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review

Apolipoprotein A-I: structure–function relationships

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Abstract The inverse relationship between high density lipoprotein (HDL) plasma levels and coronary heart disease has been attributed to the role that HDL and its major constituent, apolipoprotein A-I (apoA-I), play in reverse cholesterol transport (RCT). The efficiency of RCT depends on the specific ability of apoA-I to promote cellular cholesterol efflux, bind lipids, activate lecithin:cholesterol acyltransferase (LCAT), and form mature HDL that interact with specific receptors and lipid transfer proteins. From the intensive analysis of apoA-I secondary structure has emerged our current understanding of its different classes of amphipathic α-helices, which control lipid-binding specificity. The main challenge now is to define apoA-I tertiary structure in its lipid-free and lipid-bound forms. Two models are considered for discoidal lipoproteins formed by association of two apoA-I with phospholipids. In the first or picket fence model, each apoA-I wraps around the disc with antiparallel adjacent α-helices and with little intermolecular interactions. In the second or belt model, two antiparallel apoA-I are paired by their C-terminal α-helices, wrap around the lipoprotein, and are stabilized by multiple intermolecular interactions. While recent evidence supports the belt model, other models, including hybrid models, cannot be excluded. ApoA-I α-helices control lipid binding and association with varying levels of lipids. The N-terminal helix 44–65 and the C-terminal helix 210–241 are recognized as important for the initial association with lipids. In the central domain, helix 100–121 and, to a lesser extent, helix 122–143, are also very important for lipid binding and the formation of mature HDL, whereas helices between residues 144 and 186 contribute little. The LCAT activation domain has now been clearly assigned to helix 144–165 with secondary contribution by helix 166–186. The lower lipid binding affinity of the region 144–186 may be important to the activation mechanism allowing displacement of these apoA-I helices by LCAT and presentation of the lipid substrates. No specific sequence has been found that affects diffusional efflux to lipid-bound apoA-I. In contrast, the C-terminal helices, known to be important for lipid binding and maintenance of HDL in circulation, are also involved in the interaction of lipid-free apoA-I with macrophages and specific lipid efflux. While much progress has been made, other aspects of apoA-I structure–function relationships still need to be studied, particularly its lipoprotein topology and its interaction with other enzymes, lipid transfer proteins and receptors important for HDL metabolism.—Frank, P. G., and Y. L. Marcel. **Apolipoprotein A-I: structure–function relationships.** ***J. Lipid Res.*** **2000.** 41: **853–872.**

Supplementary key words reverse cholesterol transport • apolipoprotein A-I mutants • HDL • cholesterol efflux • LCAT

This review focuses on the identification of apoA-I structural domains and their participation to HDL RCT. We will summarize the current knowledge on apoA-I structural domains and more particularly their function in lipid binding and formation of HDL, their contributions to cellular cholesterol efflux, and their role in LCAT activation. In each case, a particular emphasis will be placed on the study of apoA-I mutants and the analysis of structural and functional domains.

1. APOLIPOPROTEIN A-I STRUCTURE AND HDL FORMATION

Apolipoprotein A-I was one of the first apolipoproteins to be identified and characterized. Yet its structural characterization, notably in the lipid-associated form, has not been achieved and the domains involved in its multiple functions are not yet fully identified.

A. Primary structure and physicochemical properties of apoA-I

The sequence of apoA-I was determined by Brewer et al. (1), and followed by cloning and characterization of its cDNA (2–5) and genomic DNA (6, 7). The gene encoding apoA-I is a member of the apolipoprotein multigene superfamily, which includes genes encoding exchange-

Abbreviations: ABC1, ATP-binding cassette transporter 1; apoA-I, apolipoprotein A-I; CE, cholesteryl ester; CETP, cholesterol ester transfer protein; DMPC, dimyristoyl phosphatidylcholine; FC, free cholesterol; GdnHCl, guanidine hydrochloride; HDL, high density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; LpA-I, apoA-I-containing lipoproteins; POPC, 1-palmitoyl 2-oleylphosphatidylcholine, RCT, reverse cholesterol transport; SR-BI, scavenger receptor class B type I.

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Human -24-MKAAVLTLAVLFLTGSQARHFWQQDEPPQSPWDRVKDLATVYVDVLKDSGRDYVSQFEGS-36
Baboon        T                          T        V    EA     K
Dog       ————————————————————————    -                AV        A   A
Pig           V                      D -          F      AI        A   A
Rabbit        V                    R   -R S  KI  F      TV     E  A   A
Cow           V                      D -  S       F     EAI        A   A
Hedgehog                           D A-K Y DQI  ML     TA    K  LTSLDT
Mouse         V  AV LV        W V       -  Q  K   F N    AV            S
Rat              AV LV     C  WE        -  Q       F      AV            S
chicken     RGVLV          T   S   H   - T L  IR MVD  LETV A  K AIA   S
Duck        RVV V   L      T   Y   H   - A L  LR  VD  LETV A  K AIA   A
Salmon        L  A TI LAA T  -FPM A A —— QLEH  AALNM IAQV LTAQRSIDLLDDT

Human 37-ALGKQLNLKLLDNWDSVTSTFSKLREQLGPVTQEFWDNLEKETEGLRQEMSKDLEEVKAK-
96
Baboon                        V
Dog                        LS  VT                        V             Q
Pig           H            LG   T V                     A             K
Rabbit     F              SLS  V   Q                        E  N   Q  RQ
Cow                       TLA  L  V                     AS     H       Q
Hedgehog  F               LS  V   Q                        E  N   Q  RQ
Mouse     S  Q     N  E   TLG  VSQ Q R   L RD          DWV    N       Q
Rat       T        N      TLG  VGR Q           A       DW  N  N    N  Q
Chicken    V    D   A  L TLSAAAA    DMA YYK VREMWL D  A  A LT       E
Duck       V    D   A  L TLGAAAA    DMA YYK VREMWL D  S  A LT       E
Salmon    EY- EYKMQ SQSL NLQQFADSTSKSWPPTPRSS-APSCDA ATV A VM  V D RTQ

Human 97-VQPYLDDFQKKWQEEMELYRQKVEPLRAELQEGARQKLHELQEKLSPLGEEMRDRARAHV-
156
Baboon                                  H  T       H         V
Dog                       V       A  GS  R       Q            L     T
Pig                 N       T    MA  G  FR      VQ         A  L   L
Rabbit            E        V R       G   R S     T         A  L  S  T
Cow               E     H  V I     A  GE FR      VQ   D     AQ L
Hedgehog          S    VE  L       A  S  WR Q    AQ   Q AGE  QQH   VRT
Mouse             E     K DV     K A  G     S     Q   GR   VA  F   M T
Rat        M  H  E  E  N  V A    L   GT  HKN ———K M RH KVVA  F   M VNA
Chicken    IR F  Q SA  T  L Q   RLT VAQ  K LTK  VELM A  T VA  A   L G
Duck       IR F  Q SA  T  L Q   RLA VAE  K LTK  VELM Q  T VA  A   L G
Salmon     LE KRAELTEVLNKHIDE  K L   IKQHI LR TEMDAFRA ID VV    AKVAVN

Human157-DALRTHLAPYSDELRQRLAARLEALKENGGARLAEYHAKATEHLSTLSEKAKPALEDLRQ-
216
Baboon                                          S
Dog          AQ       D  E       Q    G   S      R S Q  A G   R   Q
Pig       E  Q V      D    M   F     G  -S    Q   Q Q KA G
Rabbit     T  K      N  Q         SI  G   S    Q   R    V     R
Cow       ET  QQ       D     T        G  -S        S Q KA G     V
Hedgehog      D     GE A KL LQ  QDI AKS -D    QT LS   KSFG   Q T Q   H
Mouse      S   Q   H EQM ES  QR AE  S P——T N   TR KT  K  G   R       H
Rat          AKFGL   QM EN  Q  TEIRNHP——T I   T  GD  R  G       D  G
Chicken   EE  KN          K SQK  EIR K IPQAS  Q  VM Q  N R  MT LVQEF E
Duck      EE  KN          K SQK  EIR K IPQA   Q  VV Q  N R  MT LVQ FKE
Salmon    EETK K M IVEIV AK TE   E RTLAAPYAE  KEQMFKAVGEVR  VA LS  FKA

Human  217-GLLPVLESFKVSFLSALEEYTKKLNTQ-243
Baboon                            S
Dog                    L A  D A     A
Pig                NL   I A ID AS    A
Rabbit                A VQNV D A
Cow               E L   I A ID AS    A
Hedgehog      E LW GI AGAM -M  LG    S
Mouse       S M M  TL TKAQ VIDKASET TA
Rat           M    AW AKIM MID AK    A-
Chicken     R T YA NL NRLI F D LQ SVA——
Duck        R T YA NL TR I L D LQ TVA——
Salmon      RWA PPRRPSK SWLSTRPSARP———
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Fig. 1.

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able apolipoproteins (apoA-I, A-II, Cs, and E) (8). They are thought to have evolved from a common ancestor by duplication/deletion of a 33-mer motif.

Analysis of the secondary structure of mature apoA-I has allowed the identification of 11- and 22-mer repeats (9, 10). The 22-mer repeats, usually separated by Pro residues, have been associated with the formation of amphipathic α-helices (11), which allow the interaction of the protein with phospholipids through its hydrophobic face, while the hydrophilic face of the helices interacts with the aqueous phase.

ApoA-I sequences from a number of species have been determined (**Fig. 1**), with sizes ranging between 258 and 267 amino acids (mature protein plus pre-pro segment). Comparison of sequences amongst mammals indicates that the N-terminal domain of apoA-I is highly conserved while the central and C-terminal domains show conservative substitutions between species. This result is consistent with the study of Collet et al. (12), who showed, using mAbs to human apoA-I, that the antigenicity of this protein is better conserved between species in the N-terminal domain (residues 1 to 98 of the mature protein). These observations are in accord with the major role this domain plays in the structure and/or function of human apoA-I. As described in the latter sections of this review, the N- and C-terminal domains of apoA-I have been involved in the binding of lipids, an essential function of this apolipoprotein (13–15). The central domain of the protein, which has been involved in LCAT activation, may have evolved in parallel with the LCAT sequence in the corresponding species. However, in species in which this domain has evolved faster than in humans (mouse and rat), the ability of mouse apoA-I to activate mouse LCAT is reduced as compared to that of human apoA-I (16, 17). Despite changes in the primary structure of apoA-I amongst different species, the secondary structure appears unmodified, as shown by the Edmundson-wheel representation (12). Studies by Januzzi et al. (18) suggest that apoA-I has evolved about 25% faster than an average gene in mammalian lineage. However, all portions of the protein have evolved at similar rates, which suggests a global conservation of the structure.

ApoA-I is known to self-associate, a process which may stabilize the lipid-free protein. However, at low concentrations (below 0.1 mg/ml) (19), or in the presence of guanidine hydrochloride (GdnHCl) (20), only the monomeric form is detected. Denaturation studies have indicated a midpoint of denaturation close to 1 m GdnHCl for the lipid-free form of apoA-I (20, 21), and an increased stability in the presence of phospholipid (21, 22). In GdnHCl, urea, or calorimetric denaturation experiments, a low free energy of denaturation about 2.2–2.7 kcal/mol has been determined (22, 23). This value is much lower than the value determined for globular proteins (in the range of 5–10 kcal/mol) (24, 25). This observation is in agreement with a loosely folded and relatively flexible structure of the protein in the lipid-free form. This loosely folded conformation may allow rapid lipid-interaction of exposed hydrophobic portions of the protein. In support of this view, thermal denaturation experiments by Gursky and Atkinson have suggested a molten globular-like state for lipid-free apoA-I that may explain its lipid-binding properties in vivo (26). Sedimentation velocity experiments on human apoA-I indicate significant conformational heterogeneity, which also support a flexible structure (27). This observation may be relevant in the case of preβ_1-HDL, which has been shown to be the earliest acceptor of cellular cholesterol (28) and appears to contain only 1–2 molecules of partially lipidated apoA-I (29). The N-terminal sequence (residues 1–43) of apoA-I, apparently not required for lipid binding, may be important for the stabilization of lipid-free apoA-I in solution (30, 31).

Site directed mutagenesis of tryptophans and fluorescence studies show that in the lipid-free state the apoA-I monomer is a prolate ellipsoid and its tryptophans exist in nonpolar environments compatible with an N-terminal half that is organized into a bundle of helices (32).

Two cysteine mutants (D9C and A232C) have been studied by circular dichroism and fluorescence spectrophotometry. The adduct formed with the thiol reactive probe acrylodan affects the structure and stability of the lipid-free D9C mutant but not that of the mutant A234C. The fluorescence analysis showed rigid N- and C-terminal structures of the lipid-free apoA-I and a fold that brings the C-terminus close to the tryptophans of the N-terminal half (33).

B. Role of amphipathic α-helices in the interaction of apoA-I with lipids

Algorithms allowing the prediction of the secondary structure of proteins (34, 35) suggest that apoA-I may be composed of 8–9 α-helices formed essentially by the 22-mer repeats, with a less well-defined N-terminal secondary structure (**Fig. 2**) (reviewed in ref. 36). These predictions were partially confirmed by experimental data using CD and IR spectroscopy to determine the total α-helicity of apoA-I (37–39). Investigations using tryptophan fluorescence to examine the change in apoA-I conformation observed upon association with lipids led to similar conclusions (37, 40).

Fig. 1. Sequence comparison between apoA-I from 11 species. Human (2), baboon (198), dog (199), pig (200), rabbit (201), cow (202), hedgehog (203), mouse (204), rat (205) chicken (206), duck (Swiss-Prot accession number, O42296), and salmon (207) apoA-I sequences are presented in decreasing order of their similarity to human apoA-I. Blanks indicate identity to human apoA-I; dashes (–) indicate deletions inserted to maximize homology between sequences. The pre-pro sequence of dog apoA-I has not been determined. Colors indicate proline (purple, P); aspartic acid or glutamic acid (red, D and E); arginine or lysine (blue, R or K); and phenylalanine, isoleucine, leucine, methionine, valine, tryptophan, or tyrosine (green, F, I, L, M, V, W, and Y). The remaining amino acids, alanine, cysteine, glycine, histidine, asparagine, glutamine, serine, and threonine (A, C, G, H, N, Q, S, and T) are uncolored and called indifferent (8). The numbering of human apoA-I starts at −24 (signal peptide of apoA-I), residue 1 being the first one of mature apoA-I.

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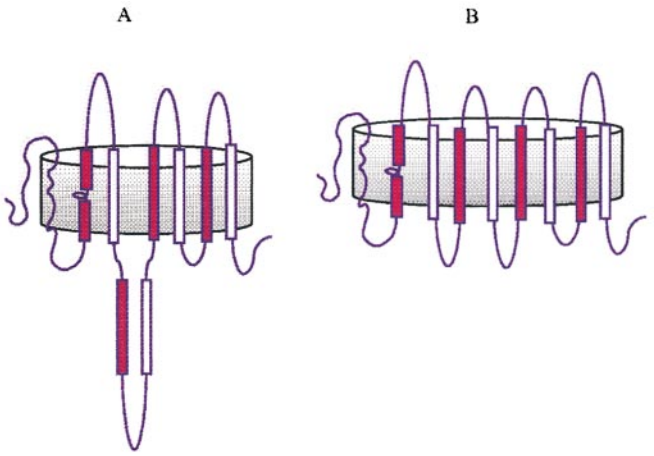

Fig. 2. Mode of interaction of apoA-I with phospholipids: picket fence model on a discoidal particle. The disc formed by a phospholipid bilayer can accommodate apoA-I amphipathic α-helices, parallel to the phospholipid acyl chains. In small discoidal LpA-I (apoA-I-containing lipoproteins), two helices do not interact with the lipid interface possibly because of a reduced affinity for lipids (A). However, in large discoidal LpA-I (B), these helices, which have been termed a hinge domain, interact with lipids and can therefore allow one molecule of apoA-I to associate with varying amounts of lipid.

Mode of interaction with lipids. Early studies have shown that isolated apoA-I can spontaneously interact with dimyristoyl phosphatidylcholine (DMPC) at its transition temperature (41–43) and form discoidal complexes (44). Reassembly of discoidal HDL and their conversion to spheres by the action of LCAT was first demonstrated by Forte et al. (45). Several groups have also shown that the binding of apoA-I to egg phosphatidylcholine or palmitoyloleyl phosphatidylcholine (POPC), in the presence of the detergent sodium cholate, resulted in the formation of discoidal lipoproteins (46, 47). The formation of spherical complexes could be obtained by incubation of LCAT and LDL, as a FC donor, with discoidal complexes (40, 45, 48, 49) or by co-sonication of a lipid–apoA-I mixture (50, 51).

A mechanism to explain the mode of apoprotein association with the lipid surface was proposed by Segrest et al. (52), who first suggested the existence of amphipathic α-helices in apolipoproteins. The involvement of α-helices in the binding of phospholipid was demonstrated by measuring the change in the ellipticity at 222 nm after binding of the apoprotein to phospholipid. Lipid-free apoA-I typically exhibits an α-helix content of 40–50% while in association with phospholipid the α-helicity increases to ~75% depending on the type of lipids and complexes formed (22, 37). This property is also shared by other apolipoproteins and suggests a very similar mechanism of association with lipids for all exchangeable apolipoproteins. ApoA-I immunoreactivity toward mAbs has also been shown to be altered by the presence of lipids (53, 54), which indicates important conformational changes affecting most domains upon association with lipids.

The Edmundson wheel representation gives some hints as to how charged residues affect the affinity of an α-helix. Segrest and colleagues (11) proposed that α-helices of apolipoproteins are not all equivalent for their affinity towards lipids. The differences in affinity do not appear to be simply related to their hydrophobic moment but also to the distribution of charge residues along the axis of the helix. Computer analysis of the different classes of amphipathic helices has led to a classification of the physicochemical properties associated with each class (11). Amphipathic α-helices of apolipoproteins form the class A. Classes L, H, M, and G have been used to identify lytic, hormone, transmembrane, and globular amphipathic α-helices, respectively. Class A is characterized by a high mean hydrophobic moment and by its unique charge distribution: positively charged residues are clustered at the polar–nonpolar interface, whereas negatively charged residues are found at the center of the polar face. For apoA-I, 6 helices have been identified with this specific amino acid distribution (helices 44–65, 66–87, 121–142, 143–164, 166–186, 187–208). Two other types of helices have been identified: class G* (helix 8–33) and class Y (helices 88–98, 99–120, 209–219, 220–241) (**Fig. 3**), each characterized by a specific charge distribution (11). These different helices may have different structural functions and lipid-binding properties but we still have a poor understanding of these relationships in apoA-I. Segrest et al. (11) had proposed that class Y had a reduced lipid affinity but, as it will be discussed below, helix 220–241 has the highest affinity for lipids amongst apoA-I α-helices (13). In class A amphipathic α-helices, the importance of the charge distribution has been examined with synthetic peptides with positively charged residues clustered at the polar–nonpolar interface and negatively charged residues at the center of the polar face. A decreased affinity for lipids was observed for peptides with a reverse charge distribution (55–57). The snorkel model of Segrest and colleagues (11) proposes that the positively charged residues, which are also amphipathic and have their long alkyl chain buried in the hydrophobic interior, extend ("snorkel") toward the polar face of the helix to insert their positive moieties into the aqueous phase. Recently, Lecompte et al. (58) have studied the steps in the binding of apoA-I to phospholipid monolayers, using alternative current polarography. This novel application of the technique identified two steps, adsorption and penetration, and supported the snorkel model (11).

The properties of synthetic peptides that correspond to each of the predicted apoA-I helical segments have been recently examined (13). Only peptides corresponding to helices 44–65 and 220–241 have been found to associate with lipids with a significant affinity. This result has been attributed to a deeper penetration of these two helices into the lipid interface, as compared to other helices. Helices 44–65 and 210–241 also present a higher total hydrophobicity on the nonpolar face. Although these isolated helices have faster kinetics of association with DMPC than apoA-I as a whole molecule, apoA-I can however reduce the enthalpy of the gel-to-liquid crystalline phase transition much more effectively, possibly due to the coop-

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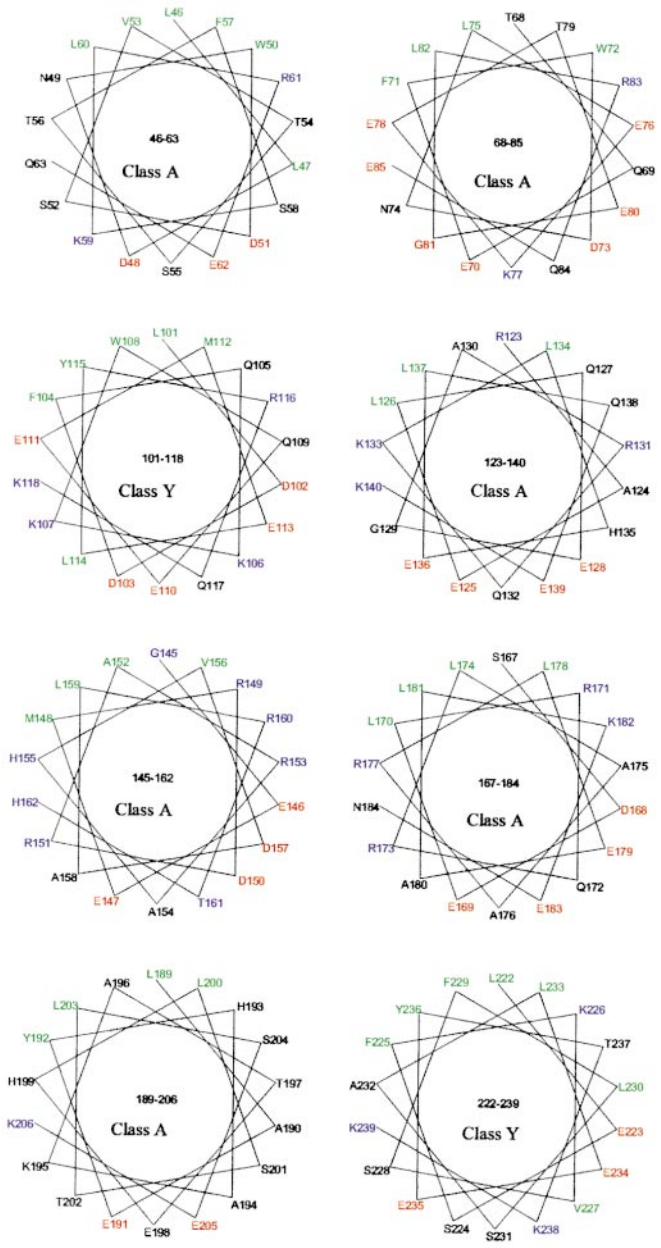

Fig. 3. Edmundson wheel representation of apoA-I α-helices. Colors indicate proline (purple, P); aspartic acid or glutamic acid (red, D and E); arginine or lysine (blue, R or K); and phenylalanine, isoleucine, leucine, methionine, valine, tryptophan, or tyrosine (green, F, I, L, M, V, W, and Y). The remaining amino acids, alanine, cysteine, glycine, histidine, asparagine, glutamine, serine, and threonine (A, C, G, H, N, Q, S, and T) are uncolored and called indifferent (8). The class of amphipathic helices (A, Y) to which each helix belongs is also indicated.

erativity between central α-helices (13). It has therefore been suggested that the two extreme helices of apoA-I may initiate the binding to phospholipid, followed by a cooperative binding of the other helices (13). This cooperativity may be facilitated by antiparallel helix–helix salt-bridges and by hydrogen bond interactions (59, 60). However, studies by Mishra et al. (14), comparing tandem and the constituent helices did not show any evidence of this cooperativity. This has first been interpreted as indications that peptides of different length cannot be directly compared and second that cooperativity effectively intervenes when the peptide is longer. Cooperativity may therefore be associated with a more complex tertiary structure of apoA-I that is yet to be determined. Alternatively, tandem helices may help in the stabilization of the lipoprotein complexes formed, avoiding their reorganization. In fact, in a recent in vitro study, we have shown that, in the central domain of apoA-I, the helix formed by residues 100–121 is very important for the stabilization of the lipid-apolipoprotein complex, while helices spanning residues 122–186 contribute to the initial rates of lipid-apolipoprotein association (61). Pro, present at the first position of almost every 22-mers (by comparison, helices of transmembrane proteins have approximately 20 amino acid residues) (62), often form β-turns or kinks between helices and are therefore essential for the secondary structure of apolipoproteins. The presence of a Pro residue appears necessary in defining the structural and functional properties of apoA-I, which associates mostly with small lipoproteins (HDL). In contrast, the apoE lipid-binding domain contains a 65-mer helix and, consequently, associates also with larger lipoproteins (11).

Structure of apoA-I on model lipoproteins. ApoA-I can form discoidal as well as spherical complexes, depending on the conditions of association. In vitro reconstituted discoidal LpA-I complexes usually have a preβ-HDL migration on agarose gel electrophoresis and change to an α-mobility following incubation with plasma factors ($d > 1.21$ g/ml) or LCAT alone (48). Discoidal LpA-I complexes can contain two, three, or more molecules of apoA-I per particle (37). As already indicated, increasing the POPC content of reconstituted discoidal LpA-I leads to an increase in the α-helical content of apoA-I (37, 39). It also leads to reduced protease accessibility suggesting a major conformational change upon association with lipids (30, 63). Studies from our laboratory using mAbs as conformational probes have also indicated that apoA-I undergoes major conformational changes upon lipid binding (53, 54, 64). In the case of discoidal complexes, specific domains are affected with increased phospholipid or cholesterol content, i.e., changes in epitope accessibility are mostly observed in the central (residues 99–148) and N-terminal domain (residues 2–8). Interestingly, the changes in immunoreactivity are in the opposite direction (i.e., central and N-terminal epitopes become less immunoreactive with increasing phospholipid and more immunoreactive with increasing cholesterol) (64).

The most studied and best-characterized model of apoA-I interaction with lipids remains the discoidal complex that will be discussed here. Several secondary structure models of apoA-I on discs have been suggested (36, 39, 41, 54, 65–67). In the first models, eight antiparallel amphipathic α-helices connected by β-turns have been proposed to interact in parallel to the phospholipid acyl chains (Fig. 2). These models were derived from investigations that combined secondary structure prediction methods and biophysical methods (39, 66, 67) or mAb immu-

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noreactivity studies (54). Several of these models suggest the existence of a hinge domain that may be responsible for the ability of apoA-I to associate with lipoprotein complexes of different sizes (37). This putative domain at first tentatively identified as either aa 100–143 or aa 122–165 (54), may be excluded from the interaction with lipid in small discoidal LpA-I, whereas it may participate in the interaction with phospholipid in large discoidal LpA-I (Fig. 2). Recent studies from this laboratory have indicated that the plasticity of the central domain of apoA-I (residues 100–186) is essential for the ability of apoA-I to associate with varying amounts of phospholipid (61). Protease accessibility studies performed with lipidated apoA-I have provided data that are consistent with this view and have identified a domain that is more sensitive to protease digestion in the central region of apoA-I in small LpA-I complexes (63).

Based on anti-apoA-I mAb competition for binding to a discoidal Lp2A-I, Bergeron et al. (64) suggested that the two apoA-I molecules be organized in a head-to-tail configuration. Another interesting conclusion emerging from these antibody competitions on models of discoidal Lp2A-I is the notion that the N-terminal domain of apoA-I may interact with the central domain of the protein, especially in small Lp2A-I (39, 54, 64). In contrast, a computer generated model of discoidal Lp2A-I from Phillips et al. (68) suggested that both the head-to-tail and the head-to-head conformations may coexist, since no particular configuration was favored. However, Phillips et al. (68) could not integrate residues 1–47 in their model, and this region has been shown to contribute significantly to the overall structure of apoA-I (39, 54, 64). Recently, fluorescence studies of cysteine mutants have shown that in discoidal Lp2A-I particles the C-terminal cysteine is close to the N-terminal tryptophans, thus excluding the possibility of a head-to-head picket fence configuration (33).

Crystal structure of apoA-I. Borhani et al. (69, 70) have recently determined the crystal structure of a truncated lipid-free form of apoA-I (lacking residues 1–43 or apoA-IΔ(1–43)). In its lipid-free form in the crystal unit, the mutant protein appears to exhibit a conformation similar to that of lipid-bound apoA-I (31), a characteristic, which justifies its discussion in the context of the structure of lipid-bound apoA-I. This protein appears as an elliptical ring-like structure consisting of four molecules of the apoprotein. These four molecules associate via the hydrophobic faces of their amphipathic helices to form a four-helix bundle (**Fig. 4**). Crystallization of the 22 kDa N-terminal fragment of apoE also showed that this fragment can adopt a helical bundle conformation that comprises 4 α-helices arranged with their hydrophobic faces brought together towards the center of the molecule (71). Similarly, the structure observed for apolipophorin III, an insect apolipoprotein, was also shown to be a helical bundle (72). These two structures are distinctly dif-

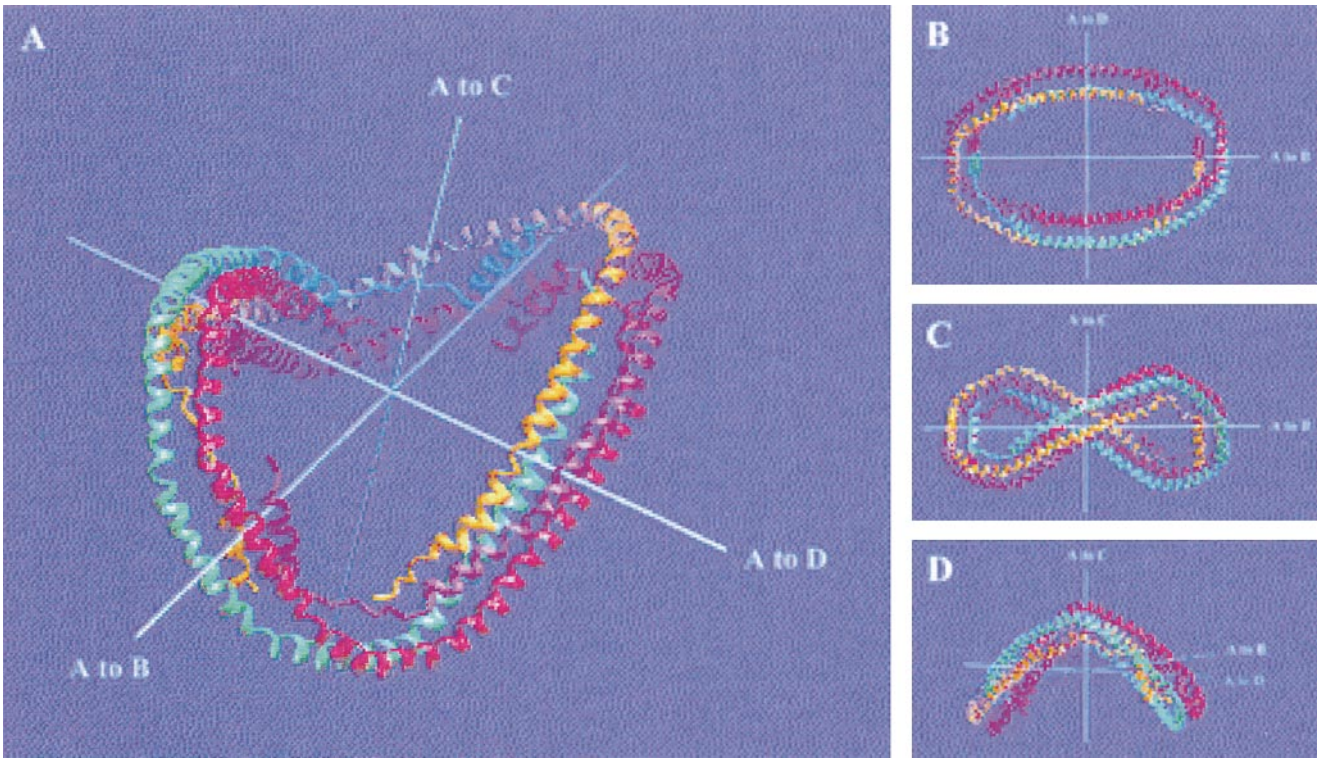

Fig. 4. Ribbon representation of the crystallized tetramer of apoA-IΔ(1–43). The elliptical and curved shape of the protein is evident from this structure. A, B, C, and D represent four different views of the same structure along different axes. Molecule A is gold, B is purple, C is pink, and D is green. Note the antiparallel orientation of molecules A and B, and C and D. The A/B dimer and the C/D dimer are also oriented in an antiparallel manner (70).

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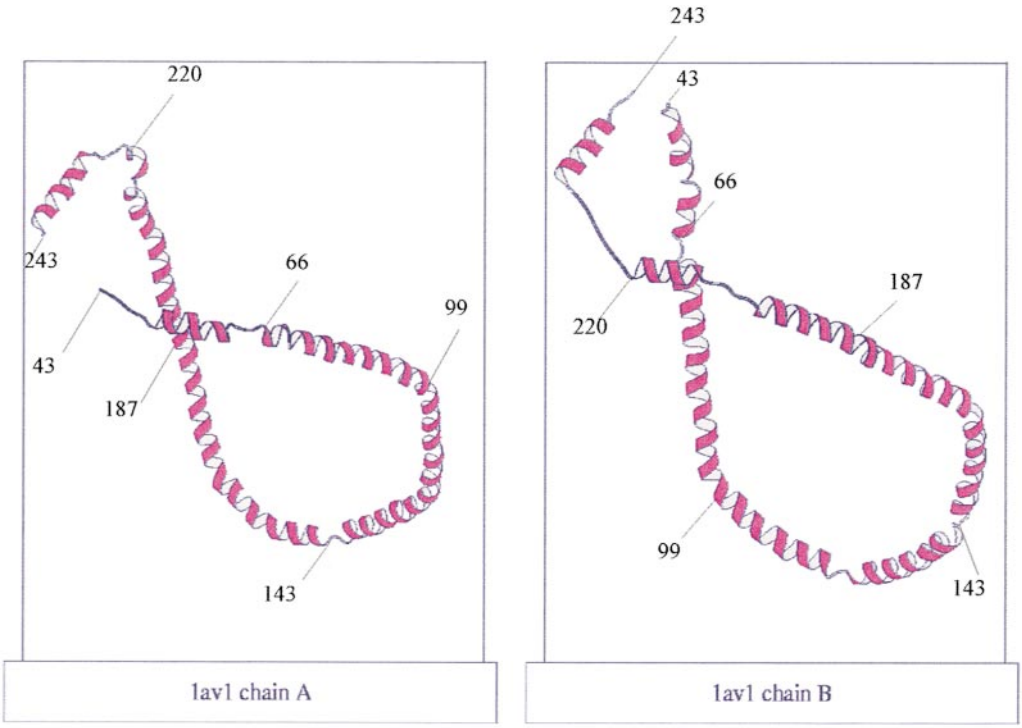

Fig. 5. Ribbon representation of two monomers of apoA-IΔ(1–43). The two basic constituents of a dimer of apoA-IΔ(1–43) are represented. The N- and C-terminals of each monomer are labeled as well as the first residues of each identified helix (70). 1av1 chains A and B refer to the PDB identification codes given by the Brookhaven protein structure database (address on the world wide web: http://www.rcsb.org/pdb/). This edited version was obtained by courtesy of Drs. David Borhani and Christie Brouillette.

ferent from that of apoA-IΔ(1–43) in that they represent intramolecular bundles whereas the apoA-IΔ(1–43) structure is an intermolecular bundle. The asymmetric unit of the mutant apoA-I structure contains two dimers, each constituted by a pair of antiparallel monomers of similar but unique conformations (**Fig. 5**). The antiparallel arrangement of the monomers brings the N-terminal and C-terminal helices of each molecule in close proximity (Fig. 5). In this crystal structure, the predicted punctuations of the 10 helices by the Pro and Gly residues are not β-turns but rather kinks occurring at these residues within the ellipsoid helical ring of each monomer. Borhani and colleagues (70) also proposed a model of a spherical HDL containing four apoA-I molecules. The spherical HDL has an ellipsoidal shape, which accommodates apoA-I in a belt mode. A model for discoidal HDL was also proposed (70), and according to this model, apoA-I may wrap around the edge of the disc with its α-helices oriented perpendicular to the phospholipid acyl chains. This model is not, however, consistent with some early results obtained by attenuated total reflection infrared spectroscopy that have shown that, in a disc, apoA-I α-helices are, rather, oriented parallel to the phospholipid acyl chain (38, 73). More recent analysis using polarized attenuated total internal reflection (PATIR) infrared spectroscopy has challenged this interpretation (74). The orientation of apoA-I on discoidal particles was examined under native conditions by adsorption of Lp2A-I particles to a phospholipid monolayer using phosphatidylserine incorporated in both the monolayer and the lipoprotein and Ca^{2+} as linkers. Under these conditions, Koppaka and colleagues (74) concluded that apoA-I helices are oriented perpendicular to the acyl chains of phospholipids and unambiguously supported a belt-model structure. The belt structure may be similar to that observed in spherical HDL (70), and, in that case, the N-terminus may modulate apoA-I conformation by directly interacting with the central domain of the protein (22, 54).

Lessons learned from biophysical techniques. In summary, many studies of apoA-I structure on model discoidal lipoproteins support the notion of a central hinge domain that is loosely bound to lipids and separates the N-terminal and C-terminal domains. These studies also generally favor the antiparallel configuration of adjacent amphipathic helices, which came to be known as the picket fence model. However, this model has now been challenged by the belt structure that is supported by the crystal structure of lipid-free apoA-IΔ(1–43) (70) and by the PATIR infrared spectroscopy of discoidal Lp2A-I cited above (74). Recently, Davidson has measured the orientation of one of apoA-I central helices in discoidal particles using nitroxide spin label-induced quenching of tryptophan fluorescence to determine the depth of helix 4 (residues 100–121) in the bilayer of the disc. The results also demonstrate that helix 4 is perpendicular to the acyl chains, an orientation

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compatible with the belt model (S. Davidson, unpublished results). Recently, computer-modeling studies performed by Segrest et al. (75) have suggested a model of discoidal complexes in which 2 molecules of apoA-I interact in an antiparallel manner and wrap around the phospholipid bilayer following a belt. Interestingly, this model also matches the crystal structure of the lipid-free apoA-IΔ(1–43) (70).

It is difficult to definitively conclude which model structure is correct and whether the belt or antiparallel models are mutually exclusive or can exist in different settings, i.e., in spherical or discoidal lipoproteins. The weight of recent evidence favors the belt structure where the amphipathic helices are perpendicular to the acyl chains. However, hybrid structures where some or most of the helices are perpendicular to the acyl chains cannot be excluded at this time.

C. ApoA-I mutagenesis and the definition of lipid binding domains

In the following section, we will analyze and compare the properties of both natural and engineered apoA-I variants that have been described, and for which, at least a partial lipoprotein phenotype and/or apoA-I structure–function characterization has been performed (**Table 1**). We will also attempt to correlate the in vivo observations with the in vitro functional apoA-I characterizations, including the works performed using site directed mutagenesis of apoA-I. To facilitate the reading, apoA-I mutagenesis is divided in three sections covering the N-terminal, central, and C-terminal domains, normally recognized in the protein. However, the reader should be cautioned that the delineation of such domains remains somewhat arbitrary.

Mutations of the N-terminal domain. Several naturally occurring mutations in the N-terminal domain have been

TABLE 1 Characterizations of naturally occurring apoA-I mutations

	Functional and Clinical Consequences					
Mutation	Lipid-binding	Cholesterol Efflux	LCAT Activation	HDL/A-I Levels	Comments	References
Pro_3→Arg	normal			normal	impaired pro apoA-I conversion	76, 172
Pro_3→His			normal	normal	impaired pro apoA-I conversion	76, 173
Pro_4→Arg	normal		normal	normal	no apparent effect	76, 76, 172, 173
Arg_{10}→Leu				normal	(?)	174
Asp_{13}→Tyr				reduced A-I levels		175
Gly_{26}→Arg					amyloidosis	176
Ala_{37}→Thr				normal	no apparent effect	177
Trp_{50}→Arg					amyloidosis	178
Leu_{60}→Arg					amyloidosis	179
60–71→Val–Thr					amyloidosis	180
Δ(70–72)					amyloidosis	181
Pro_{90}→Leu					amyloid cardiomyopathy	182
Ala_{95}→Asp				normal	no apparent effect	183
Tyr_{100}→His				normal	no apparent effect	184
Asp_{103}→Asn				normal	no apparent effect	173
Lys_{107}→0	impaired		reduced	reduced HDL-C LpA-I: A-II	possible amyloidosis	76, 185–187
Lys_{107}→Met	normal		normal	normal		76, 188
Trp_{108}→Arg				normal	no apparent effect	183
Glu_{110}→Lys			normal			189
Glu_{136}→Lys		normal		normal	$apoE_2$ phenotype	190
Leu_{141}→Arg		reduced (plasma)	reduced	reduced α-HDL	CHD (other risk factors)	128, 170
Pro_{143}→Arg			reduced	reduced A-I		191
Δ(146–160)	impaired		reduced	reduced A-I + HDL	dominant negative phenotype	77, 192
Arg_{151}→Cys			reduced	reduced A-I + HDL	similar to $apoA\text{-}I_{Milano}$	165
Val_{156}→Glu			reduced	reduced	corneal opacity	193
Ala_{158}→Glu				reduced A-I levels		194
Leu_{159}→Arg	normal	normal	reduced	reduced A-I + HDL	dominant negative phenotype	129, 195
Arg_{160}→Leu		?		reduced HDL, A-I, LpA-I:A-II		196
His_{162}→Gln					no apparent effect	184
Pro_{165}→Arg	normal	reduced	reduced	reduced A-I + HDL		76, 172
Arg_{173}→Cys	impaired	reduced	reduced	reduced A-I + HDL	increased TG longevity	78, 81, 83–85
Arg_{173}→Pro	nd			reduced	amyloidosis	197
Glu_{198}→Lys	normal	normal	normal	normal	(?)	76, 92, 93, 188
Glu_{235}→0	nd	reduced	normal	reduced	faster turnover	94

Phenotype is described as well as the properties observed in vitro. Only mutations with small modification (mutants with additional residues or with less than 90% of apoA-I sequence are not included) and for which a phenotype or functional data is available are described.

(?) indicates that reduced HDL-C levels or CHD have been associated in some patients with the mutation.

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identified and these mutations are often associated with amyloidosis (Table 1). This disease is a disorder of protein metabolism in which autologous proteins or their fragments associate with amyloid precursor proteins and are deposited as fibers in the tissues. Small N-terminal fragments of apoA-I variants have been found in some individuals with amyloidosis (Table 1). The presence of an extra positive charge in the N-terminal domain may be responsible for the formation of amyloid deposits. Defects in the metabolism of HDL resulting from mutations in the N-terminal domain have not yet been fully elucidated.

We have studied a site–directed engineered mutant of apoA-I lacking the domain 1–43. We have observed that the mutant apolipoprotein remains oligomerized after incubation with cholate (used to prepared discoidal complexes), whereas this was not the case for apoA-I and other deletion mutants, including those of the C-terminal domain (Frank, P. G., C. G. Brouillette, J. A. Engler, and Y. L. Marcel, unpublished results). Hence the N-terminal domain is not only important in the stabilization of the lipid-free protein (31) but may also favor the formation of lipid–protein complexes by maintaining a certain accessibility to the lipid-binding helices formed by residues 44–65 and residues 220–241, and possibly to the central domain. Although the domain containing helix 44–65 has not yet been fully characterized, studies by Palgunachari et al. (13) have indicated that it binds lipids almost as efficiently as the C-terminal helix 220–241. Rogers et al. (27) have recently examined site-directed engineered deletion mutants of the N-terminal domain. Their studies demonstrate the importance of the amino terminal 1–43 and lack of importance of the C-terminal aa 187–243 for the proper folding of the lipid-free protein. Moreover, they also show the presence of conformational heterogeneity observed for the first time for human apoA-I and the mutants by sedimentation velocity experiments. The dynamics and shape for these molecules provide structural information supporting a lipid binding mechanism and suggest a conformational flexibility that may help apoA-I bind lipids by allowing access to hydrophobic surface of the molecule. Deletion of residues 88–98 also resulted in an unexpected low stability and low helicity of apoA-IΔ (88–98) (5% of the residues missing results in a 50% reduction in helicity). Interestingly, the domain 88–98 represents one of the two 11-mer α-helices of apoA-I and may be important in determining the proper pairing of helices as proposed in the belt model. Work from Rogers et al. (27) that compared Δ(1–43) and Δ(1–65) suggests that residues 44–65 are completely helical in human apoA-I and may be involved in the conformational flexibility of the lipid-free molecule.

Mutations of the central domain. Several naturally occurring mutations in the central domain of apoA-I have been described (Table 1). However, few of them have been associated with clear defects in lipid binding properties. Only when the secondary structure of the protein was clearly modified [Lys_{107}→0, Δ(146–160)], were impaired lipid-binding properties observed (76, 77). Interestingly, when incubated with cells, the mutant Δ(146–160) was predominantly associated with large HDL (9–20 nm) whereas the native protein could also form smaller complexes (7.5 nm) (77). ApoA-I_{Milano} (Arg_{173}→Cys), which has been extensively studied (78–87) also appears to be associated with size restricted HDL lipoproteins (smaller HDL and reduced number of HDL subpopulations) as observed in vitro and in vivo compared to the wild-type apolipoprotein (82, 85). Apparently, this size restriction is the result of the dimerization of apoA-I induced by the presence of a Cys residue in the mutant protein. Although not demonstrated, the apoA-I_{Milano} dimer may be formed intracellularly before lipidation and generation of HDL particles.

To analyze the role and importance of the central helices in apoA-I association with lipids, three mutants corresponding to deletions of central α-helices [Δ(100–143), Δ(122–165), Δ(144–186)] have been produced (61). These deletions have been designed to characterize the lipid-binding properties of the central helices and to test the hypothesis of a possible hinge domain in human apoA-I. In the resulting mutants, the periodicity of the helices and the overall secondary structure present in native human apoA-I was maintained. We have shown that, compared to a deletion of the C-terminal domain, deletions in the central domain of apoA-I have little effect on the kinetics of association with DMPC (61, 88). Moreover, these central domain deletion mutants can still associate with cellular lipids and form lipoprotein particles in vitro (88). These results therefore suggest that these central helices may not play an essential role in the initial binding of lipid. However, the corresponding mutants appear to have a reduced lipid binding capacity (61), which may indicate that they are not capable of accommodating varying amounts of phospholipid. Taken together, these observations support the hypothesis that the central domain of apoA-I (residues 100–186) is actually responsible for the plasticity of apoA-I and allows the binding of varying amounts of lipid. This concept is also compatible with the observations of Durbin and Jonas (89) that apoA-II can displace the same central helices of apoA-I from the lipoprotein, a process that facilitates apoA-I displacement from HDL. Recent observations from this laboratory on the role of central helices in the maturation of HDL in vivo demonstrate conclusively that the helix formed by residues 100–121 and to a lesser extent the helix formed by residues 122–143 are very important in lipid binding. In contrast, the two helices between residues 144 and 186 contribute little to lipid binding (90).

Mutations of the C-terminal domain. Very few natural mutations have been described in the C-terminal domain of apoA-I (Table 1). Funke et al. (91) have described a mutant in which a frameshift mutation leads to a modification of residues 203–229 and a smaller mature protein (229 residues instead of 243). Examination of this patient revealed corneal opacity and reduced plasma LCAT activity. However, the presence of a Cys in the modified C-terminal domain of the protein may also explain the phenotype since this protein was found to form hetero-oligomers with apoA-II. A natural Glu_{198}→Lys mutation has been described by Strobl et al. (92) but it was not possible to

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firmly establish whether this mutation caused reduced HDL-C levels. In this case, half of the subjects carrying the mutation had normal HDL-C levels whereas the other half had HDL-C below the fifth percentile for age and sex. This mutant was further characterized in vitro and no particular impairment in its functional properties could be demonstrated (76, 93). However, Han et al. (94) have recently identified a new apoA-I mutant (Glu_{235}→0) that is associated with low HDL-C levels possibly because of an increased turnover of the mutant protein, which may be due to a reduced lipid-binding affinity. In support of this view, studies with rabbits (95, 96) and with transgenic mice (97) have confirmed the importance of the C-terminal domain of apoA-I in the formation of HDL lipoproteins. In rabbit studies, deletion of residues 201–243, 217–243, or 226–243 (95) and 190–243 (96) markedly increased the rates of apoA-I catabolism. Furthermore, the C-terminal truncated apoA-I proteins were mostly associated with very high density lipoprotein (VHDL), which suggests a poor ability of the mutant apoproteins to associate with lipids and form mature and fully lipidated HDL. Holvoet et al. (96) have also shown that replacement of domain 190–243 with helices of apoA-II (residues 12–77), which has a higher lipid-binding affinity than apoA-I, could not improve this association. Slightly different results were observed in transgenic mice where the same chimeric apolipoprotein, apoA-I(1–189)–apoA-II(12–77), could associate very well with lipids and form large HDL particles (97). These differences may be related to the genesis of HDL: rabbit apoA-I that is already associated with rabbit HDL has to be displaced by the injected apoprotein whereas in transgenic mice, preformed HDL containing the protein of interest were directly being studied. Moreover, studies from Laccotripe et al. (98) suggested that engineered mutants, in which hydrophobic residues of the last helix were modified, had reduced initial binding to phospholipids. These observations are consistent with our data and those of others showing that the C-terminal domain 187–243 is necessary for the initial association with lipids (88, 95, 99, 100). Moreover, we have also shown that the mutant apoA-IΔ(187–243) could not associate with cellular lipids and form lipoprotein particles (88). The C-terminal domain, probably residues 220–241, is involved in the initial association with lipids and in the formation of lipoprotein particles (99, 100).

2. INTERACTION OF APOA-I WITH CELLS

A. Mechanisms of cellular cholesterol efflux

The different steps and mechanism of cellular cholesterol efflux mediated by apoA-I and HDL have been recently reviewed (101–103). Here the emphasis is placed on the importance of apoA-I structure in the process.

In diffusional efflux, the rate-limiting step is the desorption of FC from the plasma membrane. Desorption may depend on the properties of the cell membrane as noted by several investigators, who showed that under similar experimental conditions, the rate of cholesterol efflux varies widely and depends on the cell type examined (104–106). In addition, the presence of different pools of cholesterol and their inter-exchange within the plasma membrane may affect this process (105, 107, 108). The protein composition of HDL (apoA-I/apoA-II ratio) has also been shown to affect this transfer. The lipid composition of acceptors may be a key factor in this process since several studies have shown that the phospholipid (109, 110), and FC (111, 112) content of HDL could affect cholesterol flux between cells and lipoproteins. This process appears to depend mostly on the presence of lipids in HDL since protein modifications by tetranitromethane or limited proteolysis do not affect this diffusional transfer (113, 114). Large phospholipid vesicles are also less efficient than HDL when compared on a phospholipid basis (115).

Contrary to the above, other observations suggest a significant role for apoA-I in cholesterol efflux mediated by passive aqueous phase diffusion. The role of different apoA-I domains in this process has been examined using mAbs to apoA-I or by site-directed mutagenesis. The ability of apoA-I to interact with lipid surfaces suggests a possible role for its amphipathic α-helices to promote cellular cholesterol efflux from the plasma membrane. The first studies performed with mAbs to apoA-I have indicated the involvement of a central domain in this process (116–119). Banka, Black, and Curtiss (116) found that antibodies binding to residues 74–110 could inhibit cholesterol efflux to HDL. Luchoomun et al. (118) and Sviridov, Pyle, and Fidge (119) provided evidence for the involvement of domains around residues 165 and 140–150, respectively. In a different study, Fielding et al. (117) showed that an epitope of apoA-I (region 137–144) was more exposed in preβ_1-HDL than in other subspecies of HDL. The central domain was suggested to be much more labile than other domains and, therefore, could interact more easily with the plasma membrane to promote cellular cholesterol efflux (108). However, the effects of mAbs on apoA-I are complex and not only can they interfere sterically with domains other than the epitope itself (64), but they can also modify the secondary structure of the protein (120).

Lipid efflux studies to apoA-I mutants performed by Gillotte et al. (121) and Sviridov, Pyle, and Fidge (122) in mouse fibroblasts and HepG2 cells respectively, have shown that deletions of residues 44–126, 139–170, and 190–243 (121) or residues 222–243, 210–243, 150–243, and 135–243 (122) had no effect on the ability of lipidated apoA-I to promote cellular cholesterol efflux. We have recently examined the ability of central domain deletion mutants of apoA-I to promote cholesterol efflux in LpA-I from fibroblasts (123). Our results confirm that at least in normal fibroblast the central domain of apoA-I is not required for efficient diffusional cholesterol efflux as long as the mutant protein can associate with phospholipids (123).

A second mechanism, which involves the direct binding of apoA-I to the cell membrane and causes the transfer of intracellular cholesterol to the plasma membrane, is called binding- and translocation-dependent cholesterol efflux. Whether this transfer is specifically due to apoA-I-cell interaction per se or to a transfer of cholesterol between intra-

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cellular pools and the plasma membrane as a response to the removal of FC from the plasma membrane, remains to be established. Nevertheless, cell binding of HDL and intracellular cholesterol transfer are abolished upon tetranitromethane modification or limited proteolysis of HDL (113, 114), which demonstrates the importance of intact apoA-I. Mendez (124) recently demonstrated that cholesterol efflux to lipid-free apoA-I occurs only with quiescent (growth-arrested) or cholesterol enriched cells. Several groups have reported the existence of a specific receptor for apoA-I (101), one recent candidate being the scavenger receptor class B type I (SR-BI) (125). The interesting feature of this receptor is that efflux to acceptors appears to be dependent on their phospholipid content (126), and thus appears relevant to diffusional efflux. This suggests that the ability of apoA-I (C-terminal domain) to bind phospholipid is required for efficient cholesterol efflux as was suggested by several earlier studies (127). Sviridov et al. (122), using C-terminal deletion mutants, have shown that the C-terminal domain (deletion of residues 222–243, 210–243, 150–243, and 135–243) may be important in intracellular cholesterol efflux mediated by apoA-I. However, these authors did not observe any effect of these deletions on the binding to HepG2 cells. These findings cannot be simply explained by the presence of a receptor that mediates cholesterol efflux. Other high affinity non-specific cell surface binding sites for apoA-I at the surface of HepG2 cells may mask the specific binding to the low affinity binding sites specifically involved in the transfer of intracellular cholesterol to the plasma membrane. A low binding affinity of apoA-I for cell may be required in order to allow a rapid release of apoA-I after or upon acquisition of cellular phospholipid and cholesterol.

B. ApoA-I structure and cellular cholesterol efflux

Only a few studies have characterized the efflux capability of naturally occurring apoA-I mutants (93), and most used the plasma lipoproteins formed by the mutant proteins (83, 94, 128, 129). No mutant with a mutation in the N-terminal domain has been examined (Table 1).

Most of the naturally occurring apoA-I mutants analyzed correspond to alteration of residues in the central domain of apoA-I. Three natural variants of apoA-I have been shown to have a reduced ability to promote cellular cholesterol efflux. These mutations (Pro_{165}→Arg, (93); Arg_{173}→Cys, (83)) may affect the secondary and/or tertiary structure of the C-terminal domain of apoA-I. The substitution Pro_{165}→Arg may result in the elimination of a β-turn or kink between adjacent helices, which could modify the accessibility of the C-terminal domain to lipids and/or cells in the resulting mutant. The Arg_{173}→Cys mutation induces the dimerization of apoA-I through an intermolecular covalent cystine crosslink. The monomer has a decreased lipid binding affinity (130), but the dimerization alters the conformation of the C-terminal domain and modifies the lipid binding properties of the mutant apoproteins (130). The apoA-I_{Milano} dimer has an increased ability to promote efflux from macrophages and Fu5AH compared to wild type (131).

In our studies (88, 123), the deletion of central α-helices had no effect on the ability of apoA-I to promote either diffusional or specific cellular cholesterol efflux from normal and cholesterol-loaded fibroblasts, or from cholesterol-loaded macrophages. However, these results were obtained in the absence of LCAT and other plasma factors involved in the metabolism of HDL. In particular, mutants apoA-IΔ(122–165) and apoA-IΔ(144–186) may display a reduced ability to promote cellular cholesterol efflux in plasma or in the presence of LCAT alone since they cannot activate the cholesterol esterification reaction as efficiently as recombinant apoA-I and apoA-IΔ(100–143) (123). In support of this hypothesis, the ability of a naturally occurring apoA-I mutant (Leu_{141}→Arg) to promote cellular cholesterol efflux was examined with plasma from subjects carrying this mutation (hemizygote patients) (128): A reduced cellular cholesterol efflux to plasma was observed, which may be due to a reduced transfer of cholesterol from preβ-HDL to α-HDL. This probably resulted from the reduced ability of the mutant apoprotein to activate LCAT (128). Cholesterol esterification may therefore promote the flux of cholesterol through the HDL pool by stimulating the formation of cholesteryl esters that are transferred to apoB-containing lipoproteins by CETP, and then cleared via the LDL-receptor pathway in the liver (132).

Cellular cholesterol efflux mediated by two natural variants with C-terminal mutations (Glu_{198}→Lys, Glu_{235}→0) have been examined (93, 94). The first mutation (Glu_{198}→Lys) was not associated with reduced cholesterol efflux, however, the other mutation (Glu_{235}→0) (94) was accompanied with a 54% decrease in cholesterol efflux from cholesterol-loaded mouse peritoneal macrophages. We have also observed that deletion of residues 187–243 of apoA-I was associated with a markedly reduced ability of the mutant apoprotein to promote cellular cholesterol efflux from cholesterol-loaded macrophages (88). The mutant was also unable to form lipoproteins upon incubation with the cells and this property was associated with a reduced kinetic of association with DMPC (88). Interestingly, the natural apoA-I mutant Glu_{198}→Lys did not appear to have a reduced ability to associate with lipids (76). This suggests that the ability of apoA-I to promote specific cholesterol efflux from macrophages depends on a functional C-terminal lipid binding domain.

Simple diffusional transfer between the plasma membrane and the apoA-I-containing lipoprotein acceptor (i.e., HDL) appears to require the presence of amphipathic α-helices. Diffusional cholesterol efflux to an amphipathic peptide depends on the peptide lipid affinity and ability to retain cholesterol molecules (115, 127). These concepts are supported by our observations that apoA-I mutants (deletion of the central domain 100–143, 122–165, 144–186) that can associate with lipid (61) can also, in the lipid-bound form (Lp2A-I), promote cellular cholesterol efflux (88, 123). Specific or translocation-dependent efflux involves the interaction of lipid-free apoA-I with specific cell surface binding sites, followed by the transfer of intracellular cholesterol from an ACAT-accessible pool

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to the plasma membrane and subsequent transfer to the donor. The first step also appears to be mediated by amphipathic α-helices since synthetic peptides with affinity for lipids can mediate this transfer (127, 133). In this process, apoA-I may interact with specific plasma membrane domains such as caveolae (enriched in FC and sphingomyelin) and subsequent transfer of FC and phospholipid to the apoprotein may generate preβ-HDL (134). In agreement with this proposed mechanism, the reduced affinity of peptides for the lipid surface is associated with a reduced ability to promote cellular cholesterol efflux (127, 133). The C-terminal domain of apoA-I may therefore play a major role in this interaction of the lipid-free apoprotein with the cell. Our data showing that apoA-IΔ(187–243) displays both a reduced cell surface binding and reduced cellular cholesterol efflux from cholesterol-loaded macrophages agree with this model (88). In contrast, this mutant displays similar binding and efflux with cholesterol-loaded fibroblasts. The difference in cell specificity may be due both to the lower cholesterol content of cholesterol-loaded fibroblasts and to the specific cell surface properties of cholesterol-loaded macrophages (88). That the N-terminal domain (44–65) is important to the initial binding of apoA-I to lipid (13) would suggest that it should also contribute to specific efflux (13). Further studies of apoA-I with mutations in this domain would help to determine if this domain is also involved in this important function of apoA-I. The recent characterization of mutations affecting the ABC-1 gene in Tangier disease and hypoalphalipoproteinemia (135–137) has shown the importance of this integral cell membrane protein in cholesterol efflux. Lawn et al. (138) have reported its presence on the plasma membrane, suggesting that it may directly participate in the binding of apoA-I. Differences in expression levels of ABC-1 may also account for the cell specificity in lipid efflux. Other recent studies have demonstrated the role of ABC1 in the regulation of cellular cholesterol efflux and its involvement in Tangier disease (138–140).

3. APOA-I AND THE ACTIVATION OF LECITHIN:CHOLESTEROL ACYL TRANSFERASE

The LCAT glycoprotein (EC 2.3.1.43), consisting of 416 amino acids, displays two activities: a phospholipase A_2 activity, since it can hydrolyze the *sn*-2 fatty acid from phosphatidylcholine, and an acyltransferase activity, since it can transfer the fatty acid to FC and form CE. A detailed analysis of LCAT has been previously reviewed (141–143).

A. LCAT activation

Although LCAT may be able to bind lipids directly, optimum reaction requires activation by one of the exchangeable apoproteins. ApoA-I was the first described activator of LCAT (144) and is its most potent in vivo activator (141). This reaction requires at least three steps. First, LCAT binds to the substrate (HDL) and/or the activator, and in a second step, LCAT hydrolyzes the fatty acid at the *sn*-2 position of a phospholipid. The third and final step is the transesterification of cholesterol and concomitant release of CE.

The lipid composition of HDL can affect the ability of apoA-I to activate cholesterol esterification by LCAT. In several studies, it has been suggested that this effect was directly related to a modification of apoA-I stability and/or conformation or to the ability of LCAT to bind substrates (37, 40, 145–148). In particular, Sparks et al. (51, 149) have suggested a role for the surface charge properties of apoA-I in its interaction and ability to activate LCAT.

The mechanism of activation is not simple, as LCAT can hydrolyze small soluble substrates in the absence of apolipoproteins and can directly interact with lipid interfaces, although with reduced affinity as compared to apoproteins (142). Numerous investigations have demonstrated the effect of varying the HDL apolipoprotein-lipid composition on the LCAT reaction (37, 51, 89, 145, 146, 148, 149). These changes have been in part attributed to modification of the charge and conformation of apoA-I, which suggests that in vivo, both the apolipoprotein and lipid composition of HDL can modulate the interaction between apoA-I and LCAT (37, 51, 149). In this context, several studies have shown that small complexes (low phospholipid/A-I molar ratio) were more efficient in activating LCAT (145, 149). In that case, the increased reactivity was, in part, associated with a change in the accessibility of the N-terminal and central domains of apoA-I that may be in close contact in the tertiary structure (64, 149). Other lipids such as cholesterol (increases LCAT activity with increased cholesterol content) or sphingomyelin and phosphatidylcholine (both reduce LCAT activation) may affect differently these domains of apoA-I (51, 89, 148, 149). The conformational changes of apoA-I that are associated with very specific lipid compositions appear to allow the interaction of LCAT, either directly with the lipid phase or through apoA-I.

Other apolipoproteins can also activate the LCAT reaction but below 20% of that observed for apoA-I (150, 151). Since the presence of amphipathic α-helices is required but not sufficient to activate LCAT efficiently, a specific domain of apoA-I may be responsible for this function. Studies designed to examine the sequence involved have been performed using synthetic peptides, CNBr fragments of apoA-I and, finally, site-directed mutagenesis. Studies with peptides suggested that despite a high affinity for lipids, the C-terminal domain could not activate LCAT whereas the region 148–185 could bind and activate the enzyme (152). Other synthetic peptides with a high amphipathic character, but no homology to the apoA-I sequence, could also activate LCAT (153, 154). Anantharamaiah et al. (155) have constructed a peptide corresponding to a dimer of the consensus amino acid sequence obtained for the eight 22-mer predicted α-helices of apoA-I. This peptide gave maximal LCAT activation only as a dimer and when residue 13 of the helix was glutamic acid. These results suggested that activation of LCAT was depending on a large domain of apoA-I located within residues 66–121. Inhibition of cholesterol esterification in the presence of

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Activator                          PVLDEFREKLNEELEALKQKLK

Helix 143-164 (apoA-I) 143-PLGEEMRDRARAHVDALRTHLA-164
                           ↑         ↑     ↑  ↑↑
Helix 165-186 (apoA-I) 165-PYSDELRQRLAARLEALKENGG-186
                           ↑         ↑
LCAT                       PΨ dEΨR r     leALk
Activation motif
```

Fig. 6. Alignment of the LCAT activator peptide with amino acids 143–164, 165–186 of apoA-I. The residues highlighted in bold reflect common or conserved amino acids that may be required for LCAT activation. A putative LCAT activation motif, which reflects common or conserved residues between the three aligned sequences is proposed. Lowercase letters (d, r, l, e, and k) indicate conserved residues (the preponderant residue is shown) and the symbol Ψ denotes the presence of hydrophobic residues in the three aligned sequences. Mutations of residues indicated by arrows have been identified in patients with reduced HDL levels and may result in lower LCAT activity (see text for details).

mAbs against apoA-I also suggested that a rather large domain corresponding to residues 95–175 could be involved in LCAT activation. Moreover, Meng et al. (156) demonstrated that a mAb recognizing the N-terminal domain of apoA-I (mAb 4H1 reacting with residues 2–8) could enhance cholesterol esterification. This was only observed in small cholesterol-rich Lp2A-I particles, where the N-terminal epitope shares structural relationships with specific central domain epitopes. However as noted above, the effects of mAbs on apoA-I are complex as antibodies not only interfere sterically with other domains (64) but can also modify the structure of the protein (120).

Recent studies have now clearly established that region 144–186 is the LCAT activator domain of apoA-I (123, 157–160). After alignment of these helices with other LCAT activating peptides, we have proposed an LCAT activating motif (123) (**Fig. 6**). This model is also supported by in vivo studies of naturally occurring apoA-I mutants (Table 1, Fig. 6 and see below). Interestingly as noted earlier, the central region of apoA-I may also be involved in the specific binding of cholesterol in HDL. This domain could therefore allow the presentation of cholesterol to LCAT.

B. ApoA-I mutants and identification of domains involved in LCAT activation

Numerous groups have investigated the relationship between LCAT activation and apoA-I structure and/or conformation (51, 142, 145, 149). Recent studies have identified the central and C-terminal domains of apoA-I in the activation of LCAT.

Among the natural mutants of apoA-I corresponding to modification of residues in the N-terminal domain, only a few have been studied for their ability to activate LCAT and none were shown to negatively affect cholesterol esterification (Table 1) (76). A study of the site-directed engineered mutant, apoA-IΔ(1–43), suggested that this mutant had only a slightly reduced ability to activate the LCAT reaction (31). A large body of data obtained with natural and synthetic mutants firmly shows that the central domain 144–186 is involved in this reaction (123, 157, 158, 160, 161). Since this central domain may interact with the N-terminal domain (54, 64), a specific interaction between these two domains may contribute to the stabilization of lipid-associated apoA-I and subsequently to the efficient esterification of FC by LCAT. Several natural variants with defective LCAT activation properties (Lys_{107}→0, Leu_{141}→Arg, Δ(146–160), Arg_{151}→Cys, Val_{156}→Glu, Leu_{159}→Arg, Pro_{165}→Arg, Arg_{173}→Cys) have been identified and most of them correspond to mutations in the central domain 144–186 (Table 1). Other mutations (Lys_{107}→0, Leu_{141}→Arg) may have a direct effect on the conformation of this central domain and indirectly affect the ability of apoA-I to activate LCAT (149, 156, 162, 163).

The mechanism by which apoA-I activates LCAT is still unknown but may involve a direct interaction between apoA-I helices and residues 152–169 of LCAT. Positively charged residues present in both helices 144–165 and 166–186 may interact with negatively charged residues found on this LCAT helix. Mutagenesis studies of LCAT by Wang et al. (164) have shown that Glu 154, 155, and 165 are not important for LCAT activity but may be involved in the binding of cholesterol. The region 151–174 of LCAT may therefore indirectly interact with apoA-I through the binding with HDL cholesterol, a process that may result in a conformational change of LCAT and its activation. The lower lipid binding affinity of apoA-I central helices (residues 144–186) (13) allows their displacement by apoA-II (89) and may also favor their displacement and interaction with the LCAT helix (residues 152–169).

Interestingly, mutations of positively charged residues in these apoA-I helices usually result in decreased LCAT activation (84, 165). In small discoidal LpA-I (except those rich in cholesterol (165)), where these central helices may not interact completely with lipids, LCAT activation appears to be most efficient, possibly because of an enhanced accessibility to the lipid interface and to the central domain of apoA-I (149). In support of this model, an increase in the negative surface charge of the Lp2A-I is associated with increased LCAT reactivity (149). In HDL complexes, where the lipid species have little effect on the lipoprotein surface charge, it appears that a modification of the surface charge properties is primarily due to a change in apoA-I conformation (22, 149, 166, 167). LCAT activity may therefore be a function of the exposure of apoA-I charged residues in these lipoproteins. Several studies have shown a direct effect of the surface charge of LpA-I on the structure and function of lipoproteins (22, 51, 149, 166–168). ApoA-I conformation is affected by HDL lipid composition and is the major determinant of HDL charge, even if charged lipids (phosphatidylinositol, phosphatidylserine) can directly contribute to the negative charge of HDL. Different lipids can also have different effects on the stability of apoA-I in HDL (22, 51, 149, 166). NMR and mAb immunoreactivity studies have also implicated a specific domain of apoA-I in the binding of FC in HDL (39, 64). This binding, which occurs around residue 144 of apoA-I, may also be responsible for the specific changes in the charge of HDL containing FC (166).

The C-terminal domain of apoA-I was suggested to be

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involved in LCAT activation in several studies (98, 157, 158) but it now appears that this observation was due to defective lipid-binding properties of the mutants. In these assays, lipid vesicles were incubated with apoA-I mutants and as the C-terminal mediates the initial binding, these mutants could not interact properly with lipids and therefore activate LCAT. This has been confirmed by Dhoest et al. (160) who showed that replacement of the carboxyl terminal (190–243) by residues 12–75 of apoA-II restores the lipid binding properties of the protein but does not change its ability to activate cholesterol esterification. Because apoA-II is not so efficient as apoA-I in activating the reaction mediated by LCAT (169), it follows that the C-terminal domain of apoA-I is not crucial for this activity. This notion is supported by the work of Han et al. (94) who showed that a patient carrier for the mutation apoA-I Glu_{235}→0 had normal plasma LCAT activity.

C. Physiological significance of apoA-I activation of LCAT

The N-terminal domain (residues 1–43) may be important for the stability of the protein in the lipid-free state and indirectly modulate its interaction with LCAT. The central domain (100–186) has a function in the regulation of the interaction with phospholipid and is responsible for the plasticity of the molecule by allowing its association with varying amounts of phospholipid. The domain formed by residues 100–143 and within it by the helix containing residues 100–121 has a very significant role in the binding of phospholipids, both in vitro (61) and in vivo (90). Beside this role, part of the central domain, which includes residues 144–186, forms the site involved in LCAT activation (123). However, efficient LCAT activation also requires a certain affinity for phospholipid that is contributed by the C-terminal domain (187–243), and within it, probably by the last helix (220–241). In addition, the lipid binding affinity of the C-terminal domain appears crucial for the ability of apoA-I to promote cholesterol efflux from cholesterol-loaded macrophages, a process, which may require the efficient binding of the apoA-I C-terminal domain to the cell.

As indicated in Table 1, mutations in the LCAT activating domain of apoA-I result in reduced HDL-C levels, but are not necessarily associated with increased risk of CHD, possibly because LCAT can still esterify LDL-derived cholesterol and possibly because a functional efflux capacity is more important than a functional LCAT activation. Additional risk factors (e.g., elevated LDL-C, obesity) may be required to observe an increase in CHD as observed by Miccoli et al. (170). However, cholesterol esterification may promote the flux of cholesterol through the HDL pool by stimulating the formation of cholesteryl esters that are transferred by CETP to apoB-containing lipoproteins (128). Nevertheless, it is intriguing that almost all identified mutations that affect the LCAT activating domain of apoA-I, are associated with reduced HDL-C. However, the technique used in screening for apoA-I mutations has relied, in most cases, on the presence of a charge modification of the mutant protein. Therefore, the screening procedure may not have detected mutations that did not affect the protein charge.

4. CONCLUSIONS

Much remains to be understood about apoA-I structure–function relationships. More refined mutagenesis strategies are still required to identify the specific functions of apoA-I domains. The mechanism by which apoA-I associates with phospholipid has to be examined in more detail and studies with apoA-I that contains point mutations of hydrophobic and charged residues in helices, should provide additional insight into the mechanism of apoA-I association with lipids. The role of the N-terminal domain (residues 1–43) in the lipid-free as in the lipid-bound forms has not been clearly identified; point mutations of specific charged residues may resolve this question. The mechanism of LCAT activation by apoA-I has to be further characterized, such as the functional identification of specific apoA-I residues involved.

Several major unresolved questions remain in the elucidation of apoA-I structures either in lipid-free or lipid-bound forms. Is the belt structure prevalent for lipid-bound apoA-I? Does more than one structure exist for apoA-I in different species of lipoproteins? What structural rearrangement accounts for the change of the protein charge in the preβ- and α-HDL forms?

In vivo studies of apoA-I mutants (natural mutants in human, in transgenic or adenovirus-infected animals) will also help to identify the mechanisms by which apoA-I participates in reverse cholesterol transport. The recent identification of the gene responsible for Tangier disease (135–137) and the recent demonstration that cubilin binds apoA-I and mediates its re-absorption by the kidney proximal tubule (171) have opened new avenues for the study of apoA-I metabolism and the regulation of HDL levels. The domain specificity for apoA-I interaction in these pathways still has to be established.

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