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REVIEW ARTICLE

Apolipoprotein A-I structure in high-density lipoproteins

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Abstract
High-density lipoproteins (HDL) have attracted significant attention in recent years due to their apparent role in reducing the risk of coronary heart disease (CHD). There is convincing evidence that apolipoprotein (apo) A-I, the main protein component in HDL, is a major mediator of HDL function that interacts with a host of plasma enzymes and cell surface proteins. ApoA-I is highly adaptable and may associate with, and modulate the function of, different HDL subspecies. To better understand HDL function, studies have focused on apoA-I structure in reconstituted HDL species that model different native subclasses of HDL. Powerful experimental strategies, such as X-ray crystallography and mass spectrometry, combined with chemical cross-linking and spectroscopy, have dramatically improved our understanding of apoA-I structure in reconstituted HDL. In this review, we present an overview of the current understanding of apoA-I structure and look forward to methodological developments in the near future that will allow the study of apoA-I in native subspecies of HDL found in human plasma.

Key words: *Apolipoprotein A-I, apolipoproteins, cholesterol efflux, high-density lipoproteins, reverse cholesterol transport, structure*

Background

Most therapies for treating coronary heart disease (CHD) in the past have been focused on reducing low-density lipoprotein cholesterol (LDL-C). LDL embodies the 'forward cholesterol transport' (FCT) process by which cholesterol is delivered to the peripheral tissues. When LDL-C (popularly referred to as 'bad cholesterol') is in excess, its oxidative modification in the arterial wall can contribute to arterial plaque formation which may lead to CHD. Epidemiological studies dating back to the 1950s have indicated that increased plasma high-density lipoprotein cholesterol levels (HDL-C, the 'good cholesterol') are a powerful independent negative risk factor for CHD. Data from the well known Framingham Heart Study have indicated that for a given level of LDL, the risk of heart disease increases 10-fold as the HDL levels decrease from current USA guidelines of high levels (>60 mg/dL) to low levels (<40 mg/dL). Antiatherogenic functions of HDL include: cholesterol efflux from peripheral cells such as foam cells and its transport back to the liver for catabolism (reverse cholesterol transport (RCT)); prevention of oxidative modification of LDL which reduces foam cell generation (antioxidative properties); and inhibition of expression of adhesion molecules in endothelial cells (anti-inflammatory properties). Current pharmacotherapy options to increase HDL-C include niacin, fibrates, and, to a lesser extent, some of the statin class of cholesterol synthesis inhibitors (specifically simvastatin and rosuvastatin). At present, our understanding of the underlying molecular mechanisms of such therapeutics remains at a basic level and has been hindered by the lack of information on HDL structure in its various subclasses and the molecular basis of its interactions with other proteins. Here we summarize the efforts toward understanding the structure of HDL, focusing primarily on its major protein component, apolipoprotein (apo) A-I.

Composition and subclasses of HDL

On average HDL particles contain 50% protein by mass and range from 70–120 Å in diameter. These complexes are subdivided into different subclasses

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Abbreviations

apoA-I	apolipoprotein A-I
ABCA-1	adenosine triphosphate binding cassette transporter A-I
CETP	cholesteryl ester transfer protein
CHD	coronary heart disease
EPR	electron spin resonance
FCT	forward cholesterol transport
HDL	high-density lipoproteins
HDL-C	high-density lipoprotein cholesterol
LpA-I	HDL that contain only apoA-I
LpA-I/A-II	HDL that contain both apoA-I and apoA-II
LDL-C	low-density lipoprotein cholesterol
PLTP	phospholipid transfer protein
RCT	reverse cholesterol transport
rHDL	reconstituted HDL
FRET	fluorescence resonance energy transfer

Key messages

- Taken together, data from independent techniques have indicated that monomeric apolipoprotein A-I (apoA-I) adopts a four-helical bundle structure in solution, as was seen for other apolipoproteins.
- Taken together there is solid experimental, as well as theoretical, evidence that apoA-I adopts an antiparallel double belt molecular arrangement with a 5/5 molecular registry.
- These studies indicate there may be at least two hinge domains in apoA-I on a reconstituted high-density lipoprotein (HDL) particle, a mid region including helix 5 and the extreme N-terminal regions.
- The definitive size and the fixed number of protein molecules strongly suggest that there is a protein framework holding spherical HDL complexes together just as in discoidal particles.

based on density, electrophoretic mobility, and protein composition (1). HDL is isolated in the density range 1.063–1.21 g/mL and is most commonly subclassified based on its density into HDL_1, HDL_2, and HDL_3 from least to most dense. While HDL_1 is rare except in some pathological states, the two more prevalent species are further subdivided into tighter subspecies as HDL_{2a}, HDL_{2b}, HDL_{3a}, HDL_{3b}, and HDL_{3c}. Mature HDL complexes are spherical in shape with a hydrophobic core of triglycerides and cholesteryl esters surrounded by a phospholipid and free cholesterol monolayer. Amphipathic apolipoproteins are partially embedded in this phospholipid monolayer, exposing their hydrophilic faces to the aqueous environment. Of these, apoA-I and apoA-II are the two major apolipoprotein components in HDL at a 2:1 molar ratio, making ~70% and ~20% (by mass) proteins in HDL, respectively. More minor, but functionally important, apolipoproteins, antioxidative enzymes, and even elements of the immune system constitute the remaining ~10% of HDL protein mass (2,3). Based on protein composition, HDL is separated into two subcategories using immunoaffinity chromatography: those that contain only apoA-I (LpA-I) and those that contain both apoA-I and apoA-II (LpA-I/A-II). Different functional properties have been attributed to these two subclasses. There have been several instances where LpA-I/A-II has been reported as proatherogenic with LpA-I being antiatherogenic, suggesting that apoA-II is a proatherogenic protein. Since some of these studies have been carried out in transgenic animal models and reconstituted HDL (rHDL) particles that may not represent true physiological conditions, the observations may not reflect the true effect of apoA-II on HDL (4–6).

In addition to mature spherical particles, other critically important (but lower-abundance) HDL subspecies exist in human plasma and interstitial fluid and include nascent discoidal HDL and 'lipid-free' apoA-I. Discoidal HDL particles consist of a circular phospholipid bilayer with amphipathic apolipoprotein molecules wrapping around the disc edge. It is still debated whether the component identified as lipid-free apoA-I in plasma may contain trace amounts of lipids and hence may be actually lipid-poor (1,7). These nascent HDL subspecies have been separated based on two-dimensional gel electrophoresis, based on their different electrophoresis mobilities as preβ1 (lipid-free/poor apoA-I), preβ2, and preβ3 (discoidal HDL). The most abundant mature spherical HDL exhibits an α-electrophoretic mobility.

Lipid-free apoA-I

In a sense, lipid-free/lipid-poor apoA-I in human plasma may be the most important form of apoA-I. Estimated at only 5%–10% of total plasma apoA-I concentration, lipid-free/lipid-poor apoA-I participates in the most critical 'first step' of HDL

generation by interacting with the cell surface transporter, adenosine triphosphate (ATP) binding cassette transporter A-I (ABCA-1). This process results in the net transfer of phospholipid and cholesterol from peripheral cells to apoA-I to generate nascent discoidal HDL particles.

The lipid-free/lipid-poor apoA-I in the plasma pool is mainly a result of fresh secretion of the protein from the liver and intestine or dissociation from HDL due to remodeling by plasma factors such as cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP). Most structural studies on lipid-free/lipid-poor apoA-I have focused on delipidated plasma apoA-I or recombinantly expressed apoA-I that lacks lipids. Three-dimensional structural studies of apoA-I have been hampered by its tendency for self-association and its dynamic, molten globular-like properties (8,9).

ApoA-I is a 243-amino acid single domain protein with ten putatively assigned amphipathic helices in a lipid-binding domain that encompasses residues 44–243. All the helices are of 22 residues except helices 3 and 9 which are of 11 residues. Most of these amphipathic helices belong to class A in which basic amino acids are at the boundary of the hydrophobic and hydrophilic phases of the helix, while acidic residues are in the middle of the hydrophilic phase. On the other hand, the N-terminal 43 residues form an α helix of class G*, frequently found in globular proteins, in which acidic and basic amino acids arrange randomly at the hydrophilic phase (1,2). Class G* helices are known to induce protein-protein interactions rather than protein-lipid interactions. Further, apoA-I has no known glycosylation or phosphorylation sites.

The first high-resolution crystal structure (at 4 Å resolution) of lipid-free apoA-I was published in 1997 by Borhani et al. using a deletion mutant of apoA-I (Δ(1-43)A-I) (10). The authors observed that this N-terminal truncation mutant adopted a tetrameric distorted ring-shaped structure with two outer and two inner molecules. Each molecule formed an almost continuous amphipathic helix with kinks at regularly spaced Pro residues. The two outer molecules were tightly associated with each other except at the region 220–227 amino acids(a.a.), creating a continuous hydrophobic patch along the interior phase. The two inner molecules associated in a similar fashion leading to a four-molecular cluster associated via their hydrophobic helical phases to form a distorted 'horseshoe'-shaped ring. This molecular arrangement was suggested to represent a lipid-bound form of apoA-I, despite the absence of lipids. More recently, a crystal structure of full-length lipid-free apoA-I was published by Ajees et al. at 2.4 Å resolution (11). The structure consists of two domains; three-fourths of the N-terminus forms a four-helix bundle, while the remaining C-terminal sequence forms a two-helix hairpin (Figure 1A). These two domains are held strongly in place by chromium (III) tris acetyl acetone which was used to facilitate crystal growth. The importance of the residues at the extreme N-terminus (1–43) in stabilizing the native structure may have played a role in formation of the helical bundle and stabilizing the globular structure as suggested before (12,13). In addition, there are no kinks or turns observed due to the Pro residues, since some of these residues are located in the middle of well formed helices (11). Furthermore, the apoA-I crystal structure resembles the previously published crystal structures of apoE (14) and insect apolipophorin III (15) which also acquired a four-helical bundle organization. Finally, the apoA-I N- and C-termini are near each other, supporting the prior predictions of N- and C-terminus interactions (16,17).

The apoA-I crystal structure is in general agreement with the homology model put forward 1 year earlier by Silva et al. using cross-linking/mass spectrometry/sequence-threading techniques (18). The homology model also showed two domains: an N-terminal four-helix bundle and a separately folded C-terminus (Figure 1B). Further, the homology model indicated that the N- and C-termini are near each other and Pro residues exist in the middle of the continuous helices just as was observed in the crystal structure. However, helix 4 in the homology model is terminated near residue 165 and doubles back on the main helical bundle, whereas the crystal structure shows one contiguous helix. Despite a general similarity, there are important differences between these two models. Based on circular dichroism (CD) spectroscopy, overall α-helicity for lipid-free apoA-I in an aqueous buffer is ~48%–55% (19,20). While the homology model helical content (~56%) agrees with this, the crystal structure has ~83% helical content, perhaps due to the crystallization conditions and additives which may have artificially stabilized certain elements of secondary structure. The other major difference is that most of the proteolytic sites reported for lipid-free apoA-I in aqueous buffer are at the turns or at the edge of a helix in the homology model (12,21). However, in the crystal structure, many of these sites are in the middle of α-helices, hindering proteolytic cleavage. Another observation is that two Trp residues at positions 8 and 50 in the crystal structure are in hydrophilic environments in the crystal, contradicting previous fluorescence experiments in

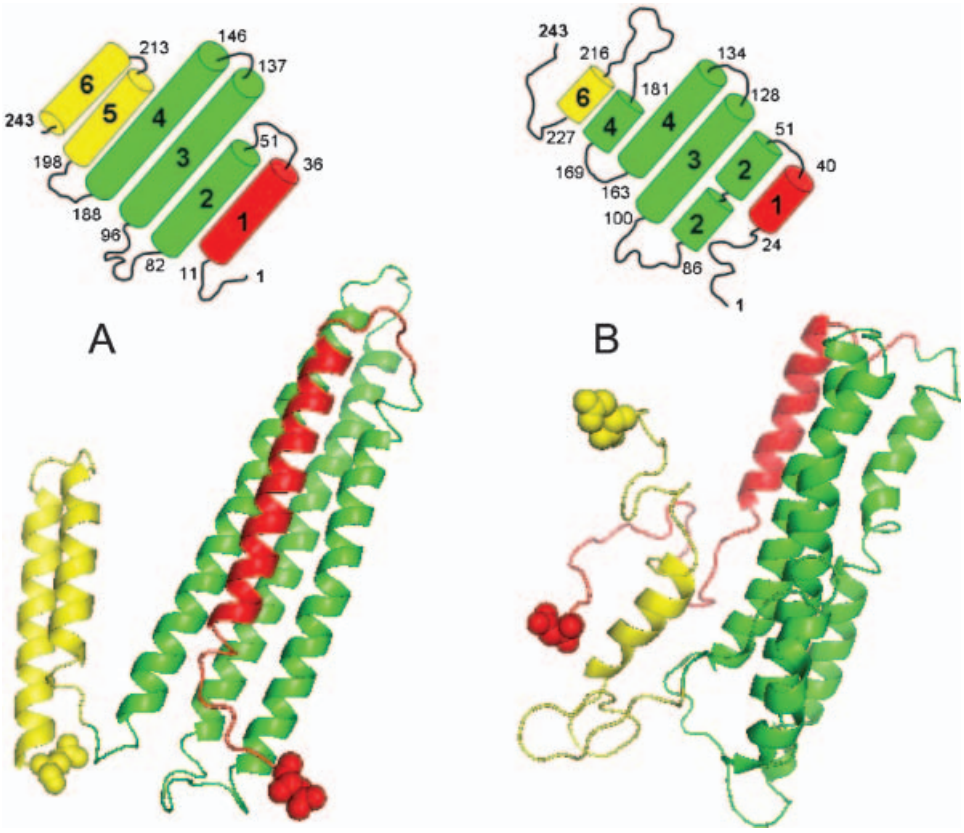

Figure 1. Models of full-length lipid-free apolipoprotein A-I. A: A ribbon diagram of crystal structure (11); and B: cross-linking/mass spectrometry/sequence treading (homology model) (18). In the ribbon diagrams, the N-terminus is shown in dark gray and the C-terminus is shown is light gray. The N-terminal four-helix bundle is clearly visible in both models. C-terminal domain is a separate entity with two dominant α-helices in the crystal structure, whereas in the homology model the C-terminal domain is dominated with unordered structure. The starting and ending residues of α-helices in each model are shown in top insets with helices labeled consecutively from N to C.

physiologic buffer indicating their relatively poor solvent exposure (22). A recent electron spin resonance (EPR) spectroscopic study based on site-directed spin labels on residues 14–98 of apoA-I has indicated that there are indeed short helices within this region (23). However, these helices are not continuous as found in the crystal structure, and only 43 of 85 residues studied are in the helical form (24). In addition, there are unstructured segments and a possible β-strand from residues 20–25 in this model. The authors have used the crystal structure as a template to build their model of solution-based apoA-I structure that encompasses residues 14–98. This model correlated well with the proteolytic sensitivity previously reported for this region. A prior study by the same group focused on the apoA-I C-terminal residues 160–240 and indicated another short β-strand in the C-terminal region (24). Even though all three models available for lipid-free apoA-I provide important information about its molecular fold, one must use them cautiously since each model inherits the strengths and weaknesses of the technique used. Furthermore the dynamic nature of apoA-I may preclude the existence of any one single structure; rather the information can be the result of different weightings from many interconverting structures based on the time response of the method. Taken together, data from independent techniques have indicated that monomeric apoA-I adopts a four-helical bundle structure in solution as was seen for other apolipoproteins.

Discoidal HDL

Discoidal HDL in human plasma are extremely short lived, but have been detected in extraplasma compartments like peripheral lymph and interstitial fluid (1). As stated above they can be reconstituted *in vitro* by combining relevant lipid components with isolated apoA-I (25). These discoidal rHDL particles possess most of the properties of native lipoprotein complexes such as enzyme activation, lipid transfer, and receptor binding (26–28). Homogeneous discoidal rHDL particles have been extremely useful in understanding the resultant effects of varying a single parameter in isolation,

such as disc diameter or lipid composition, as opposed to studying complicated heterogeneous native HDL.

The most well studied discoidal rHDL consist of two apoA-I molecules and are of ~96 Å diameter, ~47 Å thick, and consist of 150–200 phospholipid molecules (26,29). The number of phospholipid molecules changes per particle based on the type of the lipid used, i.e. different saturation and acyl chain lengths. It was reported that the lipid core of the ~96-Å diameter particle is ideal for assessing the curvature of apoA-I, assuming it forms a continuous curved amphipathic helix due to the presence of repetitive proline residues in the sequence. The reported diameter of the crystal structure of tetrameric Δ(1–43) is ~100 Å, indicating that even in the absence of lipid, apoA-I can adopt a similar curvature when the microenvironment mimics lipid-bound conditions (10).

An in-depth understanding of the molecular organization of apoA-I on these discoidal HDL complexes is extremely important since it opens the door to understanding the nature of HDL-incorporated apoA-I interaction with plasma enzymes and cell surface proteins. Two major models have been put forward for apoA-I molecular arrangement at the disc edge (2). 'Picket fence', modeled *in silico* by Phillips et al. suggests that the amphipathic helical segments of apoA-I traverse the lipid bilayer disc edge with the Pro residues creating reverse turns (Figure 2A) (30). The 'belt' model suggested by Segrest et al. indicates that the two apoA-I molecules form continuous amphipathic helices wrapping around the lipid bilayer disc edge forming two stacked rings stabilized by salt bridges (Figure 2B) (3,31–33). While the virtues of both models were hotly debated during the 1980s and 1990s, most recent evidence has lent support to the belt model. The first piece of supporting evidence came from attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) on rHDL particles in solution (34). This study clearly demonstrated that the apoA-I helical orientation is perpendicular to the acyl chains of the phospholipids in the rHDL disc, supporting the belt arrangement. The second was a Trp depth measurement study from our laboratory in which fluorescent probes were placed in each amphipathic helical segment of the protein to detect the depth with regard to acyl chains of phospholipids (35). The data clearly supported that the majority of the amphipathic helices are perpendicular to lipid acyl chains, again favoring the belt model. A fluorescence resonance energy transfer (FRET) study that used a Cys point mutant located in helix 5 indirectly supported the belt model due to strong energy transfer observed between the probes which would not be possible in picket fence arrangement (36).

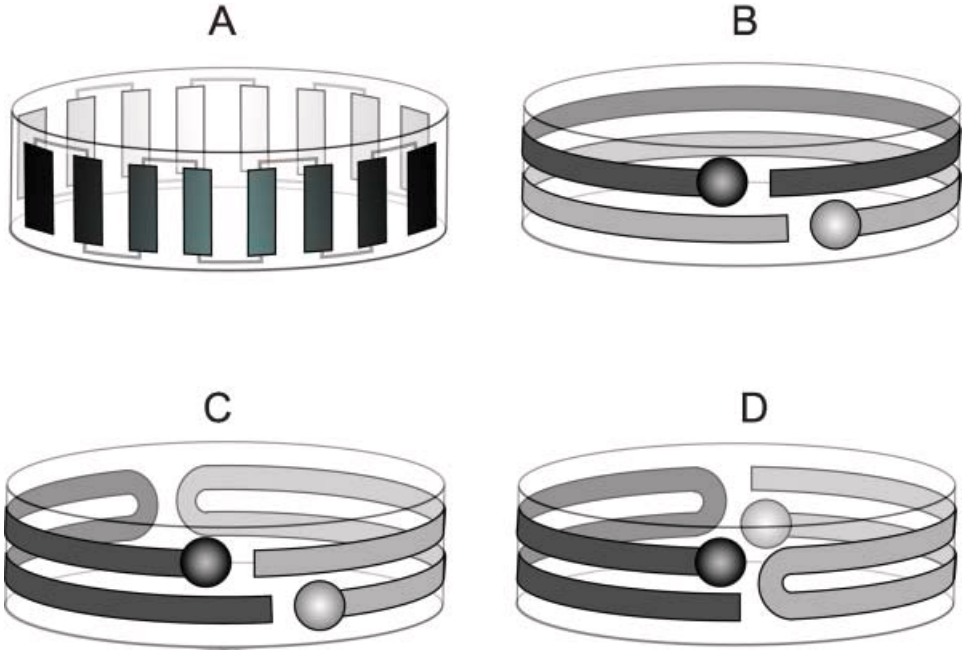

Figure 2. Competing models for discoidal high-density lipoprotein particles containing two molecules of apoA-I. A: Picket fence; B: double belt; C: head-to-head hairpin; and D: head-to-tail hairpin. Eight helical segments proposed to traverse the lipid bilayer in the picket fence model are presented as rectangles; N-termini are not shown for simplicity. In the belt variations, apoA-I molecules are shown as continuous belts or hairpins with the N-terminus presented as a small sphere.

Once the helical orientation question was addressed, attention focused on identifying different plausible variations of the belt arrangement (Figure 2). These include the so-called hairpin belts (Figure 2C and D) (2) that can either be in a head-to-head or head-to-tail orientation. In the hairpin conformation, the molecules could maintain the same salt bridges and sequence registry as in the double belt, except that they are intramolecular instead of intermolecular as in the double belt model. Tricerri et al. used site-specific Cys labels and measured FRET between specific sites in homogeneous discoidal rHDL of 96 Å (37). Their data supported the existence of a head-to-head and head-to-tail hairpin mixture, arguing against the double belt and picket fence models. We have applied chemical cross-linking combined with a high-resolution mass spectrometric approach to provide definitive distance constraints for apoA-I on rHDL (38,39). These studies generated nine intermolecular cross-links spread throughout the protein including the N- and C-terminal regions (Figure 3, top). Of these, six of nine cross-links clearly supported the antiparallel double belt molecular arrangement for apoA-I in 96-Å rHDL. In this arrangement, helix 5 of each molecule stacks next to each other (5/5 molecular registry) as suggested by Segrest et al. based on salt bridge optimizations (32) (Figure 3, top). A related study carried out by Bhat et al. on similar particles resulted in three intermolecular cross-links (40). The authors interpreted these data as generally agreeing with a double belt, except that the N-terminal region is folded back on itself forming a loop. A recent EPR spectroscopic study on 96-Å rHDL particles also indicated that the two apoA-I molecules are in an antiparallel double belt orientation with a 5/5 registry, except that the region 134–145 does not indicate spin coupling, indicative of a looped out region (41). Taken together there is solid experimental, as well as theoretical, evidence that apoA-I adopts an antiparallel double belt molecular arrangement with a 5/5 molecular registry.

The major molecular registry supported by the cross-linking data has helix 5 from each molecule in juxtaposition (referred to as the 5/5 registry, Figure 3 top). However, three of the nine cross-links found in this study supported a shifted molecular registry which would bring helix 5 and helix 2 opposite to each other (5/2 registry, Figure 3 bottom). Even though the 5/2 registry allows only 13 salt bridges in comparison with 18 in the 5/5 registry, we have seen this variation in every single sample preparation. A completely independent approach has confirmed the presence of both conformers (Davidson, unpublished data). A prior FRET study by Li and Sorci-Thomas on similar rHDL has also suggested that the apoA-I arrangement in rHDL may have a variable molecular registry rather than a fixed single registry (42).

Discoidal HDL Heterogeneity

Within the range of HDL particle diameters 70–120 Å, several discrete discoidal subclasses have been reconstituted. Li et al. have demonstrated that

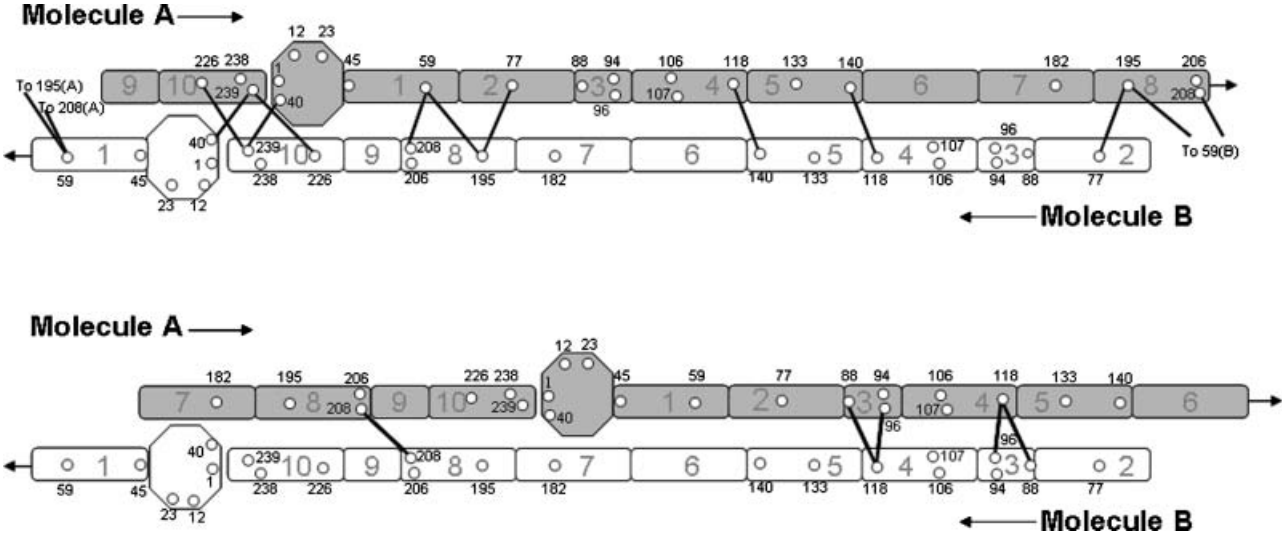

Figure 3. Two molecular registries of the double belt model supported by cross-linking/mass spectrometry experiments on reconstituted discoidal HDL particles of 96 Å that contain two molecules of apoA-I. Molecular registry 5/5 (top); and molecular registry 5/2 (bottom). The intermolecular cross-links found in the experiments are shown as black solid lines. Putative helices are shown as rectangles with the helix number. N-terminal 43 residues are shown as an octagon. The 21 lysine residues in each molecule are shown as small circles with the residue number indicated.

full-length apoA-I spontaneously interacts with 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) to generate five distinct sizes of discoidal HDL particles with sizes ranging from 98 Å to 120 Å (43). Interestingly, it was shown that the N-terminal 43 residues are required for the generation of the three larger-diameter particles, while these are not required for the smaller-diameter particles. This may suggest that one or more of the helices are not incorporated in the disc edge on the smaller particles. Further, a prior study indicated that populations of different diameter particles coexist in equilibrium, and this equilibrium state can shift to one size based on the fatty acid chains of the phospholipids (44). Another study indicated that there are definite differences in epitope binding between discoidal rHDL with two apoA-I molecules versus three apoA-I (45). Comparing two particles of 96 Å and 78 Å diameter with fluorescence spectroscopy, Mairano and Davidson have shown that there is a hinge domain consisting of the middle region encompassing helices 5, 6, and 7 in the smaller particles (46). Interestingly, this is the region reported to be important in interaction with cholesterol acyl transferase (LCAT) (47,48). Recent cross-linking/mass spectrometry data reported from our laboratory indicated that 78-Å and 96-Å particles had an overall similar cross-linking pattern with the exception of the central and N-terminal regions, consistent with the idea of a potential hinge region in the middle part of apoA-I in 78-Å particles (39). Supporting these observations, a recent EPR spectroscopic study has also suggested that there is a central loop in the 78-Å particles that is present but smaller in 96-Å particles (41). These studies indicate there may be at least two hinge domains in apoA-I on an rHDL particle: a mid region including helix 5 and the extreme N-terminal regions.

Spherical HDL

Despite the fact that the majority of HDL particles in human plasma are spherical, these particles are extremely understudied from a structural point of view. The few attempts that have been made to date were focused on apoA-I-only (LpA-I) spherical rHDL that may represent only a single population of native HDL. Even though apoA-I/A-II rHDL have been successfully reconstituted, studies have been focused primarily on their function. It is well documented that the generation of spherical HDL is a result of interaction of LCAT with one or more forms of nascent discoidal HDL in the presence of a cholesterol donor. Independent *in vitro* experiments have indicated that a single population of spherical rHDL (93 Å) with three apoA-I molecules per particle results from the LCAT reaction, irrespectively of the size of the discoidal particle used in the experiment (28,42). The definitive size and the fixed number of protein molecules strongly suggest that there is a protein framework holding spherical HDL complexes together just as in discoidal particles. In a sphere, there should be no exposure of phospholipid acyl chains as on the edge of a disc. However, it has been suggested that the amphipathic helices of apoA-I may penetrate the surface of the sphere past the phospholipid head groups to interact with the hydrophobic acyl chains (49). In a structural comparison of 96-Å disc vs. a 93-Å sphere, Mishra et al. observed that α-helicity (70%) as seen by CD spectroscopy, monoclonal antibody binding, and Trp exposure were generally comparable in both types of particles (28). However, the lipid order was somewhat higher in spherical particles, and the apoA-I in spherical rHDL was more resistant to guanidine hydrochloride denaturation. FRET analysis of apoA-I mutants fluorescently labeled in helical repeats 5 and 6 has indicated that the intermolecular distance of these labels increased when going from the disc to the sphere.

To our knowledge, there have not yet been any attempts to understand the fine structural details such as molecular structure and molecular registry of apoA-I molecules in spherical rHDL. The same cross-linking approach we have applied before on discoidal particles have indicated that the orientation and apoA-I molecular registry of apoA-I at least on 78-Å rHDL spheres may not be different from that of the discs (Silva and Davidson, unpublished data). Further, preliminary data from our laboratory on native human plasma HDL particles indicate that at least some particles may preserve the double belt molecular registry (Silva and Davidson, unpublished data).

Conclusions and challenges

It can be argued that one of the outstanding questions in vascular biology relates to how the structure of apoA-I modulates HDL metabolism and function. This highly dynamic molecule is capable of adopting an array of conformations along the RCT pathway. It is the relationship between this *structural* flexibility and the potential for *functional* flexibility that makes understanding apoA-I structure a critical problem. The crystal structures and theoretical models summarized above have provided a solid foundation for understanding apoA-I conformations. As helpful as these have been, a key component in recent advancements has been the

clever design of solution-based experiments to independently validate and/or modify these models to reflect physiological situations. As a result, we would argue that the field has a good understanding of at least the generalities of apoA-I organization in its lipid-free form and in simple discoidal particles. However, it is clear that more work needs to be done to understand the importance of the more dynamic sequences within these structural frameworks. As mentioned above, there is a critical need to derive a similar level of understanding for apoA-I in spherical particles. This may be relatively straightforward for homogeneous reconstituted spheres, but a central challenge will be to extend studies to the inherently heterogeneous particles obtained from human plasma. Does apoA-I adopt a double belt organization in actual human HDL particles? Reconstituted forms of HDL have been under intensive study for nearly three decades. Perhaps the time has come to apply that hard-earned knowledge to physiologically relevant particles that exist in the circulation. Given the inherent heterogeneity of human plasma HDL, a major hurdle to these types of experiments will be dealing with the presence of additional proteins including apoA-II. These may not only interact with apoA-I on the HDL surface, but they may also alter its conformation and thus affect the function of a given HDL subclass. These experiments will require a significant departure from traditional spectroscopic techniques that depend on a homogeneous population of particles. Meeting these challenges will be critical if we are to design, and understand the consequences of, therapies designed to manipulate HDL metabolism.

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