ApoE is a 299 residue protein of the human lipoprotein transport system and plays critical roles in protection from atherosclerosis and dyslipidemias (1). ApoE mediates the hepatic clearance of lipoprotein remnants from circulation and is involved in cholesterol efflux processes contributing to cell and tissue cholesterol homeostasis (2–5). In the human population, apoE exists in three common isoforms named apoE2, apoE3, and apoE4, differing at amino acid positions 112 and 158 (6, 7). ApoE4 is a major genetic risk factor for Alzheimer’s disease (8). In plasma, apoE is associated with chylomicron remnants, VLDLs, and some HDL subpopulations (9–11). Lipoprotein-bound apoE is the ligand for the LDL receptor as well as other receptors in vitro (12–14). The polymorphic background, as well as mutations in the apoE gene, have been linked with the pathogenesis of several diseases related to lipid metabolism, such as type III hyperlipoproteinemia, carotid atherosclerosis, diabetic dyslipidemia, and lipoprotein glomerulopathy (LPG) (15–18).

LPG is a rare kidney disorder that leads to progressive kidney failure and is characterized by lipoprotein thrombi in the glomerular capillaries (18, 19). LPG is a dominant inherited disease with incomplete penetrance (18). A series of mutations within apoE, the majority of which are point mutations in which proline or cysteine is substituted for an arginine residue, have been closely associated with LPG and it has been proposed that they may underlie disease pathogenesis, although the mechanism is unknown (18–21). Most LPG patients are heterozygous for these apoE mutations and have elevated plasma apoE levels and,
in some cases, elevated triglyceride, cholesterol, IDL, and remnant lipoprotein levels (as reviewed in Ref. 22). One of these mutations, apoE Chicago (R147P), has also been found in homozygous form in an LPG patient (23). Renal transplantation led to recurrence of LPG, suggesting that renal abnormalities are not necessary for disease pathogenesis (24). Furthermore, adenovirus-mediated gene transfer studies of apoE Sendai (R145P) in apoE-deficient mice resulted in distinct intra-glomerular deposits of apoE-containing lipoproteins, suggesting that apoE Sendai is an etiological cause of LPG (25). Although this evidence suggests a direct association between apoE mutations and LPG, the mechanism that leads to deposition of lipoproteins in the glomerulus is unknown. It has been proposed that apoE mutations may display increased affinity for the glomerulus and/or may be prone to aggregation favoring the formation of lipoprotein thrombi in the microenvironment of renal glomerulus (18). However, lack of knowledge regarding the functional and structural repercussions of LPG-associated mutations on apoE has impeded our understanding of a unified mechanism leading to LPG pathogenesis.

ApoE is highly helical with labile tertiary structure that can assume structures characteristic of a molten globule (26). It can undergo significant conformational changes during its physiological function, for both lipid and receptor binding (27, 28). Lipid-free apoE is folded to two seemingly independent structural domains that can be separated after digestion with thrombin, generating an N-terminal 22 kDa fragment and a C-terminal 10 kDa fragment (29, 30). Structural analysis has indicated that the N-terminal domain folds as a four-helix bundle of amphipathic α-helices in the lipid-free state (31). Biophysical and computational analyses suggest that the N-terminal domain must unfold during lipid binding to allow for exposure of its hydrophobic core (27). The C-terminal domain is also highly α-helical, but its tertiary structure is highly polymorphic and participates in inter-domain interactions with the N-terminal domain as well as lipids (27). Interactions between the C-terminal and the N-terminal domains can destabilize the latter and mediate its unfolding and lipid binding (27, 28, 32). A recently solved NMR structure of a monomeric apoE3 variant demonstrated that the C-terminal domain can fold over the N-terminal domain, obstructing the LDL-binding region of apoE3 from the solvent (28). apoE displays low thermodynamic stability and significant conformational plasticity, and mutations in the protein have been shown to affect these properties, impeding physiological function (27, 32–35). Furthermore, apoE has been shown to be prone to aggregation and to form oligomeric structures in solution (36, 37).

In several of the apoE mutations linked to the development of LPG, a proline is substituted for an arginine residue within the N-terminal domain of the protein. Because the N-terminal domain is highly helical, we hypothesized that these mutations may significantly perturb local structure and adversely affect apoE thermodynamic stability and, as a consequence, function. To test this hypothesis, we produced recombinant forms of three apoE3 variants linked to LPG, namely R145P (apoE Sendai) (38), R147P (apoE Chicago) (23), and R158P (apoE Osaka or Kurashiki) (20, 21) and characterized their structural, thermodynamic, and functional integrity. Our findings demonstrate that the mutations induce significant thermodynamic, structural, and aggregation-related perturbations in the molecule of apoE3 that induce unfolding of the N-terminal domain to a more molten-globule-like structure. Reconstituted HDL-like discoidal particles containing these variants show structural and thermodynamic defects and can be prone to aggregation. Our data point to common themes in the dysfunction of these mutants, pointing toward a unifying mechanism that may relate to LPG pathogenesis. Our findings provide structural insight for the causal link between these mutations and the pathogenesis of LPG.

MATERIALS AND METHODS

Expression and purification of His-tagged 3C protease
BL21-Gold(DE3) cells were transformed with the pET-24/His-3C vector (kindly provided by Dr. Arie Geerlof, EMBL, Heidelberg, Germany) carrying the gene for the 3C protease. The transformed cells were grown on Luria-Bertani (LB) agar plates containing 50 μg/ml kanamycin for 16 h at 37°C. A single colony from the plate was inoculated into a 5 ml LB medium culture containing 50 μg/ml kanamycin. The culture was incubated at 220 rpm at 37°C overnight. The following day, the culture was centrifuged at 4,500 g for 20 min at 4°C, the supernatant was discarded, and the cell pellet was resuspended in 5 ml fresh LB medium containing 50 μg/ml kanamycin. The cell suspension was added in a 500 ml LB medium culture containing 50 μg/ml kanamycin, and the cells were grown at 37°C, until A600nm = 0.6. At that point, isopropyl beta-D-thiogalactopyranoside was added to the culture in a final concentration of 0.5 mM to induce the expression of the protein. The culture was incubated at 220 rpm at 20°C for 16 h. The culture was centrifuged at 2,600 g for 20 min at 4°C, the supernatant was discarded, and the cell pellet was stored frozen at −80°C.

The cells were resuspended in lysis buffer containing 50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 20 mM imidazole, complete® mini EDTA-free protease inhibitor cocktail, and 0.2% Tween-20 (v/v). Forty milliliters of lysis buffer were used per liter of original culture. The cells were lysed using a French press (SLM-AMINCO, USA). The cell lysate was centrifuged at 100,000 g for 1 h at 4°C. Two milliliters of Ni-Nitrolotriacetic acid resin (per liter of original culture) were added in the supernatant, and the suspension was stirred gently for 1 h at 4°C to allow the binding of the His-tagged 3C protease on the resin. Subsequently, the Ni-NTA suspension was loaded onto an empty chromatography column and was washed with 50 mM Tris-HCl, pH 7.5 and 0.5 M NaCl containing 20 mM imidazole. Pure 3C protease was eluted by ≥50 mM imidazole-containing buffer. The eluted enzyme was dialyzed against 50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, and 10% glycerol v/v, aliquoted, and stored at −20°C.

Site-directed mutagenesis
The pET32-E43C plasmid containing a Trx tag, a 6x His-tag, and a 3C-protease cleavage site adjacent to the apoE4 gene has been described previously (39). To generate the apoE3 allele, we used site-directed mutagenesis to introduce the R112C mutation in the apoE4 gene, generating the pET32-E33C plasmid. All mutagenesis reactions were performed using the QuikChange™ site-directed mutagenesis kit according to the manufacturer’s

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instructions (Agilent; Santa Clara, CA). The primers used for the mutagenesis were 5'-GGCG GAC ATG GAG GTG TCG GGC CGC CTC GGT GAG-3' (sense) and 5'-CTG CAC CAG CCG GCC GCA CAC GTC TCT CAT GTC CGC-3' (antisense).

Using the same approach, we introduced the following mutations in the apoE3 background: R145P (primers: 5'-CCT CCG CAA GCT GCC TAA GCG CTT CCG-3' and 5'-GGG AGG AGC CGG TTA GGC AGC TTG CCG AGC-3'), R147P (primers: 5'-CCG CAG AGG CTT CAG AAG CCG CTC CTC GCC G-3' and 5'-GGG GGA GGA GCC GCT TAC GCA GCT TGC G-3'), and R158P (primers: 5'-GAT CAC GTC CAG AAG CCC CTG GCA GTG TAC CAG-3' and 5'-CTG GTA CAC TGC CAG GGG CTT CTC GTC GTC GAT TGC-3'). Successful mutagenesis was confirmed by DNA sequencing.

Expression and purification of apoE3

BL21-Gold (DE3) cells were transformed with the pET32-E33C vector and were grown on LB agar plates containing 100 μg/ml ampicillin for 16 h at 37°C. A single colony was inoculated into a 5 ml culture containing 100 μg/ml ampicillin. The culture was incubated at 37°C at 220 rpm for 2 h. The cells were harvested by centrifugation at 10,000 × g for 20 min at 4°C and the cell pellet was stored frozen at −80°C.

The cell pellet was resuspended in lysis buffer containing 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, complete mini EDTA-free protease inhibitors cocktail, 1 mM β-mercaptoethanol, and 0.1 mg/ml lysozyme (40 ml/l of original culture). The lysis of the cells was performed using a French press. The cell lysate was centrifuged at 10,000 × g for 1 h at 4°C. The apoE in the supernatant was purified using Ni-NTA chromatography as follows: the supernatant was adjusted to contain 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, and 10 mM imidazole and incubated with 4 ml (per liter of original culture) Ni-NTA resin, under gentle stirring, at 4°C overnight. The following day, the Ni-NTA suspension was loaded onto an empty chromatography column and washed. Pure apoE3 was found in the flow-through.

The apoE3 solution was extensively dialyzed against 5 mM NH4HCO3, lyophilized, and stored at −80°C. The purified proteins were at least 98% pure, as estimated by SDS-PAGE.

Preparation of apoE3 samples

Before all analyses, the lyophilized stocks of wild-type or mutant apoE3 forms were dissolved at a final concentration of 0.2 mg/ml in 6 M guanidine hydrochloride in Dulbecco's phosphate-buffered saline (DPBS) containing 1 mM DTT. The protein samples were incubated for 1 h at room temperature and then dialyzed extensively against DPBS, pH 7.4 (three changes, one of which was overnight). The samples were centrifuged at 12,000 × g for 20 min to remove any precipitated protein. The supernatant solutions were quantitated by measuring their absorbance at 280 nm (ε280 = 1.3 ml mg−1 cm−1). The proteins were kept at low concentrations (~0.1 mg/ml) on ice to avoid aggregation. All analyses were performed on freshly refolded protein.

Preparation of HDL-like discoidal particles

To prepare the HDL-like particles, we used a molar ratio of 100:10:1:100 of POPC:cholesterol:apoE3:sodium cholate. Cholesterol [56.5 μg (from a stock cholesterol solution, 2 mg/ml, in chloroform:methanol, 2:1)] and 1.11 mg of POPC (from a stock POPC solution, 20 mg/ml, in chloroform-methanol, 2:1) were mixed in a glass tube, vortexed gently, and dried under nitrogen gas flow. The dried POPC:cholesterol mixture was resuspended in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.01% EDTA w/v, using a dialysis membrane with molecular weight cut-off 12,000 Da. During the last step of the dialysis, the dialysis membrane was replaced by another with a molecular weight cut-off of 50 kDa, and the sample was dialyzed against the same buffer with three changes over 24 h.

All lipoprotein samples were prepared using the same phospholipid-cholesterol suspension, and the procedure was performed in parallel. The samples were stored at 4°C, under nitrogen to prevent the oxidation of lipids.

Circular dichroism measurements

We used a Jasco-715 spectropolarimeter to record the far-ultraviolet (far-UV) circular dichroism (CD) spectra of the apoE3 samples from 190 to 260 nm at 25°C. The instrument was connected to a Jasco PTC-348 W Peltier temperature controller that thermostated the cuvette chamber. We used a quartz cuvette with an optical path length of 1 mm. The protein samples were at 0.1 mg/ml in DPBS (pH 7.4) containing 1 mM DTT. The measurement parameters were as follows: bandwidth, 1 nm; response, 8 s; step size 0.2 nm; and scan speed 50 nm/min. Each spectrum was the average of five accumulations. The results were corrected by subtracting the buffer baseline.

Helical content was calculated using the molar ellipticity at 222 nm as described by Greenfield and Fasman (40) using the equation:

\[ \% \alpha = \{[\Theta]_{222} - 3,000\} / (36,000 + 3,000) \times 100. \]

For thermal denaturation measurements, we monitored the change in molar ellipticity at 222 nm, and the temperature was raised from 20°C to 80°C at a rate of 1°C/min. The curve was fitted to a Boltzmann sigmoidal model curve with the use of Graphpad Prism™ software. The relative enthalpy change was calculated as described previously (41).
For lipoprotein particle measurements, the protein component of the particle was at 0.1 mg/ml, and all measuring parameters were identical to that of the free protein, with the exception of the temperature range for thermal denaturation that varied from 20°C to 95°C.

Chemical denaturation experiments

To record the chemical denaturation profile of apoE3, 0.04 mg/ml of freshly refolded protein was added in a 4 ml quartz fluorimeter cuvette, and the intrinsic tryptophan protein fluorescence was measured at 340 nm after excitation at 295 nm. Small amounts of an 8.0 M guanidine hydrochloride (Applichem) solution were gradually added in the cuvette. The contents were continuously mixed using a magnetic stirrer. After each addition of guanidine hydrochloride, the sample was incubated in the dark for 2 min before measuring the fluorescence signal. The experimental data were fitted to a three-state denaturation model as described by Ekblad et al. (42) using the following equation

\[ y = \frac{T_n + A_1(T_i + T_u)}{1 + A_1(1 + A_2)} \]

where \( y \) is the fluorescence signal, \( T_n \), \( T_i \), and \( T_u \) is the fluorescence signal for the native, intermediate, and unfolded state of the protein, respectively, and \( A_1 \) and \( A_2 \) are:

\[ A_1 = \exp\left(\frac{m_n(x - x_{n50})}{RT}\right) \]
\[ A_2 = \exp\left(\frac{m_u(x - x_{u50})}{RT}\right) \]

where \( x \) is the denaturant concentration, \( R \) is the universal gas constant (0.001986 kcal K\(^{-1}\) mol\(^{-1}\)), \( T \) is the temperature of the experiment (298 K), \( x_{n50} \) and \( x_{u50} \) are the mid-transition points for the transition from the native to the intermediate state and from the intermediate to the unfolded state, respectively. Finally, \( m_n \) and \( m_u \) correspond to the slope of the mid-transition points from the native to the intermediate state and from the intermediate to the unfolded state, respectively. The relative change in Gibbs-free energy during the chemical denaturation was calculated as follows:

\[ \Delta G = m_n x_n \]
\[ \Delta G = m_u x_u \]
\[ \Delta G_{\text{total}} = \Delta G_1 + \Delta G_2 \text{ (kcal/mol)}. \]

ANS fluorescence measurements

1.8 ANS (1-anilinonaphthalene-8-sulfonic acid; Sigma-Aldrich) was dissolved into DMSO to a final concentration of 50 mM (ANS stock solution) and stored at \(-20^\circ\text{C}\). Freshly refolded wild-type and mutant apoE3 forms in 1× DPBS, pH 7.4, 0.1 mM DTT, 0.1 M guanidine hydrochloride, 0.1 mM EDTA. Reaction kinetics were followed by the change in absorbance at 325 nm using a Perkin-Elmer Lambda 35 UV/VIS spectrophotometer. The cuvette (1 cm path length) was thermostated at 24 ± 0.1°C using the Perkin-Elmer PCB 1500 Peltier temperature controller. The contents were mixed by repeated pipetting for 3 s every 2 min. Experimental data were fitted to a two-phase exponential decay model using Graphpad Prism™.

Dynamic light-scattering analysis

Dynamic light-scattering (DLS) experiments were performed using a Zetasizer nano series instrument (Malvern Instruments Ltd, UK) at 20°C. ApoE3 samples were at 0.1 mg/ml in DPBS, and HDL-like particle samples containing apoE3 were at 0.1 mg/ml in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.01% EDTA (w/v).

Protease sensitivity assay

To assess the sensitivity of lipid-free wild-type and mutant apoE3 forms to protease digestion, 0.08 mg/ml freshly refolded apoE3 or HDL-like particles were mixed with trypsin, chymotrypsin, or elastase (Applichem, Germany) at concentrations 0.01, 0.1, 1, and 10 μg/ml in DPBS and incubated at room temperature for 1 h. The reactions were stopped with the addition of 1.7 μM PMSF, and the samples were analyzed by SDS-PAGE.

Electron microscopy

Lipoproteins were contrasted using the drop method of negative staining (45). A 4 μl aliquot of each preparation was applied to a freshly glow-discharged (46), Formvar carbon-coated 300 mesh copper grid. After a 10 s incubation and 4 s filter paper blot, a 4 μl droplet of 1% sodium phosphotungstate (1% NaPT), pH 7.4, stain was applied for 10 s. The grid was blotted and air-dried. Images were collected under low-dose conditions at a magnification of ×56,250 on a Philips CM-12 electron microscope (Philips Electron Optics; Eindhoven, The Netherlands) with a Teitz 1 K × 1 K CCD camera (TVIPS; Gauting, Germany). For analysis of the disc diameter distribution, representative images from each sample were analyzed by measuring the diameter of 100 clearly formed discs using Adobe Photoshope™ software. The data were plotted using Graphpad prism™’s frequency distribution function.

PLA₂-induced aggregation of HDL-like discoidal particles

To induce aggregation of HDL-like particles containing wild-type or mutant apoE3, 0.1 mg/ml apoE3 in HDL-like particles was mixed with 150 ng/ml (1.956 U/mg) bee venom phospholipase A₂ (PLA₂; Sigma-Aldrich) and 1 mg/ml BSA in 5 mM Hepes buffer at pH 7.4 containing 140 mM NaCl, 2 mM CaCl₂, and 5 mM MgCl₂ (47). The samples were incubated at 37°C and analyzed by DLS as described above.

RESULTS

Protein expression and purification

All three apoE3 mutations are located on helix 4 of the N-terminal moiety of the protein (Fig. 1A). Recombinant wild-type and mutant apoE3 forms were expressed using a recently developed bacterial expression system (39). All proteins were purified by Ni-NTA chromatography as a fusion protein with thioredoxin. The fusion construct was...
The presence of the proline substitutions in the apoE3 molecule greatly destabilizes the protein. It should be noted that the thermal denaturation profiles indicate that a significant portion of the R145P, R147P, and R158P mutants is unfolded at physiological temperatures (37°C, Fig. 3A–C, gray line).

To further investigate the changes in thermodynamic stability of apoE3, we recorded the chemical denaturation profile of each variant and compared it to the wild-type protein (Fig. 4). apoE has been shown to undergo a two-stage unfolding transition when treated with the chaotrope guanidine hydrochloride, with an unfolding intermediate appearing between 1–2 M (32). Modeling of the unfolding profile to a three-state denaturation model can be used to calculate apparent values for the change in Gibbs energy during unfolding (35, 42). The R145P mutant showed an unfolding profile similar to that of wild-type apoE3, including the intermediate stage (Fig. 4A). However, the second unfolding transition was less cooperative and had a lower apparent ΔG of 5.63 kcal/mol versus 10.1 kcal/mol for the wild-type. The R147P variant had an even less-cooperative transition, with very weak formation of an unfolding intermediate (Fig. 4B). Finally, the R158P variant had a highly uncooperative transition, with no evidence for an unfolding intermediate (Fig. 4C). The second transition during the chemical denaturation of apoE has subsequently cleaved by 3C protease, and the released apoE3 was isolated using a second Ni-NTA purification step. All proteins were ~98% pure as judged by SDS-PAGE analysis (Fig. 1B). Wild-type apoE produced in this expression system has been shown to have biophysical properties identical to apoE produced by mammalian expression systems (39). Before each analysis presented here, all variants were subjected in parallel to a refolding step as previously described (32, 35).

Secondary structure

Because of the location of the mutations within the helical segment of apoE3, we hypothesized that they might interfere with the local secondary structure of the protein. To test this, we recorded the CD spectra of wild-type apoE3 and variants and calculated the helical content of the protein (32, 41). All three variants maintained the overall spectrum shape characteristic of apoE, although small deviations for R158P were evident (Fig. 2A). The intensity of the molar ellipticity was reduced in all cases, although not by the same degree. Calculation of the helical content of the variants indicated a significant loss of helicity for all three variants that was especially pronounced for R145P (~10% loss) (Fig. 2B). Such reduction of helical content cannot be attributed solely to local perturbation of the helical structure, due to the presence of the proline residue, and suggests more-extended, long-range perturbations in the folding of the protein.

Thermodynamic stability

The perturbations in the secondary structure of apoE3 prompted us to test the thermodynamic stability of the protein. apoE is a thermodynamically labile protein that displays limited cooperativity during unfolding, properties that have been associated with its ability to undergo significant conformational changes during its physiological function (26–28). We compared the thermal denaturation profile of each variant to the wild-type apoE3 while following the CD signal at 222 nm (Fig. 3A–C). In all cases, the variants unfolded at a significantly lower temperature and displayed very little cooperativity of unfolding compared with the wild-type, indicating significant thermodynamic destabilization. Although the thermal denaturation transition of apoE is not an ideal model for a two-state transition, it is still possible to calculate the apparent change in enthalpy, as previously described (32, 41). All three variants showed a marked decrease in thermal stability, losing 8–15 kcal/mol in enthalpy change during denaturation (Fig. 3D).
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...mutations, we used the hydrophobic reporter probe ANS. ANS changes its fluorescence properties when interacting with hydrophobic sites on proteins and has been used extensively for the characterization of hydrophobic area exposure on apolipoproteins (32, 41, 49). We recorded the fluorescent spectra of ANS in the presence or absence of wild-type apoE3 and variants (Fig. 5A). All variants resulted in significantly higher ANS fluorescence and a shift in fluorescence maximum toward lower wavelength, compared with wild-type (Fig. 5B), indicating a higher degree of hydrophobic area exposure in the mutated proteins. Enhancement of the signal by at least 50% was evident for variants R147P and R158P, suggesting a highly extensive exposure of hydrophobic core residues to the solvent that is consistent with partial unfolding of the N-terminal 4-helix bundle of apoE3.

**Aggregation state**

ApoE is known to be prone to self-association and to form oligomeric structures at higher concentrations (30, 50, 51). To examine whether the introduction of the mutations interferes with the hydrophobic profile of apoE3, we used the hydrophobic reporter probe ANS. ANS changes its fluorescence properties when interacting with hydrophobic sites on proteins and has been used extensively for the characterization of hydrophobic area exposure on apolipoproteins (32, 41, 49). We recorded the fluorescent spectra of ANS in the presence or absence of wild-type apoE3 and variants (Fig. 5A). All variants resulted in significantly higher ANS fluorescence and a shift in fluorescence maximum toward lower wavelength, compared with wild-type (Fig. 5B), indicating a higher degree of hydrophobic area exposure in the mutated proteins. Enhancement of the signal by at least 50% was evident for variants R147P and R158P, suggesting a highly extensive exposure of hydrophobic core residues to the solvent that is consistent with partial unfolding of the N-terminal 4-helix bundle of apoE3.
protease used. In all cases, the protease-resistant apoE3 species were absent from the R145P, R147P, and R158P digestion reactions, and the presence of novel digestion products (more evident after digestion with chymotrypsin) suggests the presence of novel protease-sensitive sites within the molecule, consistent with structural rearrangements due to the mutations. Because the proline residue does not correspond to a preferred specificity residue for any of the proteases tested, we conclude that the changes in protease sensitivity indicate local unfolding that exposes new cleavage sites to the solvent. Overall, the introduction of the mutations resulted in increased protease sensitivity for lipid-free apoE3, a property that may be physiologically relevant in sites of inflammation where proteolytic activity is increased.

**Protease sensitivity**

The structural and thermodynamic destabilization of apoE3 due to the mutations may affect its sensitivity to proteolysis. This may be relevant to sites of inflammation, where matrix proteases facilitate immune system cell migration (52, 53). ApoE4 has been demonstrated to be more sensitive to proteolysis both in vitro and in vivo, and such phenomena have been proposed to constitute important steps for disease initiation or progression (54). To examine the proteolytic sensitivity of wild-type apoE3 and variants, we incubated freshly refolded apoE3 with increasing concentrations of elastase, trypsin, and chymotrypsin and analyzed the samples on SDS-PAGE (Fig. 7). These three model proteases encompass the most-common protease specificity profiles, cleaving after hydrophobic, positively charged, and aromatic residues, respectively. Wild-type apoE3 was degraded by the highest concentrations of protease used but accumulated some protease-resistant species (marked with squares in Fig. 7). Proteolytic fragments of apoE corresponding to the more-robust N-terminal four-helix bundle of the protein (29) fall within the molecular weight range of some of the fragments observed here. All three variants were found to be overall more sensitive to proteolytic digestion, regardless of the protease used. In all cases, the protease-resistant apoE3 species were absent from the R145P, R147P, and R158P digestion reactions, and the presence of novel digestion products (more evident after digestion with chymotrypsin) suggests the presence of novel protease-sensitive sites within the molecule, consistent with structural rearrangements due to the mutations. Because the proline residue does not correspond to a preferred specificity residue for any of the proteases tested, we conclude that the changes in protease sensitivity indicate local unfolding that exposes new cleavage sites to the solvent. Overall, the introduction of the mutations resulted in increased protease sensitivity for lipid-free apoE3, a property that may be physiologically relevant in sites of inflammation where proteolytic activity is increased.

**DMPC remodeling kinetics**

One of the primary functions of apoE3 is to interact with lipids and form lipoprotein particles (27, 34, 55–58). The kinetics of these interactions are slow due to extensive conformational changes that the protein has to undergo (43). Remodeling of DMPC vesicles by apoE3 can be followed spectrophotometrically to allow kinetic analysis. We utilized this approach to investigate whether the presence of the R145P, R147P, or R158P digestion reactions, and the presence of novel digestion products (more evident after digestion with chymotrypsin) suggests the presence of novel protease-sensitive sites within the molecule, consistent with structural rearrangements due to the mutations. Because the proline residue does not correspond to a preferred specificity residue for any of the proteases tested, we conclude that the changes in protease sensitivity indicate local unfolding that exposes new cleavage sites to the solvent. Overall, the introduction of the mutations resulted in increased protease sensitivity for lipid-free apoE3, a property that may be physiologically relevant in sites of inflammation where proteolytic activity is increased.
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2.5 nm (see supplementary Fig. I). HDL-like particles containing apoE3 R158P had a distribution of diameters similar to that of the wild-type protein. However, HDL-like particles containing apoE3R145P or apoE3R147P showed slightly higher average diameters of 13.4 and 13.9 nm, respectively, indicating possible structural alterations (see supplementary Fig. I).

To better understand the differences in particle size between lipoproteins formed by different apoE3 variants, we analyzed the particles by electron microscopy (see supplementary Fig. II). Electron micrographs of particles formed by wild-type apoE3 and apoE3 variants showed rouleaux, which are indicative of discoidal particles that tend to stack on edge during the negative staining procedure, simplifying the measurement of their diameter (49). We measured the diameter of 100 particles from each preparation and limited by the unfolding of the N-terminal domain of apoE3 (35, 43, 44). We conclude that introduction of the mutations greatly affects the unfolding kinetics of the N-terminal domain of apoE3 (Fig. 8B).

Analysis of reconstituted HDL-like discoidal particles

The structural, thermodynamic, and lipid remodeling differences between wild-type apoE3 and the three variants prompted us to investigate whether the presence of each mutation affects the formation, size, or morphology of reconstituted apoE3-containing lipoprotein particles. To this end, we prepared reconstituted HDL-like particles containing either wild-type apoE3 or variants. DLS analysis indicated that the hydrodynamic diameter distribution of such lipoprotein particles made with wild-type apoE3 had an average diameter of 11.9 nm and distribution width of 2.5 nm (see supplementary Fig. I). HDL-like particles containing apoE3 R158P had a distribution of diameters similar to that of the wild-type protein. However, HDL-like particles containing apoE3R145P or apoE3R147P showed slightly higher average diameters of 13.4 and 13.9 nm, respectively, indicating possible structural alterations (see supplementary Fig. I).

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Fig. 7. Protease digestion sensitivity experiments. Wild-type and mutant apoE3 forms were incubated for 1 h at room temperature with increasing amounts of elastase (top), trypsin (middle), and chymotrypsin (bottom). Reactions were stopped by the addition of PMSF and analyzed on SDS-PAGE. Arrows indicate the bands that correspond to the protease (only visible for the highest concentration used). Squares highlight apoE3 fragments that accumulate in wild-type apoE3 but are fully degraded in the mutants.
plotted the frequency distribution of their diameters (Fig. 9). The median of each distribution closely matches the average hydrodynamic diameters measured by DLS. However, closer inspection of the electron microscopy micrographs reveals that although most of the particles that contain wild-type apoE3 or one of the variants are similar, several larger-diameter outliers exist that influence the calculated average diameter (Fig. 9, inset EM pictures). More specifically, apoE3 R145P and R147P tend to form a subset of larger-diameter discs (up to twice the normal diameter) that may be the result of the fusion of normal-sized discs (see Fig. 9, insets B and C in comparison to inset A). In contrast, R158P does not form such larger discs but has several indistinct clusters of poorly formed particles (Fig. 9D). Some small aggregates/indistinct clusters were also visible for HDL-like particles made with R147P. Overall, DLS and EM analyses suggest that all three apoE3 variants have the tendency to form some larger-sized malformed lipoprotein particles with structural defects. This property may be related to the aggregation-prone properties of the lipid-free apoE3 variants.

We subsequently investigated whether the structural and thermodynamic changes induced on lipid-free apoE3 due to the presence of the mutations persist on HDL-like discoidal particles made with the same mutants. CD spectroscopic analysis revealed that the variants have significantly lower helical content even when in lipoprotein form (Fig. 10A, B). Thermal denaturation measurements revealed that all three variants underwent unfolding transitions at lower temperatures, with the mid-point of the transition being at 8–9°C lower than that for wild-type apoE3 (Fig. 10C). Additionally, loss of helical content was obvious at lower temperatures than in wild-type apoE3. Finally, trypsin sensitivity experiments using these particles indicated some enhanced proteolytic sensitivity of apoE3 variants on those particles, as evidenced by the disappearance of proteolytically resistant fragments (see supplementary Fig. III). These results, taken together, suggest that the structural and thermodynamic destabilization induced in apoE3 due to the introduction of the R145P, R147P, and R158P mutations also applies in HDL-like discoidal particles containing these apoE3 variants.

DISCUSSION

Mutations cause N-terminus misfolding

The causative role of apoE Sendai (R145P), Chicago (R147P), and Osaka or Kurashiki (R158P) mutations (20,
Destabilization of ApoE3 by LPG-associated mutations

...support that the apoE3 variants adopt a molten-globule-like structure that perturbs normal functionality and leads to aggregation. This molten-globule structure may be similar to the apoE4-folding intermediate previously described, in which the N-terminal domain is partially opened and exposes its hydrophobic core (26). Such pronounced effects come in sharp contrast to the relatively minor perturbations found for other disease-linked mutations analyzed previously that are also located within the same structural domain (35). ApoE3 variants R136S, R145C, and K146E that have been linked with the development of type III hyperlipoproteinemia, have much milder repercussions on apoE3 structure and stability, indicating that although any mutation in this region of apoE3 may be detrimental to its structural and thermodynamic integrity, the strong effects described in this study may be the result of the exact amino acid substitution, more specifically of the introduction of the “helix-breaker” proline residue in a highly helical segment. Interestingly, the misfolding of the N-terminal domain actually enhances the kinetics of...

Fig. 10. Biophysical characterization of HDL-like discoidal particles containing wild-type or mutant apoE3 forms. A: Far-UV CD spectra (averages of three experiments). B: Percent helical content calculated based on the molar ellipticity at 222 nm as described in the Materials and Methods (Error bars indicate SD calculated from three separate experiments, *P = 0.007, **P = 0.0002, ***P < 0.0001). C: Thermal denaturation profiles normalized to correspond to the fraction of protein in the unfolded state. Black triangles correspond to HDL-like particles containing wild-type apoE3, empty circles to particles containing apoE3 R158P, dark gray circles to particles containing apoE3 R145P, and black circles to particles containing apoE3 R147P. D: Volume-normalized particle distribution profiles of HDL-like discoidal particles containing wild-type or mutant apoE3 forms, calculated by DLS measurements. ApoE3 was incubated at 37°C for 24 h in the presence of 100 ng/ml PLA2 as described in the Materials and Methods.
interactions of apoE3 with phospholipid vesicles, a finding consistent with the necessity of N-terminal domain unfolding during the process of lipoprotein particle formation. Such a change may actually be detrimental for apoE function, inasmuch as it reduces only a single kinetic component of the complex molecular assembly of a lipoprotein particle and may lead to malformed particles possibly similar to those shown in Fig. 9.

Mutations cause aggregation of apoE3 and structurally defective lipoprotein particles

Lipid-free apoE is prone to oligomerization and forms poorly characterized dimeric, tetrameric, or octameric structures (30, 50, 51). Conformational changes within the N-terminal domain (where the R145P, R147P, and R158P mutations reside) and between the N-terminal and C-terminal domains have been proposed to be part of the monomer-to-oligomer transition (50, 51). Furthermore, all three mutated positions studied here participate in specific intra- and inter-domain interactions, as seen in the recently solved structure of a monomeric variant of apoE3 (28). Accordingly, the misfolding of the N-terminal domain induced by the LPG-associated mutations had significant effects on the oligomerization state of apoE3. Although freshly refolded apoE3 variants presented hydrodynamic radii in solution that correspond to low-level oligomers similar to the wild-type protein, all three variants rapidly transitioned to higher aggregation levels upon incubation at 37°C. These phenomena are likely to be related to the significant population component of apoE3 variants that exists in an unfolded state at 37°C (Fig. 3, gray lines). Furthermore, such aggregation tendencies may contribute to the formation of abnormal lipoprotein particles and directly cause apolipoprotein or lipoprotein aggregates in the glomerular capillaries. The larger or damaged HDL-like particles formed by the apoE variants may be the result either of such processes or of direct fusion of normal discoidal particles due to the structural instability induced by mutation. Lipoprotein deposition in the glomerular capillaries may contribute to local inflammation and to the secretion of matrix remodeling proteases that can lead to apoE variant proteolysis and further protein aggregation and precipitation, exacerbating the pathophysiological process.

Biophysical analysis of reconstituted HDL-like particles containing these apoE variants suggests that the structural and thermodynamic destabilization observed in the lipid-free protein applies to a significant degree to lipoprotein particles that contain these variants. Because the mutated apoE3 may be delivered to the glomerulus in lipoprotein form, the structural and thermodynamic destabilization of lipoprotein particles carrying the mutant apoE3 may contribute to aggregation and deposition in the kidney. The highly enhanced aggregation of HDL-like particles by secretory PLA2 is indicative of their structural destabilization but may also be a component of LPG pathogenesis. Indeed, secretory PLA2 has been found in normal and infarcted kidneys (59), as well as in the arterial intima, and is implicated in atherogenesis and inflammation (60, 61), and it is therefore possible that PLA2 may be relevant to lipoprotein aggregation and deposition in the kidney.

ApoE3 R158P variant and apoE2

Of the three mutations studied here, the R158P variant warrants special attention because it resides at the amino acid location that differentiates apoE3 from apoE2. ApoE2 is a common apoE polymorphism carrying a cysteine residue at this amino acid position (7). Although apoE2 is a common allele in the human population, it is a major risk factor for type III hyperlipoproteinemia (15, 62). Structural differences between apoE3 and apoE2 have been demonstrated and suggested to revolve around changes in intramolecular bonds and, more specifically, disruptions in salt bridges within the N-terminal domain (28, 33, 51). The fact that both mutations at the same location can be pathogenic in humans highlights the importance of this position for apoE function.

A causal link between apoE3 variants and LPG

In summary, we have analyzed the effects of three apoE3 mutations that have been linked with the pathogenesis of LPG on the structural and thermodynamic integrity of the protein. We discovered that the mutations induce significant folding defects that extend to the whole N-terminal domain of the protein and lead to an overall molten-globule-like structure. These changes lead to both lipid association defects and enhanced protein and lipoprotein aggregation, leading to the generation of destabilized and defective lipoprotein particles. Our findings provide novel mechanistic insight for the role of these mutants in the pathogenesis of LPG and suggest that N-terminal domain unfolding induced by mutation may constitute an important mechanism underlying the pathogenic potential of apoE3.

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