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Effects of Disease-Causing Mutations on the Conformation of Human Apolipoprotein A-I in Model Lipoproteins

Christopher J. Wilson[†], Madhurima Das[‡], Shobini Jayaraman[‡], Olga Gursky^{‡,⊥,*}, and John R. Engen^{†,*}

[†] Department of Chemistry and Chemical Biology, Northeastern University, 360 Huntington Avenue, Boston, MA 02115, USA

[‡] Department of Physiology & Biophysics, Boston University School of Medicine, 700 Albany Street, Boston, MA 02118, USA

[⊥] Amyloidosis Research Center, Boston University School of Medicine, Boston, MA 02118, USA.

Abstract

Plasma high-density lipoproteins (HDL) are protein-lipid nanoparticles that transport lipids and protect against atherosclerosis. Human apolipoprotein A-I (apoA-I) is the principal HDL protein whose mutations can cause either aberrant lipid metabolism or amyloid disease. Hydrogen-deuterium exchange (HDX) mass spectrometry (MS) was used to study the apoA-I conformation in model discoidal lipoproteins similar in size to large plasma HDL. We examined how point mutations associated with hereditary amyloidosis (F71Y, L170P) or atherosclerosis (L159R) influence local apoA-I conformation in model lipoproteins. Unlike other apoA-I forms, the large particles showed minimal conformational heterogeneity, suggesting a fully extended protein conformation. Mutation-induced structural perturbations in lipid-bound protein were attenuated as compared to free protein, and indicated close coupling between the two belt-forming apoA-I molecules. These perturbations propagated to distant lipoprotein sites, either increasing or decreasing their protection. This HDX MS study of large model HDL, compared with previous studies of smaller particles, ascertained that apoA-I's central region helps accommodate the protein conformation to lipoproteins of various sizes. This study also reveals that the effects of mutations on lipoprotein conformational dynamics are much smaller than those in lipid-free protein. Interestingly, the mutation-induced perturbations propagate to distant sites nearly 10 nm away and alter their protection in ways that cannot be predicted from the lipoprotein structure and

*Corresponding Authors: OG: gursky@bu.edu, JRE: i.engen@northeastern.edu.

Declarations of interest. The authors declare they have no actual or potential competing financial interests.

Author contributions. CJW performed the experiments, analyzed data, and prepared the manuscript; MD and SJ designed and performed the experiments and analyzed data; OG directed the study, analyzed data, and prepared the manuscript; JRE supervised the study, analyzed data, and prepared the manuscript.

Supporting Information. Biophysical data comparing discoidal complexes reconstituted with DMPC or POPC, isolation of apoA-I:DMPC complexes and biochemical analysis, tryptophan fluorescence of apoA-I:DMPC disks, peptic peptide coverage map, deuterium uptake plots for all studied peptic peptides, example mass spectra showing EX1 kinetics.

Note added in proof: While this paper was under review, a new molecular dynamics and cross-links study reported a revised tertiary structure of apoA-I on small and large model HDL (⁷²). Large particles contained two apoA-I, 200 POPC and 20 cholesterol molecules, similar but not identical to apoA-I:DMPC complexes used in our HDX studies. The revised structure does not significantly change the interpretation of our HDX data.

stability. We propose that long-range mutational effects are mediated by both protein and lipid and can influence lipoprotein functionality.

Keywords

High-density lipoprotein nanodiscs; Local flexibility and global stability; Protein – lipid interactions; Transmission of conformational changes; Hereditary amyloidosis and atherosclerosis

INTRODUCTION

High-density lipoproteins (HDL) remove cholesterol from peripheral cells via the reverse cholesterol transport pathway and provide other beneficial properties^{1, 2}. Efforts to harness these properties require a detailed understanding of the structure, dynamics, and function of HDL¹. This is a challenging task considering plasma HDL contain heterogeneous particles differing in shape (nascent “discoidal” or mature “spherical”), size (7.7-12 nm), protein and lipid composition, and functionality^{1, 3-5}. Each particle may contain several protein molecules and up to 300 lipid molecules. The major structural protein, apolipoprotein A-I (apoA-I, 243 amino acids), also acts as a functional ligand on the particle surface⁶⁻¹⁰. Nascent HDL generation begins upon interaction of free apoA-I with the plasma membrane mediated by lipid transporters such as ABCA1^{4, 9}. Each particle consists of a cholesterol-containing phospholipid bilayer with two apoA-I molecules wrapped around the circumference in an antiparallel α -helical “double-belt” conformation^{6, 11, 12}. Although the morphology of nascent HDL and related model particles is probably non-planar¹³, the particles appear discoidal in transmission electron microscopy¹⁴. Termed “nanodiscs”, various constructs of apoA-I have been used as models of nascent HDL^{4, 5, 11, 13} and as membrane mimetics for single-molecule studies of membrane proteins¹⁵.

ApoA-I on plasma HDL directs reverse cholesterol transport by interacting with lipid-processing factors, such as lecithin:cholesterol acyltransferase (LCAT)¹⁶. ApoA-I activates LCAT which converts nascent HDL into mature spherical particles containing a core of cholesterol esters and other neutral lipids^{4, 17}. During its life cycle HDL is continuously remodeled by LCAT and other plasma factors, which can lead to an increase in the particle size and a release of apoA-I as a conformationally labile free monomer. Free apoA-I can either rapidly associate with lipids, get degraded, or misfold and deposit as amyloid¹⁸. In contrast, lipid-bound protein is protected from degradation and misfolding by kinetic barriers¹⁹.

Conformational flexibility of apoA-I, which is required for its reversible binding to the lipid surface²⁰ and for adaptation to HDL of various shapes and sizes^{20, 21}, has complicated apoA-I structural studies. Although the atomic structure of a complete HDL particle is not available, critical insights have emerged through extensive biophysical studies, including X-ray and NMR structures of truncated apoA-I in solution and in model lipoproteins^{21, 22} (Fig. 1).

ApoA-I residues 44-243 contain ten Pro-punctuated 11/22-mer tandem sequence repeats, h1 through h10 (Fig. 1a), that can form amphipathic α -helices kinked at Pro or Gly²³. Large

apolar faces of these helices form lipid surface-binding sites, while the polar faces confer solubility. Current models of HDL stem from the ~ 4 Å resolution structure of the N-terminally truncated human lipid-free apoA-I, (1-43)apoA-I, which mimics key aspects of the lipid-bound conformation²². In this structure, two antiparallel, largely α -helical protein molecules form a circular “double-belt” closed at the C-terminal end via the paired h10 repeats, with the paired h5 repeats at the center of the dimer (Fig. 1c). While the h5/h5 repeat registry on various HDL particles has been firmly established in both cross-linking and spectroscopic studies, the conformations of the N- and C-terminal regions of apoA-I on HDL are less well defined and alternative models have been proposed for various particles (8, 11, 20, 24-26 and references therein). The importance of the h5/h5 repeat registry in apoA-I dimers is supported by the 2.2 Å resolution x-ray crystal structure of lipid-free C-terminally truncated protein, (185-243) apoA-I²¹ (Fig. 1b). This structure has revealed a semi-circular dimer with a diameter $d=11$ nm commensurate with the HDL size, and a 2-fold symmetry axis passing through the middle of the h5/h5 segment pair. This segment forms a dynamic central linker connecting two globular domains, each containing a four-helix bundle encompassing residues 1-184. These helices are referred to as H_I-H_{IV} when discussing the four-helix bundle. Upon interaction with lipids this bundle is thought to open up into two helical pairs, H_I-H_{II} and H_{III}-H_{IV}, which exposes the apolar surfaces for lipid binding and converts the helix bundle into a double belt. Domain swapping around repeat h5 (residues 121-142) mediates the dimer-to-monomer interconversion upon reversible binding of apoA-I to HDL²¹. Hence, the crystal structure of (185-243)apoA-I probably depicts an intermediate conformation between the full-length free apoA-I monomer and the HDL-bound dimer. This concept is further supported by the NMR structure of a small model HDL containing the centrally and N-terminally-truncated apoA-I (Fig. 1d), the only currently available atomic structure of HDL¹¹. This structure retains the h5/h5 and h4/h6 repeat registry seen in solution but involves “right to right” helical rotation to expose apolar surfaces to lipid.

Hydrogen/deuterium exchange (HDX) mass spectrometry (MS) studies of full-length apoA-I, either as a free monomer in solution or on various model HDL²⁷⁻²⁹, are consistent with these atomic structures. HDX, which is strongly influenced by main-chain hydrogen bonding and solvent accessibility, is particularly slow for well-ordered α -helices and fast for the disordered regions³⁰⁻³³. HDX MS was used to compare local dynamics in lipid-free and HDL-bound apoA-I to identify regions undergoing lipid-induced conformational changes²⁹. HDX MS studies of small (diameter 7.8 nm) and mid-size (9.6 nm) model discoidal HDL and mid-size spherical HDL (10 nm) have established that the local protein structure and dynamics were largely conserved on particles of different diameters^{28, 29}. However, certain apoA-I regions, such as residues 115-158, showed differences in dynamics reflecting the adaptation of the protein conformation to HDL of various shapes and sizes^{28, 29}. In the current work, we extended the application of HDX MS to determine the effects of naturally occurring apoA-I mutations on the protein dynamics in model discoidal HDL. The proteins were reconstituted with a model phospholipid dimyristoyl phosphatidylcholine (DMPC), and 11-12 nm particles which resemble the size of large plasma HDL were isolated for analysis. These discoidal particles are termed “model HDL” or “discs” for brevity. Such relatively large HDL have not been previously explored by HDX MS.

We focused on how mutations influence apoA-I conformation in the model HDL. The structural bases for altered functionality of apoA-I mutants have been explored by several groups, including ours. Of more than 50 known naturally occurring human apoA-I variants, most are associated with low plasma levels of apoA-I and HDL. One group of mutations, located in repeats h5-h7 (residues 121-187) proposed to interact with LCAT, impedes HDL maturation and is often linked to an increased risk of atherosclerosis³⁴. Another group of approximately 20 mutations, clustered in residues 1-100 and 170-178, causes hereditary apoA-I amyloidosis, a life-threatening disease wherein 9-11 kDa N-terminal fragments of apoA-I deposit as fibrils in various organs and damage them³⁵⁻³⁷. Previously, we used an array of biophysical techniques to probe the structure and stability of human wild type (WT) apoA-I and its representative disease-causing mutants in solution and on model HDL. HDX MS analysis of the proteins in a free monomeric state, the precursor of amyloid, enabled us to propose a molecular mechanism of apoA-I misfolding in amyloid^{38, 39}. Here, we use the same technique to explore the conformation of human WT and variant apoA-I on model HDL. The variants included F71Y, the most subtle amyloidogenic mutation, located inside the N-terminal fragments that deposit as amyloid *in vivo*⁴⁰; L170P, an amyloidogenic mutation located outside such fragments⁴¹; and L159R (Finnish variant), a non-amyloidogenic mutation that impairs HDL maturation and increases the risk of atherosclerosis⁴²⁻⁴⁴. The results revealed unexpected effects of mutations on the protein conformation and shed new light on the mechanism of protein adaptation to lipoproteins of different sizes.

MATERIALS AND METHODS

Proteins and lipids.

Recombinant full-length human WT apoA-I and its three point mutants, F71Y, L159R and L171P, were cloned, expressed, purified, and refolded as previously described²¹. Briefly, the proteins were expressed in *E. coli* using a His6-MBP-TEV tag and purified by FPLC to 95% + purity. One additional N-terminal Gly was present in all proteins. Lyophilized proteins were refolded from 6 M guanidine hydrochloride upon extensive dialysis against a standard buffer (10 mM Na phosphate, pH 7.4) that contained 0.25 mM Na EDTA. Protein concentrations were determined by absorbance at 280 nm and by a modified Lowry assay. Each protein was characterized by liquid chromatography and electrospray MS to verify the correct molecular weight and to assess the purity (data not shown). Immediately upon refolding, the proteins were aliquoted; some proteins were reconstituted into discoidal complexes with the lipid. DMPC was 99+% pure from Avanti Polar Lipids (Alabaster, AL, USA). All other chemicals were of highest purity analytical grade.

Lipoprotein reconstitution and characterization.

To expand the range of lipids beyond palmitoyl oleoyl phosphatidylcholine (POPC) used in most previous HDX studies of model HDL²⁷⁻²⁹, we chose DMPC that can self-assemble with apoA-I to form large 11-12 nm particles. Such a spontaneous self-assembly mimics key aspects of nascent HDL formation through the ABCA1-mediated interaction of apoA-I with the plasma membrane, thus providing a suitable model for nascent HDL⁴⁵. The choice of lipid is further justified by the close similarity in the secondary structure and stability of

apoA-I complexes with DMPC and POPC, as assessed by circular dichroism (CD) spectroscopy and differential scanning calorimetry (supplemental Fig. S1), and by HDX MS showing very similar apoA-I conformations on the mid-size complexes with POPC and DMPC²⁹. By choosing DMPC over POPC we avoid the use of cholate detergent. We also avoid potential complications from the gel-to-liquid crystalline lipid phase transition that affects the hydrophobic thickness of the disk and hence, the surface available for the protein binding⁴⁶. In fact, the transition temperature in DMPC is 24 °C, but in POPC it is 4 °C, the same temperature used in our HDX experiments described below.

ApoA-I complexes with DMPC were obtained by spontaneous reconstitution as previously described³⁸. Briefly, multilamellar lipid vesicles were prepared using thin-film evaporation and re-suspension in the standard buffer. The vesicles were incubated with the protein (1:4 mg/mg protein:lipid in the standard buffer) overnight at 24 °C. Excess lipid was removed by centrifugation. Lipoproteins were isolated by size-exclusion chromatography using a Superdex-75 (10/300 GL) column controlled by an ÄKTA FPLC system (GE Healthcare) (Figure S2); the peak fractions were collected and used for further studies. Native PAGE showed that all four proteins formed particles circa 11 nm in size (Figure S2). Negative stain transmission electron microscopy using a CM2 transmission electron microscope (Philips Electron Optics, Eindhoven, the Netherlands) as previously described³⁸ ascertained that all proteins formed similar-size discoidal particles 11-12 nm in diameter (Fig. 2a, Fig. S2), consistent with previous work³⁸. Glutaraldehyde cross-linking of the variant proteins on the discs performed as described in the Supplement showed an apoA-I dimer (Fig. S3). Phospholipid assay showed that the particles containing different proteins had similar PC content, with an 1:2.8 protein:lipid weight ratio, or 235±10 DMPC molecules per particle (Fig. S3). Similarly, previous detailed studies of apoA-I:DMPC complexes obtained by this method showed that each particle contained two molecules of apoA-I and 210 to 290 molecules of DMPC depending upon the estimation method^{47, 48}. Further, all proteins showed a similar blue shift in the wavelength of maximal Trp fluorescence, from 340 nm in lipid-free proteins to 330 nm on lipoproteins, indicating sequestration of all four apoA-I tryptophans in a hydrophobic lipoprotein environment (Fig. S4). Taken together, these results showed that different apoA-I variants formed lipoproteins with similar size and stoichiometry.

Isolated lipoproteins were used within hours for HDX MS studies. All experiments in the current study were repeated using at least two independent lipoprotein preparations.

A caveat in these studies is that size exclusion chromatography enables one to separate apoA-I dimer, either free or lipid-bound, from the free apoA-I monomer; however, if apoA-I forms a dimer in solution it cannot be fully separated from the discs. Therefore, our disc preparations could potentially contain a small amount of free dimer, which would affect the HDX data. However, two lines of evidence suggest that these effects were insignificant. First, disc samples containing L159R and L170P mutants, which have increased propensity to dimerize in solution, clearly showed increased protection in the C-terminal tail (Fig. 3, Disk – Free, see below), which is a hallmark of the lipid-bound conformation. Second, the kinetic HDX data clearly showed conformational homogeneity evident from the lack of EX1 in all disc preparations (see Figs. 5b and S6 below). Third, the kinetic studies of thermal

stability showed only a slow phase in protein unfolding, which is a hallmark of lipid-bound apoA-I (Fig. 2b below). These observations suggest strongly that essentially all protein was lipid-bound in our disc preparations. Furthermore, the lack of EX1 observed in large disks (Fig. 5b below) indicates that particle size heterogeneity observed for various disk preparations (Fig. S2) had no detectable effect on the protein conformational dynamics observed in our HDX studies.

Lipoprotein stability studies.

Far-UV CD data were recorded using an AVIV 62DS spectropolarimeter with a thermoelectric temperature control as previously described⁴⁹. Kinetic stability of lipoproteins was assessed by thermal denaturation in the melting³⁹ and kinetic temperature-jump experiments following previously described protocols⁴⁹. Briefly, in temperature jumps, lipoprotein denaturation was triggered by a rapid increase in temperature from 25 °C to a higher constant value, e. g. 75 °C, and the time course of α -helical unfolding was monitored by CD at 222 nm.

Hydrogen/Deuterium exchange mass spectrometry.

Deuterium exchange experiments were performed in duplicates as previously described^{39, 50} with minor variations. Two independent lipoprotein preparations (see Lipoprotein reconstitution and characterization) were each analyzed once by HDX MS. The low variability in deuterium incorporation between the two independent biological replicates ascertains the reproducibility of our studies and shows that the effects of apoA-I mutations and lipidation observed in these studies are meaningful⁵¹. Stock solutions of lipid-free and lipid-bound apoA-I were diluted to 0.5 mg/ml in equilibration buffer (10 mM potassium phosphate, 150 mM NaCl, pH 7.0, H₂O) at 4 °C. Exchange was initiated by an 18-fold dilution into D₂O buffer (10 mM potassium phosphate, 150 mM NaCl, pD 7.0) at 4 °C; this choice of low temperature was described in²⁷. The reaction proceeded for pre-determined periods of time, from 5 s to 4 hrs, whereupon labeling was quenched by dropping the pH to 2.5 using ice-cold concentrated formic acid (Sigma Aldrich) and immediately placing the sample on ice. All following steps were performed at 0 °C and all materials were pre-chilled on ice. Sodium cholate (100 mM) was added to quenched samples to solubilize the lipoproteins, releasing free apoA-I. Immobilized pepsin^{52, 53} was added to quenched samples for 5 min for digestion. Pepsin beads were removed by centrifugation (10,000×g at 4 °C) using Corning® Costar® Spin-X® centrifuge tube filters. Flow-through was immediately introduced into a Waters nanoACQUITY with HDX technology⁵⁴. Peptides were desalted for three minutes on an Acquity UPLC BEH C18 1.7 μ m trap. After desalting the flow was reversed for chromatographic separation on an ACQUITY UPLC® HSS T3 1.8 μ m, 1.0 × 50 mm column. Peptides were eluted from the column during a 6 minute, 5-35% water:acetonitrile 0.1% formic acid gradient. Electrospray mass spectra were acquired with a Waters Synapt G2Si with mobility on. This procedure was repeated for each protein at each time-point for each replicate. Peptic peptides were identified with a combination of exact mass measurements and HDMS^E by ProteinLynx Global Server (PLGS) 3.0 software (Waters). Deuterium level incorporation was determined with the aid of DynamX 3.0 software (Waters) along with manual verification of every spectrum to ensure accurate measurements. The error of measuring the deuterium incorporation in this instrumental

system in labeling replication ⁵¹ does not exceed ± 0.15 Da ³⁹; given we performed a biological replicate and not a labeling replicate ⁵¹, we considered a difference larger of ± 0.50 Da (Figure 3), as meaningful.

RESULTS

Biophysical analysis of lipoprotein structure and stability.

Discoidal lipoproteins 11-12 nm in size, which contained WT or variant apoA-I and DMPC, were prepared and analyzed as described in Section 2.2 and Figures S2 – S4. Far-UV CD spectra of lipoproteins containing WT, F71Y and L170P closely overlapped, suggesting $70 \pm 5\%$ α -helical content, while for L159R this value decreased to $60 \pm 5\%$ (Figure 2a). Kinetic stability of lipoproteins was assessed by measuring the protein unfolding rates in temperature-jump experiments using far-UV CD at 222 nm as described in Section 2.3. Representative temperature-jump data in Figure 2b showed slow α -helical unfolding on a timescale of hours, which is a hallmark of lipid-bound apolipoproteins ¹⁹, and demonstrated large mutational effects on the unfolding rate, with the WT showing the slowest and L159R the fastest unfolding. The rank order of the kinetic disc stability emerging from these data, which is inverse of the unfolding rate, is WT > F71Y >> L170P > L159R. This rank order agrees with that previously determined in the CD melting studies of similar discs, WT > F71Y >> L170P > L159R ³⁹.

Previously we showed that destabilization of model and plasma HDL involves protein release and lipoprotein fusion ¹⁹. To probe for structural integrity of apoA-I:DMPC complexes at ambient temperatures, the complexes were incubated at 37 °C for 48 h, followed by non-denaturing PAGE. While WT, F71Y and L170P remained bound to lipid under these conditions, a fraction of L159R was released as free protein (Fig. 2c) indicating decreased disk stability. Furthermore, limited tryptic digestion at 37 °C clearly showed that complexes containing WT and F71Y were much better protected from proteolysis as compared to those containing L170P and L159R (Fig. 2d). All together, these results clearly showed that the disk stability decreases in order WT > F71Y > L170P > L159R.

Transfer from solution to the disc affects the local conformation in WT apoA-I.

HDX MS was performed on the lipid-free and lipid-bound protein state on large discs, by generating peptide fragments providing 100% sequence coverage of the protein (Figure S5). The results showed that transfer of WT apoA-I from the lipid-free monomeric state in solution to the disc surface increased the structural protection in some protein regions, yet reduced the protection in other regions (Figure 3, Disc – Free WT; Figure S6). Increased protection was observed at all exchange times in three protein segments. The first segment included the C-terminal tail (residues 186-243) that is largely disordered in free apoA-I but becomes largely helical upon lipid binding ⁵⁵. The second segment included residues 114-147 encompassing the central repeat h5 (residues 121-142), which forms a flexible hinge in apoA-I ²¹. The third segment included residues 44-55 and adjacent groups. These residues acquired an extended β -strand-like conformation in the high-resolution crystal structure of free (185-243)apoA-I but were modeled as a dynamic helix in the low-resolution crystal structure of (1-43)apoA-I ²² or as α -helical, β -strand or random coil

conformation in the electron paramagnetic resonance studies of spin-labeled apoA-I that was either free or bound to the 9.6 nm particles (⁵⁶ and references therein). In summary, protein segments that showed increased protection on the disc surface appeared to lack a stable structure in solution but acquired such a structure upon lipid binding.

Decreased protection in WT apoA-I upon transfer from solution to the disc surface was observed at long exchange times in peptides from three well-ordered regions: 1-38, 72-103, and 160-180 (Fig. 3, Disc – Free WT, 30-240 min). These segments formed the middle and bottom parts of helices H_I, H_{III} and H_{IV} in the 4-helix bundle of (185-243)ApoA-I, which are particularly well-ordered in the crystal structure. HDX MS data clearly showed that this ordering decreases upon helix bundle opening and transfer from solution to the lipid.

Comparison with previous studies of smaller WT-containing discs.

The trend emerging from our studies of WT apoA-I is that lipid binding increases the structural protection in flexible regions but decreases it in well-ordered regions. Overall, these results agree with the previous HDX MS studies of the small (7.8 nm) and mid-size (9.6 nm) discs containing WT apoA-I and either POPC or DMPC ²⁹. Both current and previous HDX MS studies consistently showed lipid-induced increase in protection of the central and C-terminal segments, which in free apoA-I are dynamic and highly labile to proteolysis. This result is consistent with the limited proteolysis studies of apoA-I showing increased protection in the central and C-terminal regions upon lipid binding ^{57, 58} (Figure 2d). Increased structural protection was also observed in segment 45-56 on the large discs (Figure 3, Disc – Free, WT) and on the mid-size discs ²⁹, while on small discs and in free protein this segment showed low protection ²⁹. These results suggest that the flexible segment 45-56 helps accommodate protein structure to various amounts of lipid in lipoproteins.

Segment 159-180, located in helix H_{IV}, is well-ordered in the helix bundle and showed the greatest decrease in protection upon protein transfer from solution to the 12 nm discs (Figure 3, Disc – Free WT; Figure 4 uptake curve 159-180). Residue segment 72-92 from helix H_{III} showed a concomitant decrease in protection (Figure 3, Disc – Free WT), which is consistent with pairing of H_{III} with H_{IV} in solution and on the lipid. A large decrease in protection of segment 159-180 observed in 12 nm DMPC discs was similar to that reported for the 9.6 nm discs containing POPC, but differed from the 7.8 nm discs where high protection in the same segment was observed resembling that in free apoA-I ²⁹. Hence, the current and previous HDX MS studies of WT apoA-I in solution and on the discs showed overall similarity but revealed distinct differences in local protein protection that depended on the lipidation degree and the disc diameter.

EX1/EX2 exchange kinetics in large WT and mutant apoA-I discs.

A significant difference between current and previous HDX studies of WT apoA-I was in the EX1/EX2 exchange kinetics. EX2 regime occurs if structural fluctuations in the protein are much faster than the exchange rate, so multiple fluctuations are required to complete the exchange; in EX1 the structural fluctuations are much slower than the H-D exchange and hence, a single visit to the unfolded state is sufficient to complete the exchange ⁵⁹. EX2 is

characterized by a unimodal distribution in the peptide isotopic envelopes throughout the HDX time scale, whereas EX1 has a characteristic bimodal pattern in the mass spectra (illustrated in Figure S6). Every spectrum for every time-point for every peptide in each state was manually inspected for signatures of EX1 kinetics. In order to visualize and understand the effects the mutants have on this phenomenon, the approximate half-lives ($t_{1/2}$)³⁹ were placed into one of seven groups, from being <0.5 s to >>240m/EX2 kinetics. The $t_{1/2}$ represents the half-life of the formation of a partially unfolded and deuterated species, a point at which in the spectra, the relative intensity of the folded, lower-mass species and the unfolded, higher-mass deuterated species are nearly equal. Unlike typical globular proteins that show mainly EX2, free apoA-I exhibits an EX1 signature, most prominently in the C-terminal tail (Figure 5)^{27, 39}. The HDX profile is consistent with a highly dynamic conformation of its molten globular structure⁶⁰. Unlike free protein, previous disc studies reported EX2 throughout most of the apoA-I molecule, except for the residue segment 44-55 and the central region that showed EX1 in residues 115-158 on the small discs and residues 125-158 on the mid-size discs²⁹. Notably, our studies of the large discs showed no EX1 in this region (Figures 5b, S6), and very little EX1 in other regions of either WT or mutant apoA-I. Consequently, apoA-I is more conformationally homogeneous on large discs studied here than in any other states previously explored.

Effects of lipid binding on the structural protection in mutant apoA-I.

Previously we showed that F71Y minimally perturbs the conformation of free apoA-I. This mutation slightly destabilized free protein causing a 3 °C decrease in the melting temperature, T_m ³⁹. The mutation also caused a small decrease in protection of several helix bundle regions, most notably in residues 0-28 encompassing segment 14-22 that has the highest amyloid-forming sequence propensity in apoA-I (Fig. 3, Lipid-free F71Y – WT). Decreased structural protection in this adhesive segment was proposed to trigger the misfolding of this and other free amyloidogenic variants^{38, 39}.

The HDX data demonstrate that, similarly to the WT, lipid binding by F71Y mutant increased the structural protection in peptides found within the central and C-terminal regions, 114-147 and 186-243, and decreased the protection in peptides within the 72-104 and 160-180 regions (Fig. 3, Disc – Free F71Y). Unlike WT, F71Y showed only increased (rather than decreased) protection at all exchange times in the N-terminal segment 0-28 upon lipid binding (Fig. 4). This difference reflected less well-ordered solution conformation of the N-terminal end in free F71Y, whereas lipid-bound F71Y and WT showed very similar N-terminal protection (Fig. 3, Disc – Disc, F71Y-WT).

Of all disease-causing apoA-I mutations explored to-date, L159R and L170P caused the greatest structural perturbations in free apoA-I (Fig. 3)³⁹. Previous biophysical studies showed that these mutations decreased the protection in the 4-helix bundle segments and increased the protection in the C-terminal tail³⁹. Similarly, HDX MS data of the current study showed that in free L159R and L170P, most helix bundle segments had vastly decreased protection while the C-terminal tail had greatly increased protection as compared to free WT (Fig. 4). Previously, we proposed that L159R and L170P substitutions widened the hydrophobic cleft between the helix pairs H_I - H_{II} and H_{III} - H_{IV} in free proteins (Fig. 1B),

and thereby helped sequester the hydrophobic C-terminal tail. Another explanation is that free L159R and L170P have an increased propensity to form dimers (SEC data not shown).

Unlike WT and F71Y apoA-I, lipid binding by L159R and L170P mutants led to increased protection throughout the entire molecule (Fig. 3, Disc – Free). Surprisingly, there was an even greater increase in protection seen in the central and C-terminal regions of L159R and L170P variants. This observation is consistent with the replacement of the solvent-accessible cleft between helix pairs H_I - H_{II} and H_{III} - H_{IV} in free L159 and L170P proteins with protective protein-lipid interactions in lipoproteins.

Effects of mutations on the protein protection in model HDL.

HDX MS data of model HDL revealed mutation-specific effects on the protein protection. Overall, these effects were consistent with the structural stability studies of lipoproteins by CD, native PAGE and limited proteolysis clearly showing that the four proteins explored fall into two groups: WT and F71Y on the disks are more stable and less susceptible to proteolysis compared to relatively labile L170P and L159R (Fig. 2). Additionally, HDX revealed subtle effects of mutations, which could not be detected by other methods.

F71Y mutation caused only slight changes, including a decreased protection in two regions: residues 72-92, which are adjacent to the site of mutation, and residues 165-192, which are juxtaposed to the 72-92 segment in the double belt (Fig. 3, Fig. 6a). Hence, the mutation-induced local perturbation in one molecule of the double belt affects the juxtaposed segment in the second molecule. In addition, either a slight increase or a small decrease in protection was detected in the N-terminal and central regions (Figs. 3, 4). Overall, these results are consistent with a small destabilization of the discs upon F71Y mutation observed by thermal denaturation: F71Y on lipoproteins showed a faster unfolding in kinetic experiments (Fig. 2b) and a low-temperature shift in the melting data, with a decrease in the apparent T_m by $\delta T_{m,app} = -5^\circ\text{C}$ ³⁹.

Compared with F71Y, L159R caused much greater disc destabilization evident from much faster unfolding (Fig. 2b) and a larger low-temperature shift in the melting data with $\delta T_{m,app} = -16^\circ\text{C}$ ³⁹. This trend continued in the HDX analysis with greater changes in protection upon L159R mutation (Fig. 3, Disc – Disc L159R). Decreased protection was observed in peptides spanning residues 115-158, adjacent to the L159R mutation site. Residues 115 and 159 from the two dimer molecules are also approximately in register, supporting the idea that the mutation-induced perturbation in one molecule of the double belt affects the juxtaposed site in the other molecule. Importantly, residues 115-159 encompass the apoA-I region implicated in binding and activation of LCAT, which facilitates HDL maturation. Hence, a large loss in protection of this region seen in L159R mutant discs is consistent with the impaired HDL maturation observed in vivo in L159R mutation carriers ^{42, 43}. Unexpectedly, the N- and C-terminal segments showed increased protection in L159R (Fig. 3, Disc – Disc L159R-WT; Fig. 6b). Most of these mutation-induced differences were observed at relatively long exchange times (30-240 min), consistent with well-ordered helical conformation in both the WT and the mutant proteins on the discs. Interestingly, the results show that the structural perturbation propagated from

L159R to the diametrically opposed region of the double belt nearly 10 nm away from the mutation site (Fig. 6b).

Like L159R, L170P also showed decreased protection in the central region. However, in L170P the region of decreased protection extended further, from residues 92 to 190, encompassing the site of mutation, its juxtaposed region in the double belt, all groups between them, and the adjacent segments (Fig. 6b). Again, the HDX MS data showed that mutation-induced perturbation in one double-belt molecule affected the juxtaposed segment from the second molecule (Fig. 6). Like L159R, L170P also showed increased protection in the N- and C-terminal regions illustrating propagation of the mutational effects to distant sites (Fig. 3, Fig. 6c). Notably, variations in the lipoprotein size can potentially influence the protein protection on the discs. Despite such variations (Fig. S2), L159R and L170P mutations had similar effects on the protein protection in discs, suggesting that these changes in protection stem mainly from the mutation. These surprising effects, which could not be predicted from other structural and stability studies, illustrate the complex relationship between the global stability and local flexibility in lipoproteins.

DISCUSSION

We used HDX MS to investigate how representative disease-causing mutations influence local conformation and dynamics in full-length human apoA-I on model discoidal lipoproteins that mimic nascent HDL. The results revealed that mutation-induced protein perturbations propagate to distant sites on lipoproteins, and that such perturbations are distinctly different in lipid-bound and in free proteins. Further, comparison of the relatively large 11-12 nm model HDL particles explored here with the previously studied smaller discoidal and spherical particles of 7.8 to 10 nm^{28, 29} provided better mechanistic insights into the structural adaptation of apoA-I to particles of varied sizes. This adaptation is essential for the cardioprotective function of apoA-I in reverse cholesterol transport during which HDL incrementally increases in size upon increasing lipid cargo^(20 and references therein), as well as for an incremental uptake of cholesterol esters from HDL by its receptor leading to an incremental decrease in the particle size⁶¹.

First, the current study revealed that the mutations had much smaller effects on the conformation of the lipid-bound as compared to free protein (Fig. 3 Disc – Free, Disc – Disc). This finding was surprising since the destabilizing effects of mutations on discs were comparable or greater than those on free proteins. For example, L159R mutation decreased the T_m by 11 °C in free apoA-I and by 16 °C in the discs^{38, 39}. Even so, the overall changes in the protein protection induced by this mutation were much greater in free apoA-I than in the discs (Fig. 3). To explain this paradoxical observation, we note that mutations which place a polar (F71Y) or a charged (L159R) group in the apolar lipid-binding face of an amphipathic α -helix and/or kink such a helix (L170P), are expected to perturb apolipoprotein-lipid interactions. We propose that such perturbed interactions decrease the overall particle stability (Fig. 2b); however, by coating the mutation sites, lipids dissipate protein perturbations. This explains why observed perturbations were muted in lipid-bound compared to free proteins (Fig. 3).

Such perturbed protein-lipid interactions may contribute to structural and stability changes in the variant lipoproteins (Figs. 2, 3, 6) as well as to their altered functionality. For example, a large loss in protein protection on the discs observed near the L159R mutation site, i.e. in the h5-h6 region implicated in LCAT binding, helps explain the impaired HDL maturation observed in carriers of this pro-atherogenic mutation [38, 39]. Further, decreased stability of lipoproteins containing L159R variant, evident from its faster protein unfolding and release as compared to any other protein explored (Fig. 2), provide a nuanced view why this most destabilizing mutation is not amyloidogenic. Our results suggest that the L159R mutation decreases protein stability on the lipid, causing protein release and proteolysis (Fig. 2c, 2d), while also destabilizing free protein and promoting its cleavage into unstable fragments that are rapidly cleared from circulation, preventing protein accumulation³⁵. In contrast, amyloidogenic mutations such as L170P and F71Y are less destabilizing on the lipid (Fig. 2) and in solution³⁹ and hence, are more likely to accumulate and ultimately deposit as amyloid. Additionally, our finding that amyloidogenic mutations such as F71Y and L170P cause a much greater structural perturbation in free as compared to HDL-bound protein is consistent the idea that free apoA-I is a direct protein precursor of amyloid.

Second, our results showed that, for all point mutations explored, the mutation-induced perturbation in one apoA-I molecule of the double belt propagated to the juxtaposed helical segment from the other molecule (Fig. 6). This observation points to tight coupling between the two juxtaposed protein molecules. The interactions leading to such coupling could involve networks of interhelical salt bridges and cation- π interactions observed by NMR and inferred from molecular modeling^{11, 62}; these interactions are amplified by the low dielectric at the lipid-water interface. We posit that other mutations may have a similar effect on the juxtaposed molecules in the apoA-I dimer, potentially influencing functional properties of the mutant-containing lipoproteins. Notably, HDL containing both WT and mutant apoA-I are present in vivo since most apoA-I mutation carriers are heterozygous; in such mixed HDL, the WT molecule is expected to be perturbed by a mutation in the adjacent molecule from the same dimer, which may contribute to the decreased plasma levels of both mutant and WT apoA-I found in mutation carriers^{34-37, 40}.

Interestingly, although all mutations studied here decreased lipoprotein stability (Fig. 2b and ³⁹), they decreased the structural protection (i.e. decreased local ordering) in some protein regions on the discs while increasing it in other regions (Figs. 3, 4 and 6). For example, L159R and L170P mutations decreased protein ordering in the central part while increasing it in the C-terminal tail (Fig. 3, Fig. 6b). This observation suggests that the belt-closing contacts between the N- and the C-terminal ends of the apoA-I molecule, which are thought to be the weakest link in the double belt, do not necessarily determine the lipoprotein stability, suggesting that the central region is important.

Another unexpected finding was that perturbations induced by point mutations propagated to distant sites nearly 10 nm away (Fig. 6b). The protein protection at these sites was altered in a manner that could not be predicted from the lipoprotein structure or stability. For example, L159R and L170P mutations increased the protection in the diametrically opposed segments of the double belt (Figs. 3, 6b). Such a long-range propagation of mutation-induced perturbations may possibly involve helical rotation and unwinding on the lipid^{11, 62, 63} as

well as altered kinking of the double belt^{13, 22}. Similarly, altered protein bending or kinking was implicated in the propagation of conformational changes to distant sites in other macromolecules, such as antibodies^{64, 65}.

Overall, our results show that the proteins fall into two distinct groups, WT and F71Y versus L159R and L170P, based on the particle stability and structural protection which do not correlate with the particle size distribution (Fig. S3). Therefore, although we cannot exclude that small variations in the particle size formed by different proteins affect their conformation on lipoproteins, these effects are not translated into major differences in the particle stability and protein protection observed by CD and HDX.

The current and previous HDX MS studies of lipid-associated apoA-I improve our mechanistic understanding of the protein adaptation to HDL of various size and HDL interactions with LCAT. The central sequence repeats h5-h6 of apoA-I, including segment 115-158, have been implicated in both these processes^{17, 29}. In the large WT discs studied here, all peptides from h5-h6 (residues 121-164) showed EX2 kinetics with a unimodal distribution. In contrast, previous HDX MS studies reported bimodal EX1 distribution in residues 115-158 on the 7.8 nm discoidal and on 10 nm spherical particles, while a shorter segment, 125-158, showed EX1 in the 9.6 nm discs^{28, 29}. The authors proposed that the bimodal distribution represented co-existing helical and disordered conformations, with the disordered loop protruding from the lipid; this loop decreased in length with increasing surface available to the protein, e.g. with increasing the particle size. This idea is reminiscent of the “hinge domain” or a “looped-belt” conformation inferred in the previous spectroscopic studies of model HDL^(29, 66, 67 and references therein). Such a “looped belt” was proposed to help adapt the apoA-I conformation to HDL of various size. Our results are consistent with this idea and show that, in contrast with less lipidated forms of apoA-I, h5-h6 loop region acquires a single well-ordered conformation on the large discs, suggesting that this whole region is now in contact with the lipid and is probably helical. We posit that such a unimodal well-ordered conformation observed in this and nearly all other regions of the apoA-I molecule on the large discs (Fig. 5) determines the limiting size of the apoA-I double belt and, hence, the limiting size of the HDL particle. We posit that this ordered conformation may provide a recognition motif for the HDL receptor that preferentially interacts with large lipid-loaded particles⁶⁸. This preferential interaction and the enhanced ability of large HDL to mediate receptor-mediated cellular uptake of cholesterol ester may contribute to the enhanced cardioprotective properties of large versus small HDL^{68, 69}. ApoA-I conformation in the h5-h6 region is also expected to directly affect the activation of LCAT whose preferred substrates are relatively small discoidal HDL^(70 and references therein).

This study shows once again that HDX MS can detect local conformational changes induced by protein point mutations in large non-covalent macromolecular assemblies containing hundreds of molecules, such as lipoproteins. The results suggest that protein mutations influence the conformation of both protein and lipid, leading to long-range effects that propagate throughout the lipoprotein particle and influence its structure, stability and, potentially, functionality. Our findings exemplify the complex relationship between the macromolecular stability and local flexibility that is difficult to predict or detect

experimentally by other methods. Such a complex relationship has been previously reported for proteins and protein complexes^{65, 71}, and the current study extends it to lipoproteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

apoA-I	human apolipoprotein A-I
HDL	high-density lipoprotein
DMPC	1,2-dimyristoyl- <i>sn</i> -glycero-3-phosphocholine
POPC	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphocholine
HDX	hydrogen-deuterium exchange
MS	mass spectrometry
CD	circular dichroism
WT	wild type
LCAT	lecithin:cholesterol acyltransferase

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Figure 1.

Available atomic structures of apoA-I. **(a.)** Linear representations of apoA-I constructs. The top cartoon shows full-length apoA-I, as analyzed in this paper. Black lines delineate helical sequence repeats: G*(1-43), h1(44-65), h2(66-87), h3(88-98), h4(99-120), h5(121-142), h6(143-164), h7(165-187), h8(188-208), h9(209-219), and h10(220-241), with the starting of each helix at the base of each line. Green bars within the cartoon indicate the location of the four amyloid hot spots in residues 14-22, 53-58, 69-72, and 227-232. The second cartoon depicts the C-terminally truncated construct, (185-243), whose high-resolution x-ray

crystal structure in lipid-free state was determined ²¹. The third cartoon depicts the N-terminally truncated construct, (1-43), whose low-resolution x-ray crystal structure was determined ²². The bottom cartoon depicts the construct with N-terminal and central truncations, (1-54), (121-142), whose structure on model discoidal HDL was determined by NMR ¹¹. **(b.)** The 2.2 Å resolution crystal structure of (185-243) shows a dimer with two four-helix bundles related via a 2-fold crystallographic axis passing through the middle of h5/h5 repeat pair. This structure probably represents an intermediate between the lipid-free monomer and the lipid-bound dimer. Two dimer-forming molecules in this and other structures are in different shades of gray. **(c.)** The ~4 Å resolution crystal structure of (1-43) shows an extended dimer in an antiparallel double-belt conformation that represents the lipid-bound state. The h5/h5 repeat pair is indicated. **(d.)** The NMR structure of (1-54), (121-142) on a small discoidal HDL. The helical sequence repeats for the front most apoA-I particle (light gray) for each of the structures are shown. The locations of the three point mutations studied in this work are color-coded. In brown is the residue segment 83-93 containing cleavage sites that generate N-terminal apoA-I fragments found in amyloid deposits in vivo.

Figure 2.

Effects of mutations on the structural and stability properties of apoA-I:DMPC discs. Protein variants are color-coded. Discoidal complexes were prepared and purified by SEC as described in Methods. **(a.)** Far-UV CD spectra at 25 °C. Based on the CD values at 222 nm, the helical content in lipid-bound apoA-I was $70 \pm 5\%$ for WT, F71Y, and L170P, and $60 \pm 5\%$ for L159R. **(b.)** Thermal stability assessed using kinetic data recorded in temperature jumps from 25 to 75 °C by CD at 222 nm for α -helical unfolding as described in Section 2.3. **(c.)** Temporal stability at an ambient temperature. The disks were incubated at 37 °C for 48 hours, and the particle integrity was assessed by non-denaturing PAGE (4-20% gradient, Denville Blue stain). **(d.)** Limited tryptic digestion of apoA-I on the disks. The disks were incubated at 37 °C for 30 min with trypsin at 1:1,000 mg:mg enzyme:apoA-I ratio in standard buffer. Tryptic digestion was quenched using 2 mM of phenylmethylsulfonyl fluoride. The reaction products were analyzed by SDS PAGE (4-20% gradient, Denville Blue protein stain). Intact apoA-I is shown for comparison.

Figure 3.

Comparisons of relative deuterium exchange in various states of apoA-I. The first three panels (left to right) show the differences in deuterium uptake between lipid-free variant and WT apoA-I, as previously reported³⁹. The middle four panels show differences in protein deuteration on 12 nm discoidal particles versus lipid-free. The last three panels show the differences in deuteration between the mutants and the WT protein on 12 nm discoidal particles. All differences are shown in Daltons and color-coded according to the scale at the bottom. The deuterium incorporation graphs used to create this figure are shown in

Supplemental Figure S6. Down the left side are the residue numbers of each peptide fragment, arranged from N- to C-terminus (top to bottom). Black horizontal lines delineate regions of the protein and are located approximately at every 25 residues. Within each panel the differences in uptake at various times, from 5 s to 240 m, are shown and indicated in the bottom of the first (left) panel; the same order is use for all panels. The error of measuring the deuterium incorporation in this HDX MS instrumental system in deuterium labeling replication ⁵¹ is under ± 0.15 Da ³⁹. Given we performed an HDX MS biological replicate ⁵¹, a difference of ± 0.50 Da was considered meaningful (Figure 3). Most differences were well above 0.50 Da.

Figure 4.

Representative deuterium incorporation curves including error bars. The graphs in the left column contain data for the lipid-free states of each protein while the graphs on the right contain the lipid-bound data. The sequence numbering begins at 0 due to an extra glycine found at the N-terminus due to a purification tag. The first four graphs show the uptake in the N-terminal region. In particular, the second plot for peptide 0-28 includes the first major amyloid hotspot, 14-22. The fifth row of uptake plots show a peptide located in the β -strand like region. The next peptide, 83-91, shows significant differences between L159R and

L170P from WT and F71Y. Slight differences are seen between L170P and WT discs. The peptide 92-104 covers the site of cleavage in amyloidogenic variants. Peptides 125-139 and 125-147 cover the flexible h5-h6 “looped belt” region, where EX1 kinetics have been seen in previous studies of smaller discs. In peptide 159-180 the discs are less protected than the lipid-free protein.

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Figure 5.

Location and approximate half-life, $t_{1/2}$, of observed EX1 kinetics in peptides from (a.) lipid-free and (b.) lipid-bound WT and variant apoA-I. All peptides are shown and labeled on the x-axis with vertical lines at approximately every 50th residue for orientation. The timescale for $t_{1/2}$ is represented in shades of green; peptides with unmeasurable EX1 on this time scale ($t_{1/2} \gg 240$ min) or with EX2 are in white. All spectra for all peptides were manually inspected for EX1 signatures and binned into one of seven groups based on their approximate half-life ($t_{1/2}$) as described in Section 3.4; representative spectra are shown in Figure S7. All mutants showed EX1 kinetics in the C-terminal tail, in agreement with previous studies³⁹.

Cartoons illustrating the effects on protein protection from (a.) the putative helical sequence repeats, (b.) lipid binding and (c.) point mutations in large model lipoproteins. Panel a is derived from Fig. 1a, panel b is derived from Fig. 3 (Disc – Free difference), and panel c from Fig. 3 (Disc – Disc difference). Two apoA-I molecules in the double belt are shown by circular lines, with arrows pointing towards the C-terminus. Color coding indicates differences in protection between the variant and WT protein on the disc, according to the legend at the lower left. The vertical arrow passing through the middle of the residue segment 121-141 (sequence repeat h5) shows the two-fold axis relating the two antiparallel protein molecules. Positions of selected Pro and Gly at the helical kinks in the double belt model are indicated (purple, 4-point stars). The double belt is shown in a fully extended helical conformation proposed for large ~12 nm particles²⁰. The molecular registry in the central part of the double belt near the h5 pair is well defined experimentally (upper half of the cartoons), while the relative positions of the N- and C-terminal ends of the two molecules are less well defined.