There are three major apolipoprotein E (apoE) isoforms. Although APOE-ε3 is considered a longevity gene, APOE-ε4 is a dual risk factor to atherosclerosis and Alzheimer disease. We have expressed full-length and N- and C-terminal truncated apoE3 and apoE4 tailored to eliminate helix and domain interactions to unveil structural and functional disturbances. The N-terminal truncated apoE4-(72–299) and C-terminal truncated apoE4-(1–231) showed more complicated or aggregated species than those of the corresponding apoE3 counterparts. This isoformic structural variation did not exist in the presence of dihexanoylphosphatidylcholine. The C-terminal truncated apoE-(1–191) and apoE-(1–231) proteins greatly lost lipid binding ability as illustrated by the dimyristoylphosphatidylcholine turbidity clearance. The low density lipoprotein (LDL) receptor binding ability, determined by a competition binding assay of 3H-LDL to the LDL receptor of HepG2 cells, showed that apoE4 proteins with N-terminal (apoE4-(72–299)), C-terminal (apoE4-(1–231)), or complete C-terminal truncation (apoE4-(1–191)) maintained greater receptor binding abilities than their apoE3 counterparts. The cholesterol-lowering abilities of apoE3-(72–299) and apoE3-(1–231) in apoE-deficient mice were decreased significantly. The structural preference of apoE4 to remain functional in solution may explain the enhanced opportunity of apoE4 isomorph to display its pathophysiological functions in atherosclerosis and Alzheimer disease.

Human apolipoprotein E (apoE) is a 299-amino acid protein with a molecular mass of 34 kDa. ApoE is encoded by the three alleles (APOE-ε2, APOE-ε3, and APOE-ε4) of a gene on chromosome 19q13.2 determining the three major isoforms, namely apoE2, apoE3, and apoE4 in six phenotypes (1). The three apoE isoforms differ from each other only by a single amino acid substitution involving cysteine-arginine replacement at residues 112 and 158, i.e. apoE2 (Cys112/Cys158), apoE3 (Cys112/Arg158), and apoE4 (Arg112/Arg158) (Fig. 1A). Genetically, the APOE-ε4 allele is associated with both familial late-onset and sporadic Alzheimer disease (AD) and atherosclerosis (2–4). AD patients carrying the APOE-ε4 allele have more profound deposition of β-amyloid peptides (Aβ) in their brains than those carrying APOE-ε2 and APOE-ε3 alleles (5, 6). Considerable evidence supports the view that apoE4 increases the risk of AD by accelerating the plaque formation and by impairing the neurons. ApoE4 appears to modulate amyloid precursor protein processing and Aβ production through both the LDL receptor-related protein pathway and domain interaction (7). Strong correlation of APOE-ε4 allele with dyslipidemia and atherosclerosis, the major underlying mechanism of coronary heart disease, has been demonstrated (8). Human apoE4 represents a dual risk factor for these two major degenerative diseases.

ApoE contains two independently folded domains (N-terminal domain, residues 20–165, and C-terminal domain, residues 225–299) that are separated by a large nonstructural segment (9) (Fig. 1A). The four helices in the N-terminal domain are amphipathic (10). The three-dimensional structures of the N-terminal domain of apoE isoforms have been determined by x-ray crystallography (11–13), which showed that this domain is arranged as an anti-parallel, elongated four-helix bundle. The C-terminal domain, which is predicted to contain three helices, is less well known. The first two helices, comprising residues 203–223 and residues 225–266, are class A type, and the third helix, consisting of residues 268 to 289, is a G* helix (9). The G* helix and part of the end of the second helix may play a key role in lipid binding and lipid interaction in apoE-containing lipoproteins (14). In the lipid-free state, full-length apoE exists as a stable tetramer, and the C-terminal domain is responsible for tetramerization (15). ApoE4 is one of the major proteins associated with Aβ plaques (16, 17). The role of apoE in the molecular pathogenesis of AD might be related to its isoform-specific interactions with lipids or Aβ aggregates.

The N terminus of apoE, containing a region spanning from residues 140 to 150 (His-Leu-Arg-Lys-Leu-Arg-Lys-Arg-Leu-Arg), is rich in basic amino acids and is involved in apoE/B receptor binding (18–20). Most of the basic amino acids in the vicinity of residues 136–158 are not involved in salt bridges and are on the solvent-accessible surface (13). ApoE in VLDL promotes the catabolism of VLDL to LDL (8). ApoE-deficient (apoE(−/−)) mice exhibit elevated plasma cholesterol because of impaired VLDL metabolism and develop atherosclerosis spontaneously (21).

To correlate the structural variation between apoE4 and apoE3 isoforms and their functional disturbances, we have expressed full-length, N-terminal, and C-terminal truncated apoE proteins tailored to interrupt the potential domain or segment interactions (22). Using analytical ultracentrifugation coupled with continuous size distribution analysis, we have demonstrated previously that apoE3-(72–299) consists of only one major species (with a sedimentation coefficient of 5.1). ApoE4-(72–299) displays a wider and more complicated species distribution. The two major species of monomer and tetramer distribution are maintained in the case of apoE3-(1–231) and apoE4-(1–231). In this study,
Human Apolipoprotein E3 and E4

we further elucidate the de-aggregation of different apoE3 and apoE4 protein in the presence of dihexanoylphosphatidylcholine (DHPC). DHPC has the chemical structure of phospholipids and exists in solution as monomers in equilibrium with micelles comprising ~40 molecules (the critical micelle concentration is 12.7–14.3 mM) (23, 24). Besides sedimentation velocity, sedimentation equilibrium experiments were also used to calculate the equivalent molar mass of different mass of apoE3 and apoE4 proteins in PBS and in the presence of lipid. Together, global analysis of both sedimentation velocity and sedimentation equilibrium data gave very reliable results on the aggregational states of apoE proteins in different solvent systems.

Furthermore, we report the functional studies of these isoform-specific, domain-truncated apoE proteins, including the interaction with lipid vesicles, binding with apoB/E receptor in HepG2 cells, and the cholesterol-lowering effects in apoE(−) mice. Our results provide novel evidence to correlate the structure variation of apoE3 and apoE4 to their biochemical functions and possible pathophysiologic consequences in atherosclerosis and AD.

EXPERIMENTAL PROCEDURES

Plasmids—The pET-29a(+) (Novagen) vectors with a C-terminal His tag sequence (Ser^2–His^6) were used. The construction of pET-apoE3, pET-apoE4(31–299), pET-apoE4(72–299), pET-apoE3(1–191), pET-apoE3(1–231), pET-apoE3(1–271), and those of apoE4 variants were amplified by PCR and the forward primer was 5′-AACTCATATGGCCTACAAATCGGA, whereas the reverse primer was 5′-AATCTGAGGGGCCCCTGCCT. The NdeI-Xhol-digested apoE3–(72–166) DNA was then ligated to the 5.2-kb NdeI-Xhol fragment from pET-29a(+). In turn, this resulted in a 5.5-kb pET-apoE3–(72–166) vector. Site-directed mutagenesis (25) was used to construct pET-apoE4(72–166). The forward primer was 5′-GAGGAGCTGCAGGGTCCGCTTG and the reverse primer was 5′-AGGCGGCCGCGACGTATCT. The pET-apoE3–(72–166) vectors were used as templates, and the primers were used to mutate the Cys112 codon for apoE4 by PCR. Their nucleotide sequences were checked by autosequencing analysis.

Purification of the Full-length, N-terminal, and C-terminal Truncated ApoE Proteins—The procedure of protein induction and purification was described previously (22). The BL21-CodonPlus(DE3)-RIPL Escherichia coli cells (Stratagene) were used for the protein expression of apoE3 and apoE4 proteins. The others were expressed by the strain of BL21 (DE3) (Invitrogen). Typical yields of the apoE3 and apoE4 proteins were 5 mg and those of apoE3 and apoE4 N- or C-terminal truncated proteins were 5–20 mg after purification from 1 liter of E. coli culture medium. The purified proteins were buffer-changed by Amicon Ultra-4 centrifugal filter devices (Millipore) with the molecular mass cutoff at 10 kDa. After repeating the concentration-dilution procedure five times, the elution buffer had been fully replaced by PBS (pH 7.3).

Sedimentation Velocity—Sedimentation velocity (SV) experiment was performed using a Beckman model XL-A analytical ultracentrifuge (Fullerton, CA). Briefly, samples (380 μl) and reference (400 μl) solutions were loaded into 12-mm double-sector Epon charcoal-filled centerpieces and mounted in an An-50 Ti rotor. Experiments were performed at 20 °C with a rotor speed of 42,000 rpm. Absorbance of the sample at 280 nm was monitored in a continuous mode time interval of 360–480 s and a step size of 0.003 cm. Multiple scans at different time points were fitted to a continuous size distribution model by using the SE DIVIT (26, 27) program as described previously (22). All size distributions were solved and regularized on a confidence level of p = 0.68 (Fig. 1, B–D) or 0.95 (Fig. 2) by maximum entropy, the best fitted average anhydrous fractional ratio (f/f_o) and a resolution N of 200 sedimentation coefficients between 0.1 and 20.0 S.

Sedimentation Equilibrium—Experiments were performed with six-channel Epon charcoal-filled centerpieces. Three different samples (0.10–0.12 ml) were loaded into the sample channels, and 0.11–0.13-ml buffers only were loaded into the reference channels. The cells were then loaded into the rotor and run at speed of 6,000, 10,000, 15,000, and 20,000 rpm each for 14–18 h at 20 °C. Ten A_280 nm scans with time interval of 8–10 min were measured for every different rotor speed to check the status of sedimentation equilibrium (SE). In our studies, all apoE proteins can achieve equilibrium state after 14 h. The scans at different rotor speeds (multispeed equilibrium data) were then globally fitted to a noninteracting discrete species model assuming a single species by using SEDPHAT (28) with Equation 1,

$$A_R = c_r e^{d e x p \left( \frac{M(1 - \nu \rho)}{2RT} \left( r^2 - r_0^2 \right) \right)} \quad \text{(Eq. 1)}$$

in which r denotes the distance from center of rotation; r_0 is an arbitrary reference radius; $\omega$ is the angular velocity; T is the absolute temperature of the rotor; R is the gas constant; $\nu$ is the partial specific volume; $\rho$ is the solvent density; $\epsilon$ is the extinction coefficient; d is the optical path length, and $c_r$ is the concentration at the reference radius. At each channel, a single base-line parameter was included as a floating parameter common to all rotor speeds. The time invariant and radial invariant noise were also fitted for the better fitting quality.

Global analyses of combined sedimentation equilibrium and sedimentation velocity data were conducted with SEDPHAT using hybrid local continuous distribution and global discrete species model (29) with Equation 2,

$$a(r,t) = \sum_i c(i) c_i(s_i, M_i, r,t) + \sum_j \int_{s_{min,j}}^{s_{max,j}} c(j)(s) c_i(s, (f/f_0)_{M_i} r,t)ds \quad \text{(Eq. 2)}$$

in which c(j)(s) denoting the population with sedimentation coefficients in different nonoverlapping intervals $I_j = [s_{min,j}, s_{max,j}]$ that can be characterized by separate frictional ratios, in combination with discrete species at loading concentration c(j) (in signal units) with s values s_{min,j} outside the intervals $I_j$. The discrete species are described by Lamm equation solutions, each with the two parameters s and M, and are not connected to the determination of (f/f_0)_{M_i} for the continuous segments. In our studies, the resolution of the local c(s) was set to zero to switch it off. Only multiple discrete species analysis was used.

DMPC Turbidity Clearance Assay—Dimyristoylphosphatidylcholine (DMPC), purchased from Sigma, was dissolved in a 2:1 (v/v) chloroform/methanol solution. The solvent was evaporated overnight under a nitrogen stream to form a thin film on the walls of a 13 × 100-mm glass tube. The clearance buffer (PBS with 3.5% KBr and 0.1 mM EDTA) was then added into the tube and vortexed vigorously for 2 min. Dilution to 0.5 mg/ml resulted in a turbid suspension of multilamellar vesicles (mLV) with an absorbance of ~0.9 at 325 nm. The solubilization of DMPC mLV by apoE proteins was determined according to modified procedures (30–32). Briefly, apoE (250 μg) was added to DMPC mLV solution (0.5 mg/ml) in a quartz cuvette, which was preincubated at 24 °C in a spectrophotometer (PerkinElmer Life Sciences, Lambda 35).
with water-circulated temperature control. The contents were mixed within 10 s by repeated pipetting. The vesicle solubilization was monitored as a decrease in absorbance at 325 nm. Experiments were repeated at least twice, and similar results were obtained. According to Segall et al. (32), the time courses for clearance were fitted by nonlinear regression to the monoeponential Equation 3,

\[ Y = A \cdot e^{-k_1 \cdot t} + C \]  

(Eq. 3)

or the biexponential Equation 4,

\[ Y = A \cdot e^{-k_1 \cdot t} + B \cdot e^{-k_2 \cdot t} + C \]  

(Eq. 4)

where \( Y \) is the absorbance at 325 nm, and \( k_1 \), \( k_2 \), or \( C \) are the rate constants for different kinetic phases of the solution clearance. \( A \) and \( B \) are the changes in turbidity for different phases (pool sizes); \( t \) is time, and \( C \) is the remaining turbidity at the completion of the reaction. Slight differences in the initial absorbance for each time course are corrected by normalizing these values to 1 and treating all others as the fractions of this initial turbidity.

Preparation of \(^{3}H\)-LDL—Unlabeled cholesterol (0.1 mg, dissolved in 1.0 ml of \( n \)-hexane) was added to a clean glass vial and dried under a stream of nitrogen to form a thin cholesterol film on the surface of 2-cm-high wall in a glass vial. \(^{7}H\)-Cholesterol (125–250 \( \mu \)Ci in ethanol) (Amersham Biosciences) was added and dried under nitrogen. Human LDL (1.4 mg/ml protein) of 1–2 ml was added and incubated at 37 °C for 22 h. Finally, the \(^{3}H\)-LDL was centrifuged at 12,000 rpm for 5 min. The upper layer and trace insoluble in the bottom were discarded. The middle clear solution containing \(^{3}H\)-LDL was transferred into a clean tube and sealed under nitrogen. Radioactivity of a small portion of \(^{3}H\)-LDL (10 \( \mu l \)) was determined by a liquid scintillation counter (Beckman).

LDL Receptor Binding Assay—The procedure was modified from previous studies (33, 34). Human hepatoblastoma cells (HepG2) (1.5 × 10\(^6\)) were cultured into a 1-cm diameter well in DMEM with 10% fetal bovine serum at 37 °C with 5% CO\(_2\). After a 48-h incubation, cells of 50–60% confluence were washed three times with PBS and then incubated in the serum-free medium for 24 h at 37 °C with 5% CO\(_2\). When the assay began, the cells were cooled on ice for 30 min, washed twice with PBS, and then incubated with DMEM containing 50 \( \mu \)g/ml \(^{3}H\)-LDL, and different receptor-binding competitors (apoE) at 4 °C for 2 h. The medium was removed followed by washing three times with chilled PBS. Cells were released from the well surface by 0.2 ml of trypsin-EDTA, pipetted into a clean vial with 2 ml of 1:3 (v/v) saturated KOH/ethanol solution, and incubated at 56 °C for 2 h. Seven milliliters of \( n \)-hexane was then added into the vial for dissolving cholesterol and dried under nitrogen to form a thin film on the vial walls. Finally, every vial was added with 10 ml of Aquasol (PerkinElmer Life Sciences), and the radioactivity was determined.

In Vivo Cholesterol-lowering Effect—The ability of apoE proteins to lower plasma cholesterol in vivo was assayed by injection of proteins into apoE(−) mice (Animal Center, National Cheng Kung University, Tainan, Taiwan). Male apoE(−) mice, 6 months old, were maintained in the specific pathogen-free animal house with a 12-h light-dark cycle. The fasting plasma total cholesterol level of each apoE(−) mouse 1 day before apoE protein injection, in the range of 650–750 mg/dl (≈2.88 × 10\(^{-5}\) mol per 20-g mice, whose total blood volume is about 8% of the body weight) (35–37), was used as its own control. The cholesterol content of VLDL + LDL of an animal was estimated to be about 2.88 × 10\(^{-5}\) mol. For each VLDL particle (~2000 cholesterol included) to accommodate at least one apoE molecule, the required amounts of injected proteins were estimated to be 490 \( \mu \)g for apoE, 420 \( \mu \)g for apoE(−)(41–299), 370 \( \mu \)g for apoE(−)(72–299), and 390 \( \mu \)g for apoE(−)(1–231). Animals were randomly assigned for each group (\( n = 6–8 \)) and were injected from tail veins with 200 \( \mu l \) for all protein solution in PBS. By determining the concentration of total cholesterol before and 24 h after injection, the cholesterol-lowering effect was determined. Because the original concentration of cholesterol associated with VLDL + LDL was high and the concentration of high density lipoprotein cholesterol in apoE(−) mice remained approximately constant (about 50 mg/dl) (35), the plasma cholesterol-lowering effect was mainly contributed by apoE proteins to accelerate VLDL clearance in the plasma.

RESULTS

Self-aggregation of ApoE Proteins Determined by Size Distribution Analysis—ApoE3, apoE4, and their truncated proteins were chosen for this study. Fig. 1A shows the model structure of apoE protein with different colors representing the truncated regions. Our previous study (22) has elucidated the structural variation in apoE3 and apoE4 at the same tendency. ApoE3 and apoE4 proteins showed the similar size distribution patterns by continuous size distribution analysis (Fig. 1B). The root mean square deviation (r.m.s.d.) of the best fitting to the continuous size distribution model was 0.00578 for apoE3 and 0.00551 for apoE4. The grayscale patterns of residual bit maps showed a high quality fitting (Fig. 1C). The best fit \( f_{ref} \) was 1.21 for apoE3 and 1.28 for apoE4. After determining the integral area by SEDFIT, apoE3 could be resolved into five major species at \( s = 2.1 \) (3%), 4.9 (13%), 6.6 (35%), 8.6 (24%), and 10.8 (13%) and those of apoE4 were \( s = 1.7 \) (4%), 4.8 (19%), 6.3 (36%), 7.9 (21%), and 9.6 (9%). Their large components (\( s > 11 \)) were lower in content and broader in distribution, were calculated as a single region. ApoE3 and apoE4 had similar percentage of larger species (\( s > 11 \)), namely 12% for apoE3 and 11% for apoE4.

Compared with the simplicity in size distribution of apoE3-(72–299), apoE4-(72–299) existed in a more complicated and aggregated pattern (Fig. 1C). The r.m.s.d. of the best fitting was 0.00440 for apoE3-(72–299) and 0.00453 for apoE4-(72–299). The best fit \( f_{ref} \) was 1.56 for apoE3-(72–299) and 1.44 for apoE4-(72–299). The species of apoE3-(72–299) appeared at \( s = 1.9 \) (11%), 5.2 (66%), 6.7 (21%), and 8.9 (3%) and larger species (\( s > 10 \)) whereas those of apoE4-(72–299) appeared at \( s = 2.2 \) (4%), 5.4 (25%), 7.1 (35%), 8.9 (17%), and 10.8 (12%), and \( s > 12.0 \) (8%).

When residues 232–299 were truncated, the two major species (\( s = 2.2 \) and 5.0) were maintained for both apoE3-(1–231) and apoE4-(1–231) (Fig. 1D). However, the contents of these two species were different between the two isoforms (apoE3-(1–231), 43% for \( s = 2.2 \) and 42% for \( s = 5.0 \) versus apoE4-(1–231), 29% for \( s = 2.0 \) and 53% for \( s = 4.9 \)). Both apoE3-(1–231) and apoE4-(1–231) had a minor species at \( s = 6.9 \), whose content was 9% for apoE3-(1–231) and 11% for apoE4-(1–231). The r.m.s.d. of the best fitting was 0.00617 for apoE3-(1–231) and 0.00611 for apoE4-(1–231). The \( f_{ref} \) of the best fit was 1.58 and 1.46 for apoE3-(1–231) and apoE4-(1–231), respectively. Monte-Carlo statistical analysis (supplemental Fig. 1) demonstrated that the peaks in the size distribution were robust against noise, indicating that they were not because of reported oscillations from noise-induced artifacts in the regularization (26).

Dissociation of ApoE Proteins in the Presence of Dihexanoylphosphatidylcholine Micelles—Because apoE proteins are intimately involved in lipid metabolism, it is ultimately important to assess the state of associ-
FIGURE 1. Structure of apoE proteins and their size distributions analyzed by analytical ultracentrifugation. A, the model structure illustrating the structural regions where deletions were made (deletion of residues 1–40 is shown in red; residues 1–71, red and yellow; C-terminal domain: residues 192–299, green, blue, and orange; residues 232–299, blue and orange; and residues 272–299, orange). The polymorphic sites (residues 112 and 158) that distinguished apoE2, apoE3, and apoE4 are also highlighted. The picture was generated with MolScript (62) and Raster 3D (63). B–D, the analytical ultracentrifugation was performed at a rotor speed of 42,000 rpm and a rotor temperature of 20 °C in PBS (pH 7.3) for 3.5 h. A fixed initial concentration at 0.5 mg/ml was used for all proteins. The A_{280 nm} was scanned, and the radial collecting interval was 0.003 cm, and the time interval of scans was 480 s. Continuous sedimentation coefficient distribution of the apoE proteins was analyzed by SEDFIT at a resolution N of 200 and the maximum entropy regularization of p = 0.68. Insets, grayscale of residual bit map showing the best data fitting quality. Red line, apoE3 isoform; blue line, apoE4. B, full-length apoE; C, apoE-(72–299); D, apoE-(1–231) proteins.
of DHPC, SE experiments were performed. First, we analyzed the SE data by SEDPHAT by assuming a single species model. The SE profiles of apoE proteins in the presence of DHPC were significantly different from those in aqueous PBS (supplemental Fig. 2S). In the presence of DHPC, all kinds of apoE proteins maintained a Meq corresponding to their monomer molar mass (Table 1). This indicated that a single species model is adequate to describe the apoE proteins in DHPC-containing PBS. The apoE3-(72–299) and apoE4-(72–299) proteins exhibited a Meq of 192 and 209 kDa, respectively. The apoE3-(1–231) and apoE4-(1–231) showed a Meq of 205 and 162 kDa, respectively. The most significant difference between apoE3 and apoE4 isoform is their corresponding apoE-(72–166) proteins, which have a Meq of 187 kDa for apoE3-(72–166) and 429 kDa for apoE4-(72–166).

Because apoE proteins are heterogeneous in aqueous PBS as shown in Fig. 1, we then performed a more robust global analysis by combining the SV and SE data. Fig. 3 showed the final results of apoE3 and apoE4 in aqueous PBS analyzed with SEDPHAT. More data are presented in the Supplemental Material (see Figs. 3S–8S). Because the aggregation of apoE proteins may not be an equilibrium system between each species, we chose to fit our data to a global heterogeneous discrete species model. These results are summarized in Table 2. According to the results of $c(s)$ (Fig. 1), the data were adequately described and fitted by a three (for apoE-(1–231)), four (for apoE-(72–299) and apoE-(72–166)), and six (for apoE and apoE4-(72–299)) species discrete model separately. The best fit calculated local root mean square errors of SE were from 0.0049 to 0.0178 and those of SV were from 0.0043 to 0.0069 (Supplemental Fig. 2S). Because the aggregation of apoE proteins may not be an equilibrium system between each species, we chose to fit our data to a global heterogeneous discrete species model. These results are summarized in Table 2. According to the results of $c(s)$ (Fig. 1), the data were adequately described and fitted by a three (for apoE-(1–231)), four (for apoE-(72–299) and apoE-(72–166)), and six (for apoE and apoE4-(72–299)) species discrete model separately. The best fit calculated local root mean square errors of SE were from 0.0049 to 0.0178 and those of SV were from 0.0043 to 0.0069 (Supplemental Fig. 2S). In this study, the calculated local concentration and sedimentation coefficient ($c(s)$) of each discrete species showed a similar content to those in $c(s)$. Most major species detected in SV were also detected in SE experiments. For apoE3 and apoE4 proteins, the discrete species of molar mass corresponded to monomer, dimer, tetramer, hexamer, octamer, and decamer. apoE-(72–299) maintained a major species of tetramer (by SV) or hexamer (by SE), and apoE4-(72–299) showed a complicated distribution of tetramer (or trimer), octamer, decamer, 14-mer, and large aggregates. apoE3-(1–231) proteins maintained more monomer, but apoE4-(1–231) had more tetramer. Both apoE3-(1–231) and apoE4-(1–231) contained significant amounts of octamer. The discrete distributions of apoE3-(72–166) and apoE4-(72–166) were similar to that of continuous distribution (data

![Figure 2. Continuous size distribution of apoE proteins in the presence of DHPC. A, apoE3, apoE4 (72–299). B, apoE-(1–231). C, apoE-(72–299). The analytical ultracentrifugation was performed at a rotor speed of 42,000 rpm and a rotor temperature of 20 °C in PBS containing 50 mM DHPC for 6 h. The protein concentration was 0.5 mg/ml. The A$_{280}$ was scanned, and the radial collecting interval was 0.003 cm and time interval of scans was 420 s. The distribution of continuous sedimentation coefficients of apoE proteins was analyzed by SEDFIT at the maximum entropy regularization of $p = 0.95$. ApoE3 proteins are shown by thin solid lines and apoE4 proteins are shown by dashed lines. Insets, grayscale of residual bit map showing the best data fitting quality.](image-url)

**TABLE 1**

| Protein (0.5 mg/ml) | Theoretical mass | Mass in PBS | Mass in PBS containing 50 mM DHPC |
|--------------------|------------------|-------------|----------------------------------|
| apoE3              | $55$ kDa         | $61$ kDa    | $52$ kDa                         |
| apoE4              | $57$ kDa         | $63$ kDa    | $54$ kDa                         |
| apoE3-(72–299)     | $192$ kDa        | $206$ kDa   | $195$ kDa                        |
| apoE4-(72–299)     | $209$ kDa        | $223$ kDa   | $211$ kDa                        |
| apoE3-(1–231)      | $187$ kDa        | $199$ kDa   | $188$ kDa                        |
| apoE4-(1–231)      | $162$ kDa        | $174$ kDa   | $163$ kDa                        |

a) Best fit calculated molar mass was from sedimentation equilibrium analysis with SEDPHAT using the noninteracting discrete species model for a single species described in Equation 1. The average fitting errors were 0.17% in aqueous PBS and 0.83% in DHPC-containing PBS.
FIGURE 3. Global analysis of the full-length apoE proteins. A, C, and E: apoE3, B, D, and F: apoE4. The speed of centrifugation for equilibrium experiments (A and B) was 10,000 rpm (circles), 15,000 rpm (triangles), and 20,000 rpm (squares) at 20°C each for 14 h. The velocity experiments (C and D) were centrifuged to 42,000 rpm (circles) at 20°C for 3 h. The solid lines in A–D are the best fit distributions from global analysis of the six discrete species model by SEDPHAT according to Equation 2. The molar mass and sedimentation coefficients of the species were floated and fitted. The residuals of each fit are shown below the panels and have a local root mean square error for each channel of 0.0143 (A), 0.0063 (B), 0.0108 (C), and 0.0027 (D). The discrete species distribution of apoE3 and apoE4 from equilibrium experiments (closed circles) and velocity experiments (open circles) are shown in E and F. The local concentration units are $A_{280}$ nm signal units.

TABLE 2
Global discrete species analysis of the combined sedimentation velocity and sedimentation equilibrium data of apoE3 and apoE4 isoforms and their truncated proteins in aqueous PBS

| Protein         | s (Svedberg) | Mass | Local concentration of SE ($A_{280}$) | Local concentration of SV ($A_{280}$) | s (Svedberg) | Mass | Local concentration of SE ($A_{280}$) | Local concentration of SV ($A_{280}$) |
|-----------------|--------------|------|--------------------------------------|--------------------------------------|--------------|------|--------------------------------------|--------------------------------------|
| ApoE            | 1.8          | 36   | 0                                    | 0.01                                 | 1.5          | 35   | 0                                    | 0.03                                 |
|                 | 5.6          | 74   | 0.10                                 | 0.21                                 | 5.0          | 84   | 0                                    | 0.21                                 |
|                 | 7.5          | 144  | 0.54                                 | 0.27                                 | 6.9          | 124  | 0.56                                 | 0.35                                 |
|                 | 9.7          | 212  | 0                                    | 0.20                                 | 8.8          | 229  | 0.31                                 | 0.15                                 |
|                 | 12.9         | 275  | 0.08                                 | 0.12                                 | 11.3         | 290  | 0                                    | 0.08                                 |
|                 | 17.4         | 330  | 0.09                                 | 0.07                                 | 15.5         | 388  | 0.25                                 | 0.04                                 |
| ApoE-(72–299)   | 2.2          | 23   | 0.04                                 | 0.05                                 | 1.9          | 25   | 0                                    | 0.05                                 |
|                 | 5.3          | 94   | 0                                    | 0.22                                 | 5.9          | 86   | 0.02                                 | 0.15                                 |
|                 | 6.8          | 165  | 0.31                                 | 0.09                                 | 7.7          | 212  | 0.07                                 | 0.12                                 |
|                 | 9.2          | 351  | 0.11                                 | 0.02                                 | 9.5          | 263  | 0.13                                 | 0.08                                 |
|                 |              |      |                                      |                                      | 11.8         | 379  | 0.03                                 | 0.05                                 |
|                 |              |      |                                      |                                      | 15.1         | 666  | 0.06                                 | 0.03                                 |
| ApoE-(1–231)    | 2.8          | 24   | 0.15                                 | 0.28                                 | 2.3          | 26   | 0                                    | 0.17                                 |
|                 | 5.3          | 99   | 0.05                                 | 0.21                                 | 5.2          | 98   | 0.12                                 | 0.33                                 |
|                 | 8.1          | 206  | 0.20                                 | 0.05                                 | 7.6          | 203  | 0.23                                 | 0.07                                 |
| ApoE-(72–166)   | 1.9          | 12   | 0                                    | 0.11                                 | 1.9          | 10   | 0                                    | 0.01                                 |
|                 | 5.1          | 63   | 0.04                                 | 0.07                                 | 4.6          | 58   | 0                                    | 0.12                                 |
|                 | 6.7          | 140  | 0.01                                 | 0.01                                 | 6.7          | 174  | 0.02                                 | 0.05                                 |
|                 | 9.2          | 243  | 0.07                                 | 0.01                                 | 9.3          | 632  | 0.09                                 | 0.02                                 |
not shown) and single species analysis. ApoE4-(72–299) showed more large aggregates in aqueous PBS.

**DMPC Turbidity Clearance Assay**—The above DHPC experiment demonstrated that lipid environment has crucial effects on apoE conformation. We further examined this by the DMPC turbidity clearance assay to illustrate the effects of various extents of N- and C-terminal truncations of apoE proteins on the interaction kinetics with DMPC mLV. Fig. 4 shows the DMPC mLV turbidity clearance by different apoE3 and apoE4 fragments. The full-length apoE3 and apoE4 proteins did not show significant isoform difference in DMPC clearance (Fig. 4A). Only the full-length and the apoE-(1–191) proteins showed monophasic kinetics. Other truncated proteins were best described by biphasic kinetics (Fig. 4 and Table 3). However, by fitting to nonlinear regression, apoE3 showed a slightly higher pool size and flux than apoE4 (Table 3). The tendency remained when comparing between apoE3-(41–299) and apoE4-(41–299) proteins in the rapid phase. ApoE3-(72–299) showed the highest values in rate constant and flux in both phases, although those values of apoE4-(72–299) were also very high (Fig. 4B and Table 3). The N-terminal truncation caused the apoE proteins to gain more DMPC turbidity clearance ability, especially when the first two α-helices were truncated.

Truncation of the entire C-terminal domain (residues 192–299) totally abolished the clearance capabilities of apoE3 and apoE4 to DMPC mLV (Fig. 4C). The rate constant was very low, and the flux was only $1.1 \times 10^{-2}$ fraction/min (Table 3). The ability of DMPC mLV clearance was partially recovered if the 192–231 segment (part of C-terminal domain) was present (Fig. 4C and Table 3). Both apoE3-(1–231) and apoE4-(1–231) showed higher flux in slow phase than those in fast phase. The clearing capabilities of the 272–299 fragment-truncated proteins, namely apoE3-(1–271) and apoE4-(1–271), were better than their corresponding full-length proteins (Fig. 4C). Compared with apoE4-(1–271), apoE3-(1–271) exhibited a higher rate constant in both rapid phase and slow phase and a higher flux in rapid phase. Although apoE4-(1–271) showed a higher rate constant in rapid phase than that in slow phase, the pool size showed the reverse pattern, leading to identical contribution in the flux of these two phases.

It is interesting to note that the two-end-truncated proteins, namely apoE3-(72–166) and apoE4-(72–166), showed significantly higher DMPC clearance abilities than those of full-length proteins. In the rapid phase, the rate constant, pool size, and flux of apoE4-(72–166) were all higher than those of apoE3-(72–166) (Fig. 4A and Table 3). ApoE3-(72–166), apoE4-(72–166), apoE3-(41–299), apoE4-(41–299), apoE3-(72–299), and apoE3-(1–271) all exhibited larger pool sizes in rapid phase than those in slow phase (Table 3). Their flux also showed a similar pattern indicating that DMPC clearance by these apoE proteins was very effective. On the other hand, apoE4-(72–299) showed a larger pool size of slow phase than that of rapid phase, but the rate constants showed the reverse pattern. However, the flux of rapid phase was still larger than that of slow phase (Table 3).

**LDL Receptor Binding Assay**—Table 4 summarizes the LDL receptor (LDL-R) binding abilities of various apoE3 and apoE4 proteins. The specific binding between LDL and LDL-R was demonstrated (Fig. 5). Twenty times more of the unlabeled LDL competed with $^3$H-LDL in binding to LDL-R and decreased the binding of $^3$H-LDL to 30–40%. The DMPC mLV itself did not show significant binding competition (Fig. 5). To compare the LDL receptor binding abilities of different apoE proteins, the ability of the apoE3-DMPC complex to replace bound $^3$H-LDL to LDL-R (defined as bound $^3$H-LDL decrease rate) was set as 100%, and the abilities of other proteins were normalized by this value (Table 4). The full-length apoE proteins with DMPC showed dose-dependent competing ability, whereas a 2.5-fold dose of apoE-DMPC complex lowered 39–42% of the bound $^3$H-LDL and 20-fold dose lowered 81–83% (Fig. 5). In accordance with previous reports (18, 38), lipid-free apoE and its truncated proteins were poor LDL competitors (Table 4). A 10-fold dose of full-length apoE protein only lowered 34% of
Comparison of the relative LDL-R binding ability of apoE3 and apoE4 isoforms and their truncated proteins

### TABLE 3

| Protein              | Remaining turbidity | Rate constant (10⁻⁵ min⁻¹) | Pool size (fraction) | Flux (10⁻² fraction/min) |
|----------------------|---------------------|-----------------------------|----------------------|-------------------------|
| apoE3 (72–299)       | 0.069 ± 0.003       | 2.66 ± 0.02                 | 0.592 ± 0.002        | 1.57                    |
| apoE4 (72–299)       | 0.001 ± 0           | 9.98 ± 0.13                 | 0.624 ± 0.006        | 0.62                    |
| apoE3 (72–299)       | 0.024 ± 0           | 31.94 ± 0.28                | 0.625 ± 0.005        | 19.93                   |
| apoE4 (72–299)       | 0.029 ± 0           | 6.35 ± 0.10                 | 0.330 ± 0.008        | 2.10                    |
| apoE3 (1–231)        | 0.236 ± 0.006       | 1.39 ± 0.46                 | 0.022 ± 0.005        | 0.03                    |
| apoE4 (1–231)        | 0.149 ± 0.009       | 1.95 ± 0.33                 | 0.038 ± 0.003        | 0.07                    |
| apoE3 (72–299)       | 0.156 ± 0.001       | 3.89 ± 0.05                 | 0.433 ± 0.003        | 1.68                    |
| apoE4 (72–299)       | 0.014 ± 0.012       | 0.55 ± 0.05                 | 0.252 ± 0.053        | 0.14                    |

### TABLE 4

| Protein              | With DMPC | Without DMPC |
|----------------------|-----------|--------------|
|                      | Decrease in H-LDL binding | LDL-R binding ability | Decrease in H-LDL binding | LDL-R binding ability |
|----------------------|-----------|--------------|
|                      | %         | %            | %                       | %                       |
| apoE3 (1–219)        | 59³       | 99           | 34                      | 100                     |
| apoE4 (1–219)        | 58³       | 99           | 34                      | 100                     |
| apoE3 (1–299)        | 46        | 78           | 15                      | 44                      |
| apoE4 (1–299)        | 47        | 80           | 20                      | 60                      |
| apoE3 (72–299)       | 27        | 46           | -8                      | ND³                     |
| apoE4 (72–299)       | 34        | 57           | 38                      | 112                     |
| apoE3 (1–191)        | 42        | 72           | 13                      | 39                      |
| apoE4 (1–191)        | 34        | 57           | 19                      | 57                      |
| apoE3 (1–231)        | 18        | 31           | 23                      | 68                      |
| apoE4 (1–231)        | 44        | 75           | ND                      | ND                      |
| apoE3 (1–271)        | 47        | 80           | 27                      | 81                      |
| apoE4 (1–271)        | 58        | 99           | 29                      | 86                      |
| apoE3 (72–166)       | 26        | 44           | 14                      | 41                      |
| apoE4 (72–166)       | 55        | 94           | 22                      | 65                      |

À Each apoE protein (0.5 mg) treated with or without 0.5 mg of DMPC and the LDL-R binding ability were determined by competitive binding assay in cultured HepG2 cells.

§ Slight differences in bound H-LDL decrease rate for each experiment were corrected by normalizing the LDL and LDL-R binding ability values to unity and treating all other experimental values toward this standardized value. The average S.E. was 14.3% with DMPC and 17.5% without DMPC.

* The decrease in H-LDL binding of apoE3 and apoE4 protein in 0.5 mg were calculated by the interpolation of those in 0.125 and 1.0 mg.

* ND indicates not determined.

When the C-terminal domain was fully removed, apoE3-(1–191)- and apoE4-(1–191)-DMPC complexes still maintained 72% and 57% relative LDL-R binding ability, respectively (Table 4). In contrast, the apoE3-(1–231)-DMPC complex lost most of its LDL-R binding ability (31%), whereas that of apoE4-(1–231)-DMPC still maintained 75% of binding ability compared with the full-length apoE-DMPC (Table 4). However, apoE3-(1–231) without DMPC did not show this functional defect. With the assistance of the 232–271 fragment, the LDL-R binding ability of apoE3-(1–271)-DMPC was largely rescued (80%) and that of apoE4-(1–271)-DMPC was fully recovered (99%; Table 4). This rescue phenomenon was also found in the lipid-free environment.

Different from that of apoE3-(72–166)-DMPC, apoE4-(72–166)-DMPC complex showed very high efficiency in H-LDL competition. The ability is 94% of full-length apoE-DMPC at the same dose and
The LDL receptor binding and competition assay of apoE and its truncated proteins. A, full-length and N-terminal truncated apoE proteins. B, C-terminal truncated apoE proteins and their 72–166-residue fragments. Human HepG2 cells were incubated with DMEM containing 50 μg of 3H-LDL in the absence or presence of competitors at 4°C for 2 h. From left to right, the bars are shown as follows: 3H-LDL binding to HepG2 treated with serum-free medium in the absence of competitor ligand (corresponding to 446 dpm/3 × 10^5 cells in A and 822 dpm/3 × 10^5 cells in B) was taken as 100% (bar 1). Binding in the presence of competitors is shown as % of control. A, incubation contained the following competitors: a 20-fold excess (1 mg) of unlabeled LDL (bar 2); 0.5 mg of DMPC (bar 3); apoE3-DMPC complex at protein concentration of 0.125 mg (bar 4) and 1 mg (bar 5); apoE4-DMPC of 0.125 mg (bar 6) and 1 mg (bar 7); and 0.5 mg of apoE3-(1–231) (bar 8); apoE3-(41–299) (bar 9); apoE3-(72–299) (bar 10); and apoE4-(72–299) (bar 11). B, incubation contained the following competitors: a 20-fold excess (1 mg) of unlabeled LDL (bar 2); 0.5 mg of DMPC (bar 3). Bars 4–11 show the following proteins-DMPC complex as the competitors: apoE3-(1–191)-DMPC; apoE3-(1–231)-DMPC; apoE4-(1–231)-DMPC; apoE3-(1–271)-DMPC; apoE4-(1–271)-DMPC; and apoE4-(72–166)-DMPC; and apoE4-(72–166)-DMPC at the protein concentration of 0.5 mg. Most values are the average of duplicated experiments with the standard errors shown.

apoE3-(72–166) was only 44% (Table 4). The ability of apoE4-(72–166) was still maintained (to 65%) in the lipid-free environment.

**In Vivo Lowering Effect**—Table 5 shows the effects of apoE proteins to lower plasma total cholesterol in apoE(−) mice. Twenty four hours after the single injection of apoE proteins through the tail vein, total cholesterol in the plasma was significantly decreased ($p < 0.05$). The decrease of total cholesterol was maintained at least to 48 h (data not shown). The cholesterol-lowering effects by apoE proteins indicated that these apoE protein fragments were functional in lipid metabolism, and the catabolism of VLDL in the circulation was assumed. In apoE3-(72–299) and apoE3-(1–231), their cholesterol-lowering effects were reduced as compared with the others.

**DISCUSSION**

The unprecedented results of SV and SE experiments provide us with important information to explain the variation of association and dissociation of apoE3 and apoE4 proteins in a lipid-free or lipid-contained environment. In the presence of DMPC, apoE3 and apoE4, including full-length and truncated proteins, showed a major monomer species. From the continuous size distribution analysis, we also found that the average $f/f_0$ values of apoE with lipid (1.9–2.2) is always higher than that of apoE in PBS (1.2–1.6). This phenomenon supports the view that apoE proteins change their conformation (elongation) when they were bound to lipids or in the presence of lipids. When apoE proteins exist in a lipid-free environment, most of apoE proteins appear as oligomers. Moreover, N- and/or C-terminal truncated apoE4 proteins showed a wider and more complicated species distribution than those of apoE3 counterparts (22). Chang et al. (39) have shown that lipid- and receptor-binding regions of apoE4 fragments act in concert to cause mitochondrial dysfunction and neurotoxicity. Their recent study also showed that apoE4 can enhance $A \beta$ production by modulating amyloid precursor protein processing in cultured neuronal cells (7). The present study suggests that the greater tendency in aggregation of apoE4 truncated proteins may be the reason. Schneeveis et al. (40) have indicated that there is no significant structural difference between the three full-length isoforms of apoE-contained lipoprotein particles. Our studies using analytical ultracentrifugation confirmed this conclusion.

As a key apolipoprotein, apoE may interact with lipids from several lipoprotein classes and initiate their catabolism. In this study, the kinetic DMPC turbidity clearance assay (30–32, 38) was chosen to explore the protein-lipid interaction. In a previous study, to explain the solubilization of DMPC mLV by apolipoprotein molecules such as apoA-I in high density lipoprotein, a simultaneous kinetic two-phase model has been proposed (32). The proposed four-stage model includes protein-lipid touching, protein adsorption, protein insertion into lipid lattice defects, and protein-lipid rearrangement. The adsorption stage is the rate-limiting step, in which the inflexible apolipoprotein may adsorb into DMPC lattice defects very slowly, whereas the flexible one will proceed more rapidly. By examining the effects of apoE domain structure and polymorphism on the kinetics of DMPC mLV turbidity clearance, Segall

---

**TABLE 5**

| Proteins       | No. | Plasma cholesterol$^a$ | Decrease   |
|----------------|-----|------------------------|------------|
|                |     | Before                  | 24 h after |
| ApoE3          | 6   | 609 ± 100$^{de}$        | 315 ± 111$^{e}$ | 49 ± 14 |
| ApoE4          | 7   | 624 ± 169$^{de}$        | 302 ± 90$^{e}$  | 52 ± 9  |
| ApoE3-(41–299) | 6   | 733 ± 144$^{de}$        | 346 ± 36$^{e}$  | 53 ± 8  |
| ApoE4-(41–299)| 6   | 721 ± 138$^{de}$        | 332 ± 77$^{e}$  | 54 ± 9  |
| ApoE3-(72–299)| 7   | 751 ± 101$^{de}$        | 480 ± 122$^{e}$ | 36 ± 16 |
| ApoE4-(72–299)| 8   | 584 ± 78$^{e}$          | 284 ± 66$^{e}$  | 51 ± 9  |
| ApoE3-(1–231)  | 8   | 621 ± 54$^{f}$          | 411 ± 41$^{f}$  | 34 ± 6  |
| ApoE4-(1–231)  | 8   | 628 ± 66$^{f}$          | 323 ± 56$^{f}$  | 48 ± 11 |

$^{a}$ Male apoE(−) mice at 39 weeks of age were injected with apoE3-(1–231) and apoE4-(1–231) and those at 29 weeks of age were injected with apoE3, apoE3-(41–299), apoE4-(41–299), and apoE3-(72–299). For apoE and apoE4-(72–299) proteins, mice at 20 weeks of age were used.

$^b$ Fasting plasma cholesterol was determined.

$^c$ The molar amount of protein injected into each animal was identical (14.4 nmol).

$^d$ Full-length apoE3 and apoE4 were injected with 490 μg apoE3-(41–299) and apoE4-(41–299) with 420 μg apoE3-(72–299) and apoE4-(72–299) with 370 μg apoE3-(1–231) and apoE4-(1–231) with 390 μg. The injection volume was 200 μl in one dose.

$^e$ p < 0.05.
Human Apolipoprotein E3 and E4

et al. (32) have indicated that a bieponential rate equation is best to describe the experimental data. The binding constant, maximum binding rate, rate constant, and pool size fit well to a bieponential model. The obtained parameters showed differences between isoforms apoE3 and apoE4. They have further suggested that the 10-kDa apoE C-terminal protein maintains the lower binding constant and the faster rate constant. On the other hand, the 22-kDa N-terminal apoE protein shows a higher binding constant and a lower rate constant. We also extensively compared our data in DMPC turbidity clearance using the same modeling approach. The major difference between the two studies is that Segall et al. (32) initially used refolded apoE proteins, and the proteins are maintained in guanidine HCl-containing buffer to avoid the protein self-association. Their approach would ignore the difference of apoE3 and apoE4 proteins in quaternary structures (22, 41). We have further demonstrated the size distribution of various truncated apoE proteins, which indicate the importance of quaternary structure. The significance of the present study is that full-length and truncated apoE proteins, while maintaining the isoform variation, including quaternary structures, were used for structural correlation in protein-lipid interaction.

Our results indicated that apoE-(72–299), especially apoE3-(72–299), showed very efficient DMPC mLV clearance. This suggests that the first and second α-helices in the N terminus play an important role in the inhibition of DMPC mLV solubilization by full-length apoE. One of the possibilities in causing inhibition is the interaction of N- and C-terminal domains of apoE, and deletion of two helices in the N terminus removes the interaction. We have reported a size distribution change when the first two α-helices are truncated, and we demonstrated that apoE4-(72–299) maintains a more complicated and larger size distribution (self-association) in a lipid-free environment (22). It is compatible with the trend that apoE4-(72–299) showed a lower DMPC mLV clearing effect than that of apoE3-(72–299) in this study.

The LDL receptor binding assay is focused on the interaction of the LDL-R and apoE. The LDL-R-binding region in apoE has been mapped to residues 140–150, a basic region that can interact with the negatively charged ligand-binding domain of the LDL-R (42). This study shows that the N-terminal domain alone of apoE maintained 60–70% LDL-R binding ability. Although apoE3-(1–231) almost completely lost its LDL-R binding, apoE4-(1–231) still maintained 75% binding capacity (Table 4). This functional loss was also detected in the apoE-induced plasma cholesterol lowering with apoE-deficient mice as an in vivo model. Our study using analytical ultracentrifugation suggests that apoE4-(1–231) has a greater propensity to aggregate than apoE3-(1–231) (Fig. 1D). Choy et al. (43) suggested that there is an intermolecular coiled-coil formation in the apoE C-terminal domain. It is possible that oligomerization of apoE4-(1–231) releases the intramolecular interactions between the N and C terminus, leading to the exposure of the basic region 140–150 for LDL receptor binding.

As to C-terminal truncation, there are several reports indicating that the apoE-(1–271) fragment, produced by a chymotrypsin-like serine protease, occurs in vivo and appears to be neurotoxic (44, 45). In our study, both apoE3-(1–271) and apoE4-(1–271) showed higher DMPC mLV clearance rates than those of apoE3-(1–231) and apoE4-(1–231). This indicates that the efficiency of DMPC solubilization by apoE depends on the existence of the 232–271 regions. This is conceivable because this region is a known lipid-binding domain (20, 46). Furthermore, given that apoE4-(1–271) shows similar LDL-R binding ability to that of the full-length protein, this suggests that residues 272–299, which form a G” helix (9, 11, 47), are not necessary for receptor inter-

actions. Nevertheless, a fragment based on this C-terminal region has been demonstrated to inhibit the neurotoxicity of apoE4 (39, 45).

Another interesting finding is that the apoE-(72–166) fragment still maintained significant apoE function, especially apoE4-(72–166). The flexibility of apoE4-(72–166) should be higher than that of the apoE3 counterparts because of the absence of disulfide bonds. The DMPC clearance by apoE4-(72–266) is very efficient, and the LDL receptor binding ability of apoE4-(72–166) is nearly equal to that of full-length apoE. The plasma cholesterol-lowering effects of apoE3-(72–166) and apoE4-(72–166) have also remained.2 The two helices in 72–166 residues may behave like a flexible hinge, which can be adsorbed into DMPC lattice defects very rapidly (Fig. 6). This feature may help to expose the 140–150-residue basic region leading to a higher efficiency in LDL receptor binding. This fragment seems to be a plausible target for the design of a peptide drug.

Results based on the DMPC turbidity clearance assay help to explain the trend of molecular interaction of full-length and truncated apoE proteins with a phospholipid bilayer domain. The analytical ultracentrifugation analysis of apoE variants examined in this study showed that these proteins exist as monomers in the presence of the short chain phospholipid, DHPC, which is consistent with a previous study (41). These features help us propose a molecular model to explain the three kinds of apoE protein partitions into the lattice defects in the DMPC bilayer (Fig. 6). In the slow binding mode, the apoE-(1–191) and apoE-(1–231) proteins behave as molten globules (48), which expose their hydrophobic regions (the core of the four α-helix bundle) and interact with lipids very slowly (49–51). The rate-limiting step is the conformational change of apoE molten globule. In the moderate association mode, full-length apoE, apoE-(1–271), and apoE-(41–299) proteins interact with DMPC through their C-terminal region, especially the major lipid-binding region residues 245–270 (20, 46). In the fast binding mode, apoE-(72–299) and apoE-(72–166) proteins behave as a flexible apolipoprotein (32). The hydrophobic region of the N terminus is exposed very rapidly because only two amphipathic α-helical structures are left. The C terminus of apoE-(72–299) also accelerates the partition of apoE-(72–299) into lattice defects. This can explain the differences in DMPC turbidity and kinetic properties of the truncated variants examined in this study (Table 3). After rearrangement, the final DMPC-apoE discoidal complex may exist as a lipoprotein disk or belt, and each apoE molecule is folded into a helical hairpin with the binding region for the LDL-R at its apex (40, 52, 53).

The in vivo functional study showed that both the full-length and truncated apoE proteins were still active in lowering cholesterol. The animal studies were conducted using apoE isoform proteins in a parallel manner. Full-length apoE3 and apoE4, apoE3-(41–299), and apoE4-(41–299) lowered plasma cholesterol to a similar extent (50%). The efficacy of apoE4-(72–299) protein to lower plasma cholesterol was about equal to that of the full-length protein. However, the efficacy of apoE3-(72–299) (36%) was significantly lower than that of apoE4-(72–299). It strongly suggests that helix 2 is involved in LDL-R binding. The observation of low LDL-R binding ability (Table 4) is compatible with the findings that the efficacy of apoE3-(1–231) to lower plasma cholesterol was also low (34%). The more stabilized apoE4 isoform, which may be assisted by the domain-domain interaction, is less affected by helix 2 or C-terminal truncation. The interaction of Arg171 with Glu255, which may stabilize an extended helical structure in the C terminus to accommodate a larger, less curved VLDL surface (54, 55), cannot explain our observations. An alternative explanation is that stabilization by helix 2

---

2 C.-Y. Chou, Y.-H. Hsieh, M.-S. Shiao, and G.-G. Chang, unpublished data.
to LDL-R binding of an apoE isoform is more important than that of domain-domain interactions, especially for the apoE3 isoform. However, more detailed information on the structure of the truncated forms is needed to support this hypothesis.

The results of the LDL-R binding assay presented in this study demonstrate that truncated apoE4 variants possess a greater propensity than the equivalent apoE3 proteins to interact with the LDL-R, both in the presence and absence of phospholipid (Table 4). In addition, the results of the in vivo functional assay show that apoE4 and the various truncated variants employed in this study have a greater effect at lowering cholesterol levels than their apoE3 counterparts (Table 5). According to Farkas et al. (56), the recycling of apoE and its fragments showed multiple, redundant pathways. Upon partitioning into triglyceride-rich lipoproteins (such as VLDL), apoE is refrained from degradation and recycled (57, 58). Truncated apoE4, as a functional protein, is still able to assist the internalization of lipoproteins and spared the apoE molecules from degradation.

Recent studies have suggested that ubiquitin-proteasomal degradation of proteins is impaired in AD (59–61). Moreover, the greater tendency for truncated variants of apoE4 to aggregate (22) may reduce the exposure of apoE4 to proteases and thus prolong its half-life in vivo relative to apoE3. Together, prolonged functional stability and increased half-life because of aggregation may result in an accumulation of damaged apoE4 in vivo and therefore enhanced neuronal damage.

On the basis of the structural and functional elucidation, we suggest that the potential structural stability of apoE4 protein to remain functional in solution may explain the enhanced opportunity of apoE4 to display its pathophysiologic functions. The speculation can be extended to the higher tendency of apoE4 isoform to aggregate and co-aggregate with Aβ in the molecular pathogenesis of AD. The still functional, even extensively truncated, apoE4 may still preferentially partition into chylomicron and VLDL and assist the catabolism of chylomicron and VLDL resulting in elevated LDL-cholesterol and therefore adversely accelerate atherosclerosis. The structural abnormality and correlated functional disturbances of apoE4 isoform may shed light on the future development of peptide and protein drugs for the treatment of atherosclerosis and AD.

REFERENCES

1. Mahley, R. W., and Rall, S. C., Jr. (2000) Arnaus. Genomics Hum. Genet. 1, 507–537
2. Greenow, K., Pearce, N. J., and Ramji, D. P. (2005) J. Med. Med. 83, 329–342
3. Lane, R. M., and Farlow, M. R. (2005) J. Lipid Res. 46, 949–968
4. Tanzi, R. E., and Bertram, L. (2005) Cell 120, 545–555
5. de la Torre, J. C. (2002) J. Alzheimers Dis. 4, 497–512
6. Faller, L. A., Cupples, L. A., Haines, J. L., Hyman, B., Kukull, W. A., Mayeux, R., Myers, R. H., Pericak-Vance, M. A., Ritch, N., and van Duijn, C. M. (1997) J. Am. Med. Assoc. 278, 1349–1356
7. Ye, S., Huang, Y., Mullendoreif, K., Dong, L., Giedt, G., Meng, E. C., Cohen, F. E., Kunz, I. D., Weisgraber, K. H., and Mahley, R. W. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 18700–18705
8. Gregg, R. E., and Brewer, H. B., Jr. (1988) Clin. Chem. 34, 828–832
9. Wolter, R. T., and Atkinson, D. (1992) Biophys. J. 63, 1221–1239
10. Segrest, J. P., Jones, M. K., De Loof, H., Brouillet, C. G., Van de Voorde, T., Coen, F. E., and van Duijn, C. M. (1992) J. Lipid Res. 33, 141–166
11. Wilson, C., Wardell, M. R., Weisgraber, K. H., Mahley, R. W., and Agard, D. A. (1991) Science 252, 1817–1822
12. Dong, L. M., Wilson, C., Wardell, M. R., Simmons, T., Mahley, R. W., Weisgraber, K. H., and Agard, D. A. (1994) J. Biol. Chem. 269, 22358–22365
13. Wilson, C., Mau, T., Weisgraber, K. H., Wardell, M. R., Mahley, R. W., and Agard, D. A. (1994) Structure 2, 713–718
14. Sparrow, J. T., Sparrow, D. A., Fernando, G., Culwell, A. R., Kovar, M., and Gotto, A. M., Jr. (1992) Biochemistry 31, 1065–1068
15. Aggerbeck, L. P., Wetterau, J. R., Weisgraber, K. H., Wu, C. S., and Lindgren, F. T.
