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Charged residues in the C-terminal domain of apoA-I modulate oligomerization

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Abstract

Charged residues of the C-terminal domain of human apolipoprotein A-I (apoA-I) were targeted by site-directed mutagenesis. A series of mutant proteins were engineered in which lysine residues (Lys 195, 206, 208, 226, 238, and 239) or glutamate residues (Glu 234 and 235) were replaced by glutamine. The amino acid substitutions did not result in changes in secondary structure content or protein stability. Cross-linking and size-exclusion chromatography showed that the mutations resulted in reduced self-association, generating a predominantly monomeric apoA-I when five or six lysine residues were substituted. The rate of phosphatidylcholine vesicle solubilization was enhanced for all variants, with a ~3-fold rate enhancement for apoA-I lacking Lys 206, 208, 238 and 239, or Glu 234 and 235. Single or double mutations did not change the ability to protect lipolyzed low density lipoprotein from aggregation, but variants lacking >4 lysine residues were less effective in preventing lipoprotein aggregation. ApoA-I mediated cellular lipid efflux from wild-type mice macrophage foam cells was decreased for the variant with five lysine mutations. However, this protein was more effective in releasing cellular phosphatidylcholine and sphingomyelin from *Abca1*-null mice macrophage foam cells. This suggests that the mutations caused changes in the interaction with ABCA1 transporters, and that membrane microsolubilization was primarily responsible for lipid efflux in cells lacking ABCA1. Taken together, this study indicates that ionic interactions in the C-terminal domain of apoA-I favor self-association, and that monomeric apoA-I is more active in solubilizing phospholipid bilayers.

Graphical Abstract

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Apolipoprotein (apo) A-I is a multifunctional protein with an established role in reverse cholesterol transport, wherein cholesterol is transported from peripheral tissues to the liver and steroidogenic tissues. ApoA-I is the main protein component of high-density lipoprotein (HDL), which circulates through plasma promoting cholesterol efflux from macrophages and has been extensively reviewed [1–5]. The 28 kDa protein is made of a series of amphipathic α -helices, offering a hydrophobic surface to facilitate reversible association with lipids [6]. The N-terminal (NT) domain, residues ~1–189, is composed of a bundle of helices providing structure and stability to the protein and harboring lecithin cholesterol acyl transferase (LCAT) activity [7–10]. The C-terminal (CT) domain (residues ~190–243), is structurally less defined and is responsible for self-association; it contains short α -helices that provide high lipid binding affinity to apoA-I [11–16]. In addition, the protein contains sites responsible for binding to the ATP binding cassette (ABC) AI transporter and hepatic SR-BI scavenger receptor [17, 18]. Lipid-free or lipid-poor apoA-I matures into discoidal and spherical HDL with the aid of LCAT and ABCA1. Several models of lipid-free apoA-I have been documented [19–21] and recently a consensus model was developed based on a wide variety of new and old structural data [22]. Further, deletion variants have provided key insight into the three-dimensional structure of the protein [23, 24]. The NMR solution structure of truncated murine apoA-I (Protein Data Bank entry 2LEM) shows a bundle of α -helices and small CT helices, similar to proposed models of human apoA-I. In contrast, X-ray structures of N- and CT deletion mutants of human apoA-I lost their helix bundle arrangement and formed an open circular helical structure and may represent a lipid-bound form of the protein. Indeed, a recent high-resolution structure of a lipid bound apoA-I variant, lacking two segments not involved in lipid contacts, confirmed such an arrangement of an extended α -helix circumscribing the discoidal particles, aligning their α -helices perpendicular to the phospholipid acyl chains, and is known as the belt model [25, 26]. Attempts to acquire a high-resolution structure of full-length human apoA-I have been hampered, likely due to the flexible nature of the protein and the existence of multiple oligomeric states [2]. The protein self-associates at concentrations above 0.1 mg/mL forming dimers, tetramers and octamers [27]. However, since serum concentrations of lipid-free are likely below 0.1 mg/mL, most of the protein may be monomeric although local concentrations may be higher, for example at the cellular surface [28]. Apolipoprotein E

(apoE) is another well-characterized apolipoprotein functioning in lipid homeostasis and is structurally similar to apoA-I [29]. X-ray structures demonstrated that the independently folded 22 kDa NT domain of apoE is a classic bundle of four amphipathic α -helices [30, 31]. While lacking X-ray structural analysis, biophysical analyses showed that the 10 kDa CT domain can be portrayed as an arrangement of loosely folded short α -helices [32, 33]. When several residues in full-length apoE were changed, a monomeric version was created, facilitating elucidation of an NMR high-resolution structure in which the CT domain was wrapped around the NT helix bundle [34]. Thus, self-association of apolipoproteins may need to be minimized in order to gain insight in the structural details of the full-length protein.

ApoA-I contains 21 lysine residues, which upon acetylation showed reduced self-association based on the presence of a single protein band on non-denaturing polyacrylamide gel electrophoresis (PAGE) [35]. Since self-association is governed by the CT domain, the six lysine residues present in this domain may affect the oligomerization state of the protein. Therefore, we targeted the CT lysine residues by site-directed mutagenesis to create a version of the protein with reduced self-association properties. Since many biophysical characterizations are carried out at concentrations at which apoA-I self-associates, this would allow for structural characterization of monomeric apoA-I. In addition, a monomeric version of the protein may not only provide insight in factors that govern self-association, but also in apoA-I functionality.

Experimental Methods

Protein expression, purification, site-directed mutagenesis

Wild type and apoA-I variants were expressed using the pET-20b expression vector. BL21 (DE3) pLysS *Escherichia coli* cells (Agilent Technologies) were transformed with plasmid containing recombinant apoA-I with an NT 6xHis-tag. A single colony was used to inoculate an overnight culture, which was used to seed a 2 L expression culture at 37 °C. When cell density reached an OD₆₀₀ value of 0.6, protein expression was induced with 0.5 mM IPTG for 3.5 h. Cells were collected by centrifugation at 8,000 g for 15 min (Sorvall RC5C Plus), and subsequently resuspended in phosphate buffered saline (PBS; 150 mM NaCl, 10 mM NaH₂PO₄, 10 mM Na₂HPO₄, pH 7.4). The cell suspension was sonicated on ice five times with 30 s on/off intervals (Branson). Samples were then centrifuged twice at 20,000 g for 30 min at 4 °C to remove cell debris. The supernatant was filtered through a 0.45 μ m syringe-driven filter unit (Millex), and the filtrate was mixed with an equal volume of loading buffer (2x PBS, 6 M guanidine-HCl, pH 7.4) and passed through a 5 mL Hi-trap chelating column (GE Healthcare) pre-charged with Ni⁺. Recombinant apoA-I was eluted using elution buffer (500 mM Imidazole in PBS), and the eluent was dialyzed against 4 L of 10 mM NH₄HCO₃ and 1 mM EDTA over 48 h with three buffer changes. Protein samples were then subjected to size-exclusion chromatography using a XK-16/70 column (GE Healthcare) filled with Sepharose CL-6B resin (Sigma Aldrich); proteins were eluted with 10 mM NH₄HCO₃. The apoA-I peak was collected, freeze-dried (Labconco), and stored at -20 °C until further use. The purity of the samples was verified by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). For protein analysis, 1 mL of 6 M guanidine-HCl was added to lyophilized protein and dialyzed for 48 h against 2 L of buffer, depending on the analysis,

with three buffer changes. Site-directed mutagenesis was employed to replace lysine or glutamate residues with glutamine in CT-apoA-I using the Quick-Change-II method (Agilent Technologies). Complementary primers were designed and ordered from Eurofins MWG Operon. To verify the desired variant, plasmid DNA was sequenced (Gene-Wiz, San Diego, CA). Protein concentrations were determined using the bicinconinic acid assay (ThermoFisher) or the absorbance at 280 nm. Verification of the desired mutations was determined by MALDI-TOF analysis of trypsin digests. Proteins were digested with trypsin for 16 h at 37 °C (trypsin gold mass spectrometry grade, Promega) at a mass ratio of protein to trypsin of 150:1 in 50 mM NH_4HCO_3 buffer (pH 8.0). The fragmentation pattern and sequence of target fragments were determined using a 4800 MALDI TOF/TOF Analyzer (Applied Biosystems) at the CEPA Proteomics core facility at CSU Long Beach.

Cross-linking and electrophoresis

Ten μg of each apoA-I variant was incubated for 2 h at 24 °C with 50 μg dimethyl suberimidate (DMS; Sigma-Aldrich) in 63 mM triethanolamine, pH 9.0 (30 μL total volume). Upon crosslinking, 10 μL of 4x Lithium dodecyl sulfate sample loading buffer (Invitrogen) was added and incubated at 70 °C for 10 min. Samples were loaded onto a 10% Bis-Tris SDS-PAGE (Invitrogen) and electrophoresed at 200 V for 35 min in MES-SDS running buffer. Gels were stained for 30 min using 0.5% (m/v) naphthol blue-black in 45% methanol and 10% glacial acetic acid and destained in 45% methanol and 10% glacial acetic acid. To determine the ratio between monomer and oligomer, the density of the apoA-I bands was determined using Image J software. For non-denaturing electrophoresis, 20 μg of protein was loaded in each lane of a 4–20 % gradient Tris-glycine gel (Invitrogen) and electrophoresed at 125 V for 120 min. Gels were stained and destained as described for SDS-PAGE.

Fast protein liquid chromatography

Elution characteristics of each apoA-I variant were analyzed by fast protein liquid chromatography on an ÄKTA Purifier (GE Healthcare) using a Superdex 200 10/300 GL size-exclusion column (GE Healthcare) in the appropriate buffer. PBS was used for experiments at physiological pH, while for pH 3 20 mM glycine 150 mM NaCl was used and for pH 12 20 mM NaP 150 mM NaCl. Five-hundred μL of 0.5 μg protein/ μL was loaded onto the column and eluted at a flow rate of 0.5 mL/min. The elution profile was obtained by continuously measuring the sample absorbance at A_{280} . Each protein variant was analyzed three times and the average of the retention volume was determined.

Multi-angle light scattering

Protein samples at a concentration of 10 mg/mL in PBS were applied to a Superdex 200 10/300 gel filtration column (GE Healthcare) coupled to DAWN HELEOS-II light scattering and Optilab T-REX refractive index detectors (Wyatt Technology). ASTRA 6 software was used to analyze the data to obtain the molecular weight of the protein sample. To determine the oligomeric state, the experimental molecular weight was compared to the molecular weight of the apoA-I variant based on the amino acid sequence.

Circular dichroism

Protein samples were prepared in 25 mM sodium phosphate pH 7.4 and diluted to 0.2 mg/mL. Ellipticity was recorded from 185 to 260 nm in a Jasco 810 polarimeter using a circular cuvette with a 1 mm pathlength. The ellipticity of each sample was collected with 4 repetitions using a scanning rate of 50 nm/min to obtain the average scan. This was repeated three times for each protein variant. Molar ellipticity and α -helical content was obtained as described previously [35]. To obtain the denaturation profile of each variant, proteins (0.2 mg/mL) were denatured with guanidine-HCl and incubated overnight at room temperature at the appropriate concentration of denaturant, after which the ellipticity at 222 nm was recorded.

DMPC vesicle solubilization

Lipid films of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC; Avanti Polar Lipids) were formed by dissolving 10 mg of dry lipid in 500 μ L of 3:1 chloroform: methanol (by volume) and subsequently evaporating all solvent using a gentle stream of N₂ gas. Films were then dried further in a Labconco freeze dryer for 24 h and stored at -20 °C until use. Lipid films were then suspended in PBS, vortexed vigorously for 1 min, and large unilamellar vesicles (LUV) were obtained by extrusion 21 times through a 200 nm polycarbonate membrane (Whatman) using the Avanti Mini-Extruder at 45 °C. To measure the rate of DMPC solubilization, LUVs (500 μ g) in PBS were equilibrated at 24.1 °C after which 500 μ g protein was added (1 mL final volume) and the turbidity of the sample was monitored at 325 nm in a Shimadzu UV-2401PC spectrophotometer. The plots were fit to an exponential decay equation using SigmaPlot version 11.0 to determine the first order rate constants of vesicle solubilization. The rate enhancement of vesicle solubilization of each apoA-I variant was determined by dividing the rate constant of the apoA-I variant by the rate constant of the wild-type protein.

Lipoprotein aggregation

Fifty μ g of each apoA-I variant was mixed with 50 μ g protein of low density lipoprotein (LDL; Sigma-Aldrich) in 50 mM Tris-HCl, 150 mM NaCl, 2 mM CaCl₂, pH 7.4, in a 96-well plate (Greiner Bio-One). Two hundred mU of phospholipase C from *Bacillus cereus* (Sigma-Aldrich) was added to initiate the reaction, keeping the final volume at 200 μ L. The formation of lipoprotein aggregates was measured by the absorbance at 340 nm for 2 h in a Thermo Varioskan plate reader; the temperature was kept at 37 °C.

Isolation and modification of lipoprotein

Lipoprotein was isolated from human plasma by stepwise density ultra-centrifugation and the LDL fraction was collected density between 1.006 and 1.063 g/mL as described elsewhere. LDL was dialyzed for 3 days against PBS (4 L) with 2 changes at 4 °C. For acetylation, LDL concentration was adjusted to 1 mg protein/mL in PBS and 15 mL (15 mg protein) was mixed with an equal volume of saturated sodium acetate solution at 0°C. Anhydrous acetic acid was added to the solution 2 μ L each for 12 times total over 1 h with stirring on ice. The solution was kept stirring on ice for 30 min, followed by dialysis against PBS (4 L) for overnight at 4 °C to obtain acetylated-LDL (Ac-LDL) [36,37].

Animals

C57BL/6 mice were purchased at local suppliers. *Abca1*-null mice breeding pairs were obtained from The Jackson Laboratory (Stony Brook, NY) and backcrossed to C57BL/6 mice for 8 generations. All animals were bred at 25°C, 45–65% humidity under a standard 12-h light/12-h dark cycle with chow and water *ad libitum* at the Center for Experimental Animal Science Nagoya City University Graduate School of Medical Sciences. Animal handling and experimental procedures were followed based on standard methods of The NCU Animal Care and Use Committee (H15–21, H18–34). Mouse genotype was determined by tail DNA as templates. The primer sequence for 5'-TGCAATCCATCTTGTTCAAT-3' and 5'-TTTCTCATAGGGTTGGTCA-3' for *Abca1*-null allele and 5'-TGGGAACCTCTGCTAAAAT-3' and 5'-CCATGTGGTGTGTAG ACA-3' for *Abca1*-wild type allele were used.

Mouse primary peritoneal macrophage foam cells

Primary peritoneal macrophages were cultured from 12- to 20-week old wild type and *Abca1* null mice as described previously [36]. Briefly, the harvested macrophage cells were plated on 6 well plates and incubated for 18 h in 15% FBS containing RPMI-1640 supplemented with 5 units/mL penicillin, and 5 µg/mL streptomycin (Wako Pure Chemical Industries, Ltd) at 37°C in a 5% CO₂ incubator. Unbound cells were washed by PBS and macrophages were further incubated in 100 µg/mL acetylated LDL, 0.2% BSA (fatty acid free, BioReagent A8806, Merck KGaA) containing RPMI-1640 supplemented with 5 units/mL penicillin, and 5 µg/mL streptomycin for 48 h. In some assays, cells were also labeled with [1,2-³H(N)]-cholesterol (2 µCi/mL, Moravek, Inc.). Macrophage foam cells were further labeled by [1-³H]-choline chloride (2 µCi/mL, American Radiolabeled Chemicals Inc.) containing RPMI-1640 + 0.2% BSA for 24 h and incubated 24 h in fresh medium of RPMI-1640 containing 0.2% BSA. The indicated concentration of apoA-I or mutated apoA-I containing RPMI-1640 supplemented 0.02% BSA were pre-incubated for 30 min prior to loading the cells. The conditioned medium was collected after 24 h and the released cellular lipids were extracted by 4 times volume of chloroform: methanol (2:1) for overnight at 4°C. Cells were dried-down from the remaining solution and lipids were extracted by hexane: isopropanol (2:3) for 1 h. Cellular protein was dissolved in 1 mL of 0.5 N NaOH for 1 h to determine the protein concentration. Extracted lipids were developed on a TLC silica gel plate 60 (20 cm × 20 cm, Merck KGaA, Germany). The radiolabel in phosphatidylcholine, sphingomyelin, and cholesterol spots were analyzed by the Aloka liquid scintillation counter LSC-6100 (Aloka, Japan).

Results

Design of apoA-I variants

The CT domain of apoA-I contains six lysine residues at positions 195, 206, 208, 226, 238, and 239, which were replaced by glutamine using site-directed mutagenesis. Several rounds of mutagenesis were necessary to substitute all lysine residues, and single variants K238Q- and K239Q-apoA-I and a double variant K238/239Q-apoA-I (2KQ) were produced first. Using plasmid DNA of the double lysine variant as a template for additional mutations, K206/208/238/239Q- (4KQ), K206/208/226/238/239Q- (5KQ), and

K195/206/208/226/238/239Q- (6KQ) apoA-I were engineered. The structural and functional analysis of this series of apoA-I variants allowed for a better understanding of the contribution of lysine residues towards protein structure and lipid binding. Because of potential salt bridge formation with the eight negatively charged residues that also reside in the CT domain (D213 and E190, 198, 205, 212, 223, 234, 235) three additional variants were generated: single variants E234Q- and E235Q-apoA-I, and the double mutant E234/235Q-apoA-I (2EQ). These residues are relatively close to K238 and K239 and have potential to form intermolecular or intramolecular salt bridges. Upon verification by DNA sequencing of plasmid DNA to confirm that the correct nucleotide change resulted in the desired amino acid change, proteins were over-expressed in *E. coli* and purified by Ni²⁺-affinity and size-exclusion chromatography. The mutations were then verified on the protein level by mass spectrometric analysis of trypsin digests of wild-type, 2EQ, and 6KQ apoA-I.

Self-association properties

The KQ apoA-I variants were first analyzed by native PAGE (Fig. 1). This showed that, compared to the wild-type protein, the electrophoretic mobility of the variants increased when K238 and/or K239 was substituted, while an additional increase was observed for 4KQ-apoA-I. Removal of 5 or 6 lysine residues did not lead to further changes in gel migration, and the protein was visible as a discreet band at the bottom portion of the gel. The change in pI from 5.27 (wild-type) to 4.87 (6KQ) likely contributed to the increase in electrophoretic mobility. Further, wild-type, single, and double lysine mutants were visible as heterogeneous protein bands while 4KQ, 5KQ and 6KQ-apoA-I protein bands were much more defined, indicating decreased self-association and presence of a more homogeneous apoA-I population.

Next, the proteins were cross-linked with DMS, which forms covalent bonds with amines of closely spaced lysine side chains. The cross-linking pattern was analyzed by SDS-PAGE, which showed extensive cross-linking for wild-type apoA-I with distinct protein bands at ~30, 60, 90, 120, and 150 kDa (Fig. 2). When single lysines were substituted, the intensity of the multimers decreased, which was even more visible in the double mutant. The multimers virtually disappeared for 4KQ-, 5KQ- and 6KQ-apoA-I, which predominantly appeared as intra-molecular crosslinked monomers (see Table 1 for the ratio of monomers to oligomers). Removal of lysine residues may have decreased DMS cross-linking efficiency since reactive amines were removed from the CT domain. The crosslinking results for the EQ mutants were less conclusive, although the amount of crosslinking may have decreased for the E234Q and the E234/235Q mutant.

The ratio of monomer to oligomer was based on densitometric scanning of the apoA-I bands of SDS-PAGE gels, elution volume was determined by FPLC, % helix and midpoint of guanidine denaturation [Gdn-HCl]_{1/2} were determined by circular dichroism, and the rate enhancement compared to wild-type apoA-I was determined for solubilization of DMPC vesicles (NM = not measured).

To determine the oligomeric state of the apoA-I variants more precisely, the proteins were eluted on a Superdex-200 size-exclusion FPLC column. While wild-type apoA-I eluted around 11 mL, the elution volume increased incrementally with each lysine substitution until

five lysines were replaced (Fig. 3A). 5KQ- and 6KQ-apoA-I were visible as a single narrow peak with a 14.4 mL elution volume (Table 1). Changes in the elution volume were also observed for the EQ mutants, with a 0.4 mL increase in elution volume for E235Q-apoA-I, 1.1 mL increase for E234Q-apoA-I, and a 3.1 mL increase for 2EQ-apoA-I (Fig. 3B). Thus, the degree of oligomerization decreased as more lysine or glutamate residues were removed from the CT domain, similar as observed for the DMS crosslinking analysis. The elution profile of wild-type apoA-I was also analyzed at acidic and basic pH to change the ionization state of charged amino acid side-chains (Fig. 3C). At pH 12 and pH 3, the protein eluted as a single peak with a retention volume similar to 5KQ-apoA-I. At these extreme pH values the protein remained α -helical based on circular dichroism analysis, with estimated helical contents of $44.7 \pm 0.4\%$ at pH 3 and $62.8 \pm 3.0\%$ at pH 12. For pH 10, the elution profile was very similar to the wild-type protein. These results confirm that salt bridges form between oppositely charged residues in apoA-I, and their disruption may lead to weakening of intermolecular interactions resulting into oligomer dissociation. Multi-angle light scatter (MALS) analysis of 5KQ-apoA-I revealed a molecular mass of 30 kDa, indicating the protein is predominantly present in a monomeric form (Fig. 3D).

Structural and stability analyses

The overall secondary structure of the mutant proteins was determined by circular dichroism far-UV scans to ensure that the substitutions made to apoA-I did not perturb the predominantly α -helical character of the native protein. Wild-type apoA-I and all variants displayed a peak at 190 nm and a double trough at 208 and 222 nm, characteristic of a predominantly α -helical structure (not shown). The α -helical content was estimated to be in the range of $60 \pm 4\%$ for all variants (Table 1), indicating the mutations did not cause perturbation of the secondary structure of the protein. In addition, there was no measureable difference in ellipticity values between wild-type and 5KQ-apoA-I at a protein concentration range of 2 – 20 $\mu\text{g}/\text{mL}$ which were later used in the lipid efflux studies.

The stability of the variants was assessed by measuring the ellipticity at 222 nm with increasing concentration of guanidine-HCl, determining the midpoint of denaturation to assess protein stability. The denaturation plots of each mutant protein looked similar (Fig. 4), producing denaturation midpoints that were comparable (Table 1). This indicated that mutations made in the CT domain did not have an adverse effect on the overall structure and stability of apoA-I. However, small differences were observed at low guanidine-HCl concentrations between 0 – 0.6 M. Wild-type and single KQ and EQ variants clearly displayed a more complex unfolding pattern with an initial unfolding maximum at 0.5 M, and a second transition state at denaturant concentrations of > 0.5 M resulting in a complete unfolding of the protein. In contrast, 5KQ-apoA-I and 6KQ-apoA-I followed a sigmoidal shaped curve indicative of a simple two-state unfolding process. Denaturation at > 0.5 M denaturant likely represents unfolding of the NT domain, and was not affected by the mutations. The changes in secondary structure at low denaturant concentrations observed for wild-type apoA-I may represent disruption of subunit interaction in the quaternary structure, since measurements were carried out with a protein concentration of 0.2 mg/mL at which the protein forms oligomers. When five or six lysine residues of the CT domain of apoA-I were

substituted, intermolecular interactions were not detected by circular dichroism, indicating the protein is in a monomeric state, in agreement with the FPLC and cross-linking analysis.

Lipid binding analyses

The ability of the mutant proteins to solubilize phospholipids was analyzed using LUVs of DMPC produced by extrusion. ApoA-I rapidly solubilized LUVs to form small discoidal protein-lipid complexes, which was monitored by measuring the decrease in light scatter intensity when the LUVs were converted into the much smaller protein-lipid complexes. Fig. 5 showed that all variants were able to solubilize DMPC vesicles at a faster rate compared to the wild-type protein. 4KQ- and 2EQ-apoA-I displayed the fastest solubilization kinetics, see Table 1 for the rate enhancement compared to wild-type apoA-I. For the lysine variants, the solubilization rate was reduced when more than four lysines were removed (5KQ and 6KQ), although these variants remained more potent compared to the wild-type protein. It is of interest to note that the solubilization rate closely followed the FPLC elution profile shown in Fig. 3. The order of solubilization rate enhancement was 2EQ>E234Q>E235Q>WT, while the same order was observed in the elution volumes of these proteins. This was also observed for the KQ mutations, with the exception of 5KQ- and 6KQ-apoA-I. This result suggests that when the equilibrium shifted from an oligomeric to a monomeric state, the ability to solubilize DMPC vesicles increased.

When LDL was incubated with phospholipase-C, hydrolysis of phosphatidylcholine created surface localized diacylglycerol as described earlier [38]. When sufficient amounts of phosphatidylcholine were converted into diacylglycerol and reached a critical mass, LDL aggregated resulting in a turbid appearance of the sample, as can be seen at the 10-min time point in Fig. 6. In the presence of wild-type apoA-I, phospholipase-C-induced LDL aggregation was prevented during the 2 h incubation period, which results from apoA-I association with the LDL surface. Similarly, all single and double variants were able to provide full protection during the 2 h incubation time. However, 4KQ-, 5KQ- and 6KQ-apoA-I lost some of their protective properties as the sample turbidity started to increase after 40 min, with 6KQ-apoA-I providing the least protection.

Apolipoprotein A-I mediated lipid efflux from mouse peritoneal macrophage foam cells

The previous analysis demonstrated that lysine substitutions promote a monomeric state of apoA-I, but may also have an adverse effect on lipoprotein binding. 5KQ-apoA-I was selected for their role in lipid efflux, both in ABCA1 dependent and independent pathways. This analysis was carried out at low protein concentrations at which wild-type and 5KQ-apoA-I are predominantly in a monomeric state, and thus the effect of the reduced number of charges in the CT domain on lipid efflux was measured. As shown in Fig. 7, phosphatidylcholine (PC, panel A), sphingomyelin (SM, Panel B), and cholesterol (Panel C) release increased in a dose-dependent manner for both wild-type apoA-I (open circles) and 5KQ-apoA-I (solid circles) from wild-type Ac-LDL-loaded macrophage foam cells. Consistent with the typical apoA-I mediated cellular lipid efflux profiles, apoA-I lipid release was saturated between 5 to 10 µg/mL while the release of PC mediated by 5KQ-apoA-I followed a more linear pattern. After an initial lag, SM and cholesterol were also released dose dependently but with lower efficacy.

Foam cells harvested from *Abca1*-null mice indicated no lipid release by wild-type apoA-I under these experimental conditions (Fig. 7, panels D and E). On the other hand, 5KQ-apoA-I was able to mediate release of cellular PC and SM from *Abca1*-null mice macrophage foam cells. These results showed that the majority of the cellular lipid efflux mediated by 5KQ-apoA-I seems to follow an ABCA1 independent pathway.

Discussion

In order to study apoA-I oligomerization, all six lysine residues in the CT domain of apoA-I were substituted by glutamine, which led to a decreased tendency to oligomerize. This effect was additive, with each successive mutation leading to an increase in the monomer to oligomer ratio. 5KQ- and 6KQ-apoA-I were predominantly monomeric, showing a discrete protein band on non-denaturing PAGE, loss of DMS intermolecular cross-linking, and eluting as single peak on size-exclusion FPLC (with a molecular mass of 30 kDa determined by MALS). Despite the five or six mutations, apoA-I maintained an overall α -helical secondary structure similar to that of wild-type apoA-I. In addition, the core stability of the variants was largely unaffected by the amino acid changes.

Characterization of chicken apoA-I and insect apolipoprotein III showed that they are monomeric proteins [39, 40], however, many other apolipoproteins including human apoA-I are known to self-associate. Several studies have been undertaken to change the oligomeric character of these proteins. For example, mouse apoA-I forms oligomers similar to human apoA-I [41] and two clusters of mutations in a deletion variant of mouse apoA-I led to a monomeric version of the protein. Human apoA-IV is present as monomers and dimers, which form when two apoA-IV molecules associate through helix swapping forming two successive 4-helix bundles [42]. Introduction of a flexible linker group in the elongated helix allowed the protein to fold back on itself, creating a monomeric 4-helix bundle. In human apoE3, five mutations in the CT domain changing bulky hydrophobic to small polar side-chains resulted in a monomeric form of the protein, both in isolated CT domain and in the full-length protein, which was then used to obtain the NMR solution structure [34, 43]. The nature of the amino acid changes was considerably different in the various apolipoproteins, implying differences in the protein-protein interactions that are responsible for self-association. Further, mutations in the NT domain of apoA-I were also shown to change self-association properties. When four NT domain tryptophan residues were replaced by phenylalanine the extent of self-association increased. This also led to reduced functionality: decreased DMPC solubilization rates and ABCA1 dependent uptake of phospholipids and free cholesterol from ABCA1 transfected HEK293 cells [28]. In addition, a S36A mutation led to changes in self-association and lipid binding properties [44]. When several leucine residues were introduced in the nonpolar face in the CT helix, the helix packing of the NT domain was affected [45]. Together, these studies suggest there is considerable communication between the NT and CT domain of apoA-I. However, other studies suggest that the CT domain can also act independently from the NT domain, as lipid binding and self-association were successfully transferred to apoLp-III in a chimeric protein composed of apoLp-III and residues 179–243 of apoA-I [46].

The lysine residues of the CT domain of apoA-I are located in two putative amphipathic α -helices spanning residues 187–208 and 220–241 [6]. The first helix (residues 187–208) is considered class A (negatively charged residues clustered at the polar-nonpolar interface with positively charged residues at the center of the polar face), while the second helix (residues 220–241) is considered class Y (positively charged residues distributed at the polar-nonpolar interface and center of the polar face, with negatively charged residues located between the negatively charged clusters). The hydrophobicity and lipid binding affinity are significantly higher for the 220–241 helical segment compared to other apoA-I helices [6, 47]. The lysine substitutions of the present study resulted in increased hydrophobicity and a small decrease in hydrophobic moment for both helices, with the net charge becoming negative for both helices (Figure 8). This change likely promoted the dissociation of the apoA-I oligomers, possibly disrupting inter-molecular ionic interactions between CT helices from neighboring apoA-I molecules.

Current apoA-I structures do not provide sufficient molecular detail, but lipid bound apoA-I or lipid bound-like structures have indicated E234, E235, K208 and K226 can participate in salt bridges [23, 25]. Since a number of negatively charged amino acids are positioned one helical turn away from the CT lysine residues, these residues may participate in intrahelical salt bridges. This would stabilize formation of amphipathic α -helices, and promote helix-helix interactions between adjacent apoA-I molecules. Thus, disruption of intrahelical salt bridges may also favor the monomeric state of the protein.

The CT domain helices of apoE3 are thought to form an interhelical coiled-coil leading to formation of a tetrameric protein [49]. Monomeric apoE3 exhibits increased lipid binding activity; dissociation of tetramers and dimers into monomers is thought to be the rate-limiting factor in phospholipid vesicle solubilization [50]. Self-association of apoA-I may be different from that in apoE3 since there was no indication of coiled-coil helices in apoA-I, based on molar ellipticity ratios at 222 and 208 nm [28]. Nevertheless, when apoA-I is present in an oligomeric state, it is conceivable that the protein dissociates into monomers similar to apoE. This would allow replacement of protein-protein interaction with protein-lipid interaction. This is a critical step as the CT domain is mainly responsible to initiate apoA-I lipid binding [7, 8, 51].

In wild-type apoA-I, lysine residues are located at the polar-nonpolar interface of class A and Y helices and likely contribute to the lipid binding properties of the helix according to the “snorkel model” with the methylene groups interacting with the phospholipid acyl chains, while the charged ϵ amino group is in close proximity to the phospholipid head group [6, 47]. However, 4KQ-apoA-I, lacking two lysine residues in the polar/nonpolar interface of each of the two helices, displayed the highest phospholipid solubilization rate. This suggests that lysine residues may not be critical for lipid binding affinity of the CT helices. As discussed above, a shift from an oligomeric state towards a monomeric state may be the determining factor responsible for enhanced interaction with the phospholipid vesicle bilayer. Also, when glutamate 234 and 235 were substituted, it led to a shift towards monomeric protein and increase in vesicle solubilization rates.

While interaction with the phospholipid vesicles was enhanced in the mutant proteins generated in the present study, there was a decreased capability to protect lipolyzed LDL from aggregation. Although it appears contradictory, it is likely that the apolipoprotein may interact differently with the hydrophobic defects generated on the vesicle and LDL surface. The rate-limiting step of the phospholipid vesicle solubilization may be determined by remodeling of the phospholipid bilayers, while the ability to protect the LDL surface from aggregation is dependent on the association of the hydrophobic face of the α -helices to the DAG-enriched lipoprotein surface. It is possible that the lysine substitutions may have altered the association equilibrium; further studies are needed to address this behavior in more detail.

The current study also showed that lysine substitution decreased the efflux of cholesterol, phospholipid, and sphingomyelin from macrophages compared to the wild-type protein. The dissociation into monomers does not appear to play a role in the lipid efflux studies, as the concentration used in the efflux studies was well below values at which the protein self-associates (~ 0.1 mg/mL). The changes in lipid efflux are likely a consequence of the lysine substitutions, altering the amphipathic nature of the CT helices. The CT helix is critical for ABCA1 mediated cholesterol efflux, as others have shown that deletion of residues 220–243 or 231–241 severely reduced the cholesterol efflux [52, 53]. The decrease in lipid efflux of 5KQ-apoA-I from wild-type macrophages indicates that there is an electrostatic component in transporter binding interaction. It has been shown previously that poly-lysine and heparin led to inhibition of the interaction between apoA-I and ABCA1, suggesting ionic interactions may play a role [54]. Further, ABCA1 lysine modification decreased binding interaction between the two proteins and apoA-I acetylation led to a decrease in cholesterol acceptor activity, indicating a contribution of apoA-I lysine residues to ABCA1 receptor binding [55]. In contrast to the studies using wild-type macrophages, 5KQ-apoA-I facilitated release of phospholipids and sphingomyelin from foam cell macrophages deficient in ABCA1 transporters. Thus 5KQ-apoA-I may be able to microsolubilize cell membrane lipids in the absence of ABCA1 protein, and these results seem in agreement with the increased phospholipid vesicle rates observed for model bilayer membranes. It is possible that the 5KQ-apoA-I variant is able to accelerate cellular lipid transport at advanced plaque lesions where ABCA1 expression is low, but future analysis will be required to verify this concept [56].

In summary, our studies demonstrate that lysine to glutamine mutations in the CT domain of apoA-I cause a shift from oligomers towards monomers which is more amenable to high-resolution structural analysis. Structural characterization of monomeric apoA-I may provide new insights in the structure-function relationship of this plasma protein to better understand the molecular basis of apoA-I function.

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Figure 1.

Native PAGE analysis of apoA-I lysine variants. The apoA-I mobility increased and protein bands were more homogeneous when multiple lysine residues were replaced by glutamine. The variants used are indicated at the top of the gel.

Figure 2. SDS-PAGE of crosslinking of apoA-I. ApoA-I was incubated with DMS crosslinker and subjected to SDS-PAGE to determine the oligomerization state of the protein. The apoA-I variants are indicated at the top of the gel, + or – indicates addition or exclusion of DMS during the incubation.

Figure 3.

FPLC elution profiles of lysine and glutamine variants. Panel A shows the following selected lysine variants: profile 1 = wild-type, 2 = K238Q, 3 = 2KQ, 4 = 4KQ, and 5 = 6KQ-apoA-I. The elution volumes of all lysine mutants are listed in Table 1. The elution profiles of 5KQ- and 6KQ-apoA-I are very similar with elution volumes that are indistinguishable. The glutamate variants are shown in panel B: profile 1 = wild-type, 2 = E235Q, 3 = E234Q, 4 = 2EQ-apoA-I. Panel C shows the elution profile of wild-type apoA-I at the pH indicated, and panel D shows the MALS analysis of 5KQ-apoA-I.

Figure 4.

Denaturation plots of apoA-I. The protein was incubated in the presence of Gdn-HCl and the molar ellipticity measured at 222 nm. The % change in ellipticity was plotted against the denaturant concentration. Panel A shows wild-type (closed circles), K238Q- (open circles), 2KQ- (open triangles), 4KQ- (closed squares), and 5KQ-apoA-I (open squares). The inset shows the denaturation of wild-type and 5KQ-apoA-I between 0 and 0.7 M Gdn-HCl. Panel B: wild-type (closed circles), E234Q- (open circles), E235Q- (closed triangles), and 2EQ-apoA-I (open triangles).

Figure 5. Solubilization of DMPC LUVs by (A) lysine variants and (B) glutamate variants. All variants displayed faster solubilization kinetics compared to wild type protein. In both panels wild-type is shown as a solid line and vesicles without protein addition as medium dashed lines. Panel A: 2KQ-(dash-dot-dot), 4KQ- (dotted), 5KQ- (short dashes), and 6KQ-apoA-I (long-short dashes). Panel B: E234Q- (long dashes), E235Q- (dash-dot-dot), and 2EQ-apoA-I (dash-dot).

Figure 6.

ApoA-I prevents LDL aggregation induced by phospholipase C. Light scattering at 325 nm was used to measure the formation of LDL aggregates, which form rapidly in the absence of apolipoprotein (closed circles). Inclusion of wild-type apoA-I, single and double mutants provided full protection, however, the variants with 4 or more mutations were less effective as seen by the increase in sample turbidity after 40 min.

Figure 7.

Apolipoprotein A-I mediated lipid efflux from peritoneal macrophage foam cells isolated from wild type or ABCA1-deficient (*Abca1-null*) mice. Lipid efflux to wild-type apoA-I (open circles) and 5KQ-apoA-I (solid circles) from wild-type mice macrophages (A-C) or ABCA1-deficient mice macrophages (D, E) was determined for 24 h at the indicated protein concentrations. Phosphatidylcholine (PC, panels A and D), sphingomyelin (SM, panels B and E), and cholesterol (panel C) radioactivity was adjusted for cellular protein content. The data are indicated as fold increase in lipid efflux relative to that noted in the absence of added apoA-I. Asterisks indicate the statistical significance (unpaired two-tailed t-test) between wild-type apoA-I vs. 5KQ-apoA-I. *, $P < 0.05$, **, $P < 0.01$.

Figure 8.

Helical wheel diagrams of the two CT helices of apoA-I. Shown on the left is the helical wheel for the class A amphipathic helix (residues 187–208). Based on calculations with HeliQuest ^[48], the following values were obtained: hydrophobicity 0.110, hydrophobic moment 0.363, net charge +1. The lysine to glutamine mutations changed this to 0.215 (hydrophobicity), 0.331 (hydrophobic moment), and –2 (net charge). For the class Y helix (residues 220–241), the hydrophobicity changed from 0.430 to 0.535, hydrophobic moment from 0.395 to 0.383, and net charge from 0 to –3. The lysine and glutamate residues that were mutated in the current study are numbered.

Table 1:

Characteristics of wild-type and apoA-I variants used in the present study

Protein	Elution volume (mL)	Monomer to oligomer ratio	% helix	[Gdn-HCl] _{1/2} (M)	Rate enhancement
Wild-type	11.0	0.4	62.9	0.97	1.0
E234Q	12.1	0.8	56.3	1.02	2.0
E235Q	11.4	0.4	56.7	1.00	1.6
2EQ	13.2	0.6	60.8	0.98	2.8
K238Q	11.7	0.6	55.6	0.98	NM
K239Q	11.3	0.7	58.2	0.96	NM
2KQ	13.2	1.8	59.6	0.98	2.2
4KQ	13.7	4.3	59.6	0.97	2.7
5KQ	14.3	8.4	59.6	0.97	1.3
6KQ	14.5	11.5	58.1	0.97	1.5