically with the polar face of the predicted amphipathic \(\alpha\)-helical segment found close to the active site of the LCAT enzyme (residues 151 and 174 of LCAT), as it was proposed by Fielding (14). Ionic interactions between LCAT and apoA-I have also been suggested by the quantitative dissociation of LCAT from HDL in concentrated salt solutions (58). Alterations in the ionic interactions between apoA-I and the LCAT enzyme may lead to a less efficient activation.

The 42% decrease in the LCAT activation ability of the deletion (Δ209–243) mutant could be attributed mainly to the change in the apparent \(V_{\text{max}}\). This is consistent with previous studies (29, 30, 31), which showed that deletion of residues 209–219, 220–241, 212–233, and 213–243 resulted in LCAT activation of 11.2, 16, 28, and 13%, respectively, as compared with the wild type apoA-I. The variation in the extent of inhibition is probably due to either the difference in the phospholipid used for the formation of the reconstituted particles (egg phosphatidylcholine in the previous studies, versus POPC in this study), or the difference in the size of the particles formed. In addition, the deletion (Δ213–243) mutant used in the previous study contained 12 residues of unrelated carboxyl-terminal sequence (29). Substitution of valines for leucines 211, 214, 218, and 219 or substitution of lysines for leucine 222 and phenylalanines 225 and 229, resulted in 32 and 68% reduction, respectively, in the capacity of the mutant proteins to activate LCAT. This impairment was the result of a decrease in the apparent \(V_{\text{max}}\) for the former mutant and mainly due to an increase in the apparent \(K_m\) for the latter mutant. The combined data from this and the previous studies (29–31) suggest that domains and residues within the carboxyl-terminal region of apoA-I contribute to the optimal activation of LCAT. It is possible that the presence of the carboxyl-terminal domain allows apoA-I to acquire a proper conformation, which facilitates the postulated specific interaction of the middle region (residues 145–183) of apoA-I with the LCAT enzyme.

A mutant in the putative helix 6 containing amino acids in positions that are expected to destabilize the bundle structure in solution (49) binds normally to HDL and DMPC and also activates LCAT normally. These findings suggest that potential structural alterations of the bundle structure in solution have no consequences for lipid binding and LCAT activation, where the lipid bound protein assumes a new conformation.

Substitution of alamines for charged residues in putative helix 8 enhanced slightly the capacity of the variant protein to activate LCAT as compared with the wild type proapoA-I. The significance of this increased activation is not clear. It is possible that the removal of charged residues in putative helix 8 may allow a stronger electrostatic interaction to occur between the putative \(\alpha\)-helical segment of LCAT (residues 151 and 174) and putative helices 6 and 7 of apoA-I. Alternatively, the absence of these charged residues may allow the cholesterol substrate to position itself more favorably relative to the active site serine residue of the LCAT enzyme. The catalytic efficiency of the last two mutants was comparable with that of the wild type proapoA-I (Table II) due to concomitant increases in both the \(V_{\text{max}}\) and the \(K_m\).

The lack of dramatic changes in the apparent \(V_{\text{max}}\) for any of the mutations tested strongly suggests that none of the apoA-I residues altered participate in the catalytic mechanism of LCAT.

Domains of apoA-I Involved in Self-association—In addition to its role in the initial anchoring of the protein into the phospholipid bilayer, the carboxyl-terminal domain of apoA-I also appears to participate in the self-association of the protein. The deletion mutants (Δ185–243) and (Δ209–243) existed only as monomers and dimers in solution rather than as a mixture of oligomers. Similar conclusions were reached with the aminoterminal proteolytic fragment (1–192) of apoA-I (34). It has been suggested that self-association of apoA-I may promote stabilization of the potential amphipathic \(\alpha\)-helical segments of the carboxyl-terminal region which is less organized in the monomeric form (53). Overcoming the oligomerization problem and achieving high concentrations of the monomeric apoA-I fragment in solution can facilitate efforts to derive the three-dimensional structure of apoA-I by x-ray crystallography or NMR spectroscopy.

Overall, the present study shows that specific hydrophobic residues in the predicted random coil region between helices 8 and 9, and in the putative helix 8 (Fig. 3), are critical for the ability of apoA-I to bind to HDL and to lyse multilamellar vesicles of DMPC. It is possible that the formation of a “leucine zipper-like” structure between the putative helices 8 and 9 may stabilize this random coil region during its interaction with lipids and lipoproteins and allow its association with the phospholipid surface. Although the carboxyl-terminal region of apoA-I is required for both the lipid binding and self-association, the residues which participate in these two functions appear to be different.

Several amino acid substitutions in the carboxyl-terminal domain of apoA-I cause a moderate reduction in the catalytic efficiency of LCAT, suggesting that residues in this region contribute to optimum activation of LCAT, without a direct participation in the catalytic mechanism.

In addition to their use in the present study, the variant apoA-I forms generated will serve as valuable reagents for future studies to identify the domains and residues of apoA-I involved in binding to scavenger class B1 receptor, in promoting cholesterol efflux from cells, as well as for the structural analysis of apoA-I.

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