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Fluorescence Study of Domain Structure and Lipid Interaction of Human Apolipoproteins E3 and E4

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Abstract

Human apolipoprotein E (apoE) isoforms exhibit different conformational stabilities and lipid-binding properties that give rise to altered cholesterol metabolism among the isoforms. Using Trp-substituted mutations and site-directed fluorescence labeling, we made a comprehensive comparison of the conformational organization of the N- and C-terminal domains and lipid interactions between the apoE3 and apoE4 isoforms. Trp fluorescence measurements for selectively Trp-substituted variants of apoE isoforms demonstrated that apoE4 adopts less stable conformations in both the N- and C-terminal domains compared to apoE3. Consistent with this, the conformational reorganization of the N-terminal helix bundle occurs at lower guanidine hydrochloride concentration in apoE4 than in apoE3 as monitored by fluorescence resonance energy transfer (FRET) from Trp residues to acrylodan attached at the N-terminal helix. Upon binding of apoE3 and apoE4 variants to egg phosphatidylcholine small unilamellar vesicles, similar changes in Trp fluorescence or FRET efficiency were observed for the isoforms, indicating that opening of the N-terminal helix bundle occurs similarly in apoE3 and apoE4. Introduction of mutations into the C-terminal domain of the apoE isoforms to prevent self-association and maintain the monomeric state resulted in great increase in the rate of binding of the C-terminal helices to a lipid surface. Overall, our results demonstrate that the different conformational organizations of the N- and C-terminal domains have a minor effect on the steady-state lipid-binding behavior of apoE3 and apoE4: rather, self-association property is a critical determinant in the kinetics of lipid binding through the C-terminal helices of apoE isoforms.

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Keywords

apolipoprotein E; domain structure; lipid interaction; Trp fluorescence; fluorescence resonance energy transfer

1. Introduction

Apolipoprotein E (apoE) plays a key role in regulating lipid transport and cholesterol homeostasis in the cardiovascular and central nervous systems [1–3]. In humans, there are three major isoforms of the protein, apoE2, apoE3, and apoE4, each differing by a single amino acid substitution [4]. ApoE3, the most common isoform, contains Cys-112 and Arg-158, whereas the less common apoE2 and apoE4 contain Cys-112/Cys158 and Arg-112/Arg-158, respectively (Fig. 1). ApoE2 displays defective binding to the low-density lipoprotein (LDL) receptor superfamily and is associated with type III hyperlipoproteinemia [5]. Although apoE3 and apoE4 bind similarly to the LDL receptor [6], apoE4 reduces plasma cholesterol less in humans compared to apoE3, giving rise to a more proatherogenic lipoprotein-cholesterol distribution [7, 8]. ApoE4 is also known to be a major genetic risk factor for Alzheimer's disease [9, 10]. Despite the profound differences in the outcomes of these diseases, the differences in the molecular properties of the apoE isoforms are still unclear [11].

ApoE contains two independently folded functional domains: a 22-kDa N-terminal domain (residues 1–191) and a 10-kDa C-terminal domain (residues 216–299) linked by a hinge region [4, 12]. The N-terminal domain is folded into a four-helix bundle of amphipathic α -helices [13, 14] and contains the region (residues 136–150) that binds to the LDL receptor [15]. The C-terminal domain contains amphipathic α -helices that are involved in binding to lipoproteins with high affinity [16–18] and self-association to form predominantly tetramer in solution [16, 19, 20]. Studies of apoE3 and apoE4 variants containing a progressively truncated C-terminal domain demonstrated that the region spanning residues 260–299 is important for determining self-association and ability to bind to lipoprotein particles of apoE [20–23]. Recent hydrogen/deuterium exchange coupled with electron-transfer dissociation mass spectrometry indicated that residues within region 230–270 are critical for oligomer formation of apoE isoforms [24], overlapping the major lipid binding region in the C-terminal domain. This suggests that the lipid binding and self-association in apoE isoforms are closely linked [25], consistent with the kinetic analysis of turbidity clearance of phospholipid vesicles by apoE [26].

A recent NMR structure of a monomeric variant of full-length apoE3 demonstrated that the C-terminal domain folds over and around the N-terminal domain through extensive interactions of salt-bridges and hydrogen bonds between the two domains [27]. Consistent with this, our previous deletion mutagenesis study indicated that the segment 261–272 contributes to the helix stabilization of the N-terminal domain, with this effect being much greater in apoE3 than apoE4 [28]. ApoE has a much lower thermodynamic stability compared to other globular proteins [29], and mutations in the protein have been shown to alter these inter-domain interactions, causing defective physiological functions [30–32]. The

overall stability of the entire apoE molecule exerts a major influence on its lipid- and lipoprotein-binding properties [33].

In this study, we examined the conformational stability and lipid binding properties of the N- and C-terminal domains of apoE3 and apoE4 using selectively Trp-substituted variants, in which Trp residues in either the N- or C-terminal domains were mutated to Phe to allow us to evaluate each domain separately. In addition, we extended our previous site-directed fluorescence labeling approach in the N- or C-terminal helices of apoE [34] to directly compare the conformational reorganization of each domains upon binding to spherical lipid particles for apoE3 and apoE4 variants that have different tendencies to self-associate in solution.

2. Methods

2.1. Materials

Human apoE3 and apoE4 variants and their 12-kDa (residues 192–299) fragment were expressed in *E. coli* as thioredoxin fusion proteins and isolated and purified as described previously [17, 35]. Cleavage of the thioredoxin fusion protein with thrombin leaves the target apoE with two extra amino acids, Gly and Ser, at the N terminus. The QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was employed to introduce the S94C/C112S or C112S/S290C mutations in apoE3 and the S94C or S290C mutations in apoE4 so that a single cysteine residue was present in each molecule. To generate monomeric apoE3 and apoE4 variants, additional mutations (F257A/W264R/V269A/L279Q/V287A) in the C-terminal domain were introduced [19]. In addition, Trp-substituted apoE3 and apoE4 variants in which Trp residues were selectively substituted to Phe in the N-terminal (W20F/W26F/W34F/W39F, W-NT) or C-terminal (W210F/W264F/W276F, W-CT) domains were prepared. The apoE preparations were at least 95% pure as assessed by SDS–PAGE. In all experiments, the apoE sample was freshly dialyzed at 4°C from a 6 M guanidine hydrochloride (GdnHCl) and 1% β -mercaptoethanol solution into Tris buffered saline (TBS; 10 mM Tris, 150 mM NaCl, 0.02 % NaN_3 , pH 7.4) before use. Egg yolk phosphatidylcholine (PC) was kindly donated from Kewpie (Tokyo, Japan). *N*-(1-pyrene)maleimide and 6-acryloyl-2-dimethylaminonaphthalene (acrylodan) were purchased from Invitrogen (Eugene, OR).

2.2. Fluorescence labeling

Cysteine-containing apoE variants were incubated with 10-fold molar excess of tris(2-carboxyethyl)phosphine hydrochloride (Thermo Scientific, Rockford, IL) for 1 h to reduce the sulfhydryl group. A 10 mM stock solution of *N*-(1-pyrene)maleimide (in dimethylsulfoxide) or acrylodan (in dimethylformamide) was added so that a final molar ratio of probe to protein was 10:1. The reaction mixtures were then incubated at room temperature for 3 h in the dark, and unreacted probe was removed by extensive dialysis at 4 °C in TBS. The degree of labeling was determined using the extinction coefficients of $38,200 \text{ M}^{-1} \text{ cm}^{-1}$ at 338 nm for pyrene and $19,200 \text{ M}^{-1} \text{ cm}^{-1}$ at 391 nm for acrylodan, respectively.

2.3. Preparation of small unilamellar vesicles (SUVs)

Egg PC SUVs were prepared as described [36, 37]. Briefly, a film of egg PC on the wall of a glass tube was dried under vacuum overnight. The lipid was then hydrated in TBS and sonicated on ice under nitrogen. After removing titanium debris, the samples were centrifuged in a Beckman 70.1Ti rotor for 1.5 h at 15 °C at 40,000 rpm to separate any remaining large vesicles. The PC concentration was determined using an enzymatic assay kit from Wako Pure Chemicals (Osaka, Japan).

2.4. Fluorescence measurements

Fluorescence measurements were carried out with a Hitachi F-4500 fluorescence spectrophotometer at 25 °C in TBS (pH 7.4). Trp fluorescence emission spectra of apoE variants were recorded from 300 to 420 nm using a 290 nm excitation wavelength to avoid tyrosine fluorescence. For monitoring chemical denaturation, proteins at concentrations of 25–50 µg/ml were incubated overnight with GdnHCl or urea at various concentrations. K_D at a given denaturant concentration were calculated from the change in Trp fluorescence intensity. The free energy of denaturation, G_D° , the midpoint of denaturation, $D_{1/2}$, and m value which reflects the cooperativity of denaturation in the transition region, were determined by the linear equation, $G_D = G_D^\circ - m[\text{GdnHCl}]$, where $G_D = -RT \ln K_D$. In fluorescence quenching experiments, the Trp emission spectra were recorded at increasing concentrations of KI (0–0.56 M) using a 5 M stock solution containing 1 mM NaS₂O₃ to prevent the formation of iodine. After correction for dilution, the integrated fluorescence intensities were plotted according to the Stern-Volmer equation, $F_0/F = 1 + K_{sv} [\text{KI}]$, where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively, and K_{sv} is the Stern-Volmer constant. Quenching parameters were obtained by fitting to the modified Stern-Volmer equation, $F_0/(F_0 - F) = 1/f_a + 1/f_a K_{sv} [\text{KI}]$, where f_a is the fraction of Trp residues accessible to the quencher.

In SUV binding experiments, binding parameters of apoE Trp variants to SUV were derived as described [38]. Briefly, the fraction of bound apoE, θ was calculated according to $\theta = P_b/P_T = (\text{WMF} - \text{WMF}_0) / (\text{WMF}_{\min} - \text{WMF}_0)$, where P_b and P_T are bound and total apoE concentrations, respectively, and WMF and WMF₀ are wavelength of maximum fluorescence (WMF) for apoE variants in the presence and absence of SUV, respectively, and WMF_{min} represents the WMF when apoE completely binds to SUV. Assuming that binding of apoE to lipid particles is described by a one-site binding model, $P_b/[\text{PC}] = B_{\max} P_f / (K_d + P_f)$, where P_f is unbound apoE concentration, $[\text{PC}]$ is PC concentration of SUV, and K_d and $B_{\max}$ are the dissociation constant and the maximal binding capacity, respectively, binding data were analyzed by linear regression based on the Hanes-Woolf equation, $[\text{PC}]P_f / P_b = K_d / B_{\max} + P_f / B_{\max}$.

For fluorescence resonance energy transfer (FRET) experiments, the emission spectra of acrylodan-labeled and unlabeled apoE variants were measured from 300 to 600 nm with excitation of 290 nm. FRET efficiency (E) was calculated according to $E = 1 - F_{DA}/F_D$, where F_{DA} is the fluorescence intensity of the donor with acrylodan attached and F_D is the fluorescence intensity of the donor lacking acrylodan. The FRET distance (R) was calculated

according to $E=R_0^6/(R_0^6+R^6)$, where R_0 is the Förster radius for energy transfer from Trp to acylodan in a protein (2.7 nm) [39].

Pyrene emission fluorescence was recorded from 360 to 560 nm using a 342 nm excitation wavelength. Excimer to monomer ratios were calculated by dividing the area under the excimer emission peak from 450 to 490 nm by that for the monomer emission peak from 370 to 410 nm. In experiments to measure binding to SUV, the increase in emission fluorescence intensity of pyrene-labeled apoE variants was monitored at 385 nm with excitation at 342 nm. The kinetics of increase in pyrene fluorescence upon SUV binding were analyzed using the two-phase exponential association model, $F = A[1 - \exp(-k_{\text{fast}} \cdot t)] + (1 - A)[1 - \exp(-k_{\text{slow}} \cdot t)]$, where F is the fluorescence intensity, A is the amplitude for the fast phase, and k_{fast} and k_{slow} are the apparent rate constants for the fast and slow phases, respectively.

3. Results

3.1. Evaluation of domain structure and stability using Trp-substituted variants of apoE isoforms

Human apoE possesses seven Trp residues, located at positions 20, 26, 34, and 39 in the N-terminal domain, 210 in the hinge region, and 264 and 276 in the C-terminal domain (Fig. 1). To evaluate structure and stability of the N-terminal and C-terminal domains of apoE isoforms using Trp fluorescence, Trp-substituted variants of apoE isoforms in which Trp residues in the N-terminal half (W20, W26, W34, and W39) or in the C-terminal half (W210, W264, and W276) were mutated to Phe were prepared. Thus, apoE W20F/W26F/W34F/W39F (W-NT) variants possess Trp residues only in the C-terminal half region including the C-terminal domain whereas apoE W210F/W264F/W276F (W-CT) variants possess Trp residues only in the N-terminal domain. Such conservative substitutions of Trp to Phe in apoE were shown to have little effect on the protein structure and stability [20, 22, 40]. Indeed, characteristic biphasic behavior in GdnHCl denaturation of apoE3 [41] was retained in these Trp-substituted variants (Supplementary Fig. 1).

We first performed GdnHCl-induced denaturation experiments of apoE isoform variants monitored by Trp fluorescence. Fig. 2A shows denaturation curves of apoE3 variants derived from the change in Trp fluorescence intensity at 335–340 nm. The denaturation curve for intact apoE3 displayed biphasic behavior, in which the first and the second phases (the midpoints of denaturation are 0.6 and 2.3 M GdnHCl, respectively) correspond to denaturation of the C-terminal and N-terminal domains, respectively [20, 22, 23, 42]. The midpoints of denaturation for apoE3 W-NT (W20F/W26F/W34F/W39F) and W-CT (W210F/W264F/W276F) were 0.7 and 2.3 M GdnHCl, respectively. This agrees well with those for the C-terminal and N-terminal domains in intact apoE3, indicating that the denaturation behavior of Trp-substituted variants indeed reflects the conformational stability of each domains in full-length apoE. Interestingly, the denaturation behavior of apoE3 W-NT (W20F/W26F/W34F/W39F) reflecting the conformational stability of the C-terminal domain was much less cooperative than that for the isolated C-terminal 12-kDa fragment, suggesting that the intermolecular interactions of the C-terminal helices of apoE [43, 44] is less pronounced in the full-length protein than in the isolated C-terminal fragment.

Figs. 2B and 2C compare GdnHCl denaturation behaviors for the N- or C-terminal Trp-substituted variants of apoE3 and apoE4 isoforms. GdnHCl denaturation parameters summarized in Table 1 demonstrate that while the free energy of denaturation G_D° values are not significantly different in full-length apoE W-CT (W210F/W264F/W276F) variants, the midpoint of denaturation $D_{1/2}$ in apoE4 variant is significantly lower than that in the apoE3 counterpart, consistent with the prior finding that the N-terminal helix bundle in apoE4 is less stable than that in apoE3 [29, 42]. Removal of residues 261–272 caused large reductions of the conformational stability G_D° both in the W-CT (W210F/W264F/W276F) variants of apoE3 and apoE4 (Table 1), consistent with the notion that the segment spanning residues 261–272 contributes to the stabilization of the N-terminal helix bundle through the domain-domain interaction [28]. Urea denaturation results for these apoE variants (Supplementary Fig. 2 and Table 1) indicate that such a stabilizing effect of residues 261–272 on the structure of the N-terminal helix bundle is greater in apoE3 than in apoE4 [28]. Comparison of GdnHCl denaturation behavior between the W-NT (W20F/W26F/W34F/W39F) variants of apoE3 and apoE4 (Fig. 2C and Table 1) demonstrated that the organization of the C-terminal domain in apoE4 is less stable than that in apoE3.

Trp quenching behavior by the aqueous quencher KI for apoE W-CT (W210F/W264F/W276F) variants (Fig. 3A) demonstrated that apoE4 W-CT variant is somewhat more quenched than the apoE3 counterpart, indicating the N-terminal domain in apoE4 being relatively more exposed to the aqueous phase than that in apoE3. Consistent with this idea, Met-108 in apoE4 was shown to be more solvent-exposed than in apoE2 and apoE3, as demonstrated by mass spectrometry-based protein foot-printing methods coupled with photochemical oxidation of the proteins [45]. In contrast, similar quenching behavior was seen for the W-NT (W20F/W26F/W34F/W39F) variants of apoE3 and apoE4 (Fig. 3B). Removal of residues 261–272 resulted in marked increases in the accessibility parameter K_{SV} in both the W-CT (W210F/W276F) variants of apoE3 and apoE4 (Table 2), consistent with residues 261–272 stabilizing the N-terminal helix bundle.

3.2. GdnHCl-induced conformational reorganization of apoE isoforms monitored by FRET

We next explored the conformational reorganization of the N-terminal helix bundle in apoE isoforms by monitoring FRET from intrinsic Trp residues in apoE to acrylodan attached at S94C in the N-terminal helix. This S94C mutation was previously shown not to disturb the structural property of apoE4 [34]. In addition, we introduced in this study the further mutation S94C/C112S into apoE3 to leave only one Cys residue for acrylodan labeling. The C112S mutation in apoE3 was shown not to alter global folding structure and stability of the N-terminal domain of apoE3 [46], and we also confirmed that apoE3 S94C/C112S has similar secondary and tertiary structure to apoE3 (Supplementary Fig. 3). Based on the NMR structure of monomeric apoE3 [27], residues S94 and C112 located in Helix 3 are both facing in the same direction and exposed to solvent.

Fig. 4A shows the fluorescence emission spectra of unlabeled and acrylodan-labeled apoE3 S94C/C112S variant in solution. A large reduction in Trp emission fluorescence at around 340 nm and the concomitant appearance of an acrylodan fluorescence peak at around 480 nm indicates the occurrence of FRET from Trp residues to acrylodan in the folded protein.

Although the observed FRET is thought to be the sum of not only intra-domain but also inter-domain energy transfer between Trp residues and acrylodan attached at the N-terminal domain, the contribution from the domain-domain proximity seems to be relatively small because the change in FRET efficiency was less than 20% at 1.5 M GdnHCl (Fig. 4C) where the C-terminal domain is almost fully unfolded (Fig. 2C). Thus, the observed FRET can be considered as mainly intra-domain involving only Trp residues in the N-terminal domain [26, 34]. When completely denatured in the presence of 4.6 M GdnHCl, the difference in Trp fluorescence between unlabeled and acrylodan-labeled protein and acrylodan fluorescence peak in apoE3 S94C/C112S variant became remarkably small (Fig. 4B), indicating a great reduction in the FRET efficiency due to increased separation of the Trp residues and acrylodan. Fig. 4C compares the denaturation curves of apoE3 S94C/C112S and apoE4 S94C variants derived from the change in the FRET efficiency. This demonstrates that the conformational unfolding of the N-terminal helix bundle occurs at lower GdnHCl concentration in apoE4 than in apoE3, consistent with the result of Trp-substituted variants (Fig. 2B). It should be noted that the contribution from self-association of apoE to the observed FRET appears to be negligible because the FRET efficiency hardly changes upon addition of up to 1 M GdnHCl, at which point the self-association of apoE through the C-terminal domain is eliminated [34, 44].

3.3. SUV binding of apoE isoform variants monitored by Trp fluorescence or FRET efficiency

Using Trp-substituted variants of apoE3 and apoE4, we next examined the lipid binding behaviors of apoE isoforms by monitoring Trp fluorescence. Upon binding to egg PC SUV, a significant blue shift of WMF with increased fluorescence intensity in Trp emission fluorescence spectra was observed for the Trp-substituted variants of apoE, indicating that Trp residues in each domains of apoE are transferred into a more hydrophobic lipid environment [40]. As shown in Fig. 5A, such blue shifts of WMF greatly increased with increasing SUV PC/apoE ratio for the W-NT variants of apoE3 and apoE4 (Trp residues are located in the C-terminal domain) whereas the shifts of WMF were within 2–3 nm for the W-CT variants (Trp residues in the N-terminal domain). This indicates that the change in the local environment for Trp residues through the conformational reorganization of apoE upon lipid binding is much greater for the C-terminal domain compared to the N-terminal domain. Taking changes in WMF for the apoE W-NT variants upon SUV binding as an indicator of the fraction of apoE bound, binding isotherms of apoE3 and apoE4 to SUV were derived (Fig. 5B). As listed in Table 3, the resultant K_d and B_{max} values obtained from the linear regression lines in Fig. 4B indicate that apoE3 and apoE4 bind to the SUV surface similarly, with somewhat higher affinity for apoE4 than apoE3 [47]. Since conformational rearrangement of the N-terminal helix bundle occurs upon lipid binding [17, 48], binding titration curves were also derived from the change in FRET from Trp residues to acrylodan upon SUV binding of the S94C-acrylodan variants of apoE isoforms (Fig. 5C). The obtained binding parameters for the S94C-acrylodan variants (Table 3) are similar to those for the corresponding Trp-substituted variants, indicating that these parameters reflect the lipid binding of apoE with the N-terminal helix bundle adopting an open conformation [34]. We note that the difference in K_d values from Trp fluorescence and FRET may be due to the different aspects of the apoE binding process these two signals monitor; Trp fluorescence

monitors lipid contact of the C-terminal domain whereas FRET monitors opening of the N-terminal helix bundle.

3.4. Self-association behavior of apoE isoform variants

We previously investigated the self-association property of apoE4 by gel filtration chromatography and pyrene excimer fluorescence assay [34]. The excimer/monomer fluorescence ratio of pyrene attached at the C-terminal helices of apoE molecule provides information about spatial proximity between the C-terminal domains [44]. In this study, we extended these approaches to compare the self-association behaviors of apoE3 and apoE4 isoforms.

Gel filtration elution profiles of apoE3 C112S/S290C-pyrene, apoE4 S290C-pyrene, and their variants with monomeric F257A/W264R/V269A/L279Q/V287A mutation in comparison with intact apoE3 and apoE4 were shown in Supplementary Fig. 4. Compared to the monomeric variants of apoE3 C112S/S290C-pyrene and apoE4 S290C-pyrene which predominantly exhibit a single sharp peak (Supplementary Figs. 4E and 4F), a broader and lower-elution volume peak was seen in apoE3 and apoE4 variants (Supplementary Figs. 4A–4D), indicating that both apoE3 and apoE4 self-associate in solution, in which the degree of oligomerization is greater in the former case [20]. It should be noted that neither the introduction of cysteine residue nor the addition of pyrene label at S290 position alters the self-association property of apoE [34].

Fig. 6 shows pyrene fluorescence emission spectra (Fig. 6A) and excimer/monomer ratios for the pyrene-labeled S290C variants at protein concentrations ranging from 5–75 µg/ml (Fig. 6B) where apoE is predominantly in oligomeric form [49]. The relatively higher excimer/monomer ratio of apoE4 S290C-pyrene [34] is consistent with the notion that apoE self-associates through the C-terminal domain. In contrast, apoE3 C112S/S290C-pyrene exhibited a much lower excimer/monomer ratio comparable to the values for the corresponding monomeric variants despite the fact that this apoE3 variant self-associates to a greater degree than its apoE4 counterpart (Supplementary Figs. 4C and 4D). This suggests that the C-terminal region around residue 290 in apoE3 has different organization from that in apoE4 such that this region is not available for the intermolecular interaction, perhaps due to the extensive inter-domain interactions between the N- and C-terminal domains [27]. Previous hydrogen/deuterium exchange experiments demonstrated that the C-terminal residues 271–279 in the monomeric form of apoE3 are less solvent exposed and more protected than that in apoE4 [25].

3.5. Effect of self-association on binding kinetics of pyrene-labeled apoE variants to SUV

It has been suggested that differences in the self-association behavior of apoE isoforms result in differences in their lipidation kinetics based on analysis of the turbidity clearance of phospholipid vesicles [26]. Thus, we next explored the effect of the monomeric mutation on the binding behavior of pyrene-labeled variants of apoE3 and apoE4 isoforms to SUV. Taking advantage of the fact that the fluorescence intensity of pyrene attached at the N- or C-terminal helices in apoE significantly increases upon lipid binding of apoE [34, 38], we monitored the lipid binding behaviors of the N- and C-terminal domains of apoE isoforms

independently. Fig. 7 shows the time course of the increase in pyrene fluorescence upon binding of the pyrene-labeled apoE3 and apoE4 variants to SUV. As demonstrated previously [34], apoE4 S290C-pyrene exhibited faster kinetics than apoE4 S94C-pyrene (Fig. 7B), consistent with the two-step mechanism for lipid binding of apoE4: initial binding occurs rapidly through the C-terminal α -helices (the S290C-pyrene) followed by relatively slow conformational reorganization of the N-terminal helix bundle (the S94C-pyrene). In contrast, the S290C-pyrene did not show faster binding than the S94C-pyrene in apoE3 (Fig. 7A), perhaps because apoE3 C112S/S290C-pyrene self-associates with a great degree of oligomerization (Supplementary Fig. 4C). To support this, the monomeric mutation in the S290C-pyrene variants of apoE3 and apoE4 caused 5–10 fold increases in the rate of binding whereas similar binding kinetics were observed for the S94C-pyrene variants (Figs. 7C, 7D, and Table 4). These results clearly demonstrate that dissociation of oligomers to monomers is the rate-limiting step for the rapid lipid binding of apoE through the C-terminal domain.

4. Discussion

ApoE displays low thermodynamic stability and significant conformational plasticity [50, 51], and mutations associated with lipoprotein glomerulopathy [31] and Alzheimer's disease [32] have been shown to induce structural and aggregation-related perturbation, impeding its physiological function. Among three major isoforms in human apoE (apoE2, apoE3, and apoE4), apoE4 is distinguished by a lower stability [42] and a greater tendency to form a molten globule state [29], which may contribute to the isoform-specific effects of apoE in diseases [10]. Since the mutations in the N-terminal domain of apoE are thought to be propagated through the structure to the C-terminal domain [11, 50], it is important to have information about the structural differences of the N- and C-terminal domains in apoE isoforms to understand their structure-function relationships.

In this study, we evaluated the structural stabilities of the N- and C-terminal domains by introducing selective substitutions of Trp to Phe into each domain of apoE3 and apoE4. The GdnHCl denaturation data in Figs. 2B and 2C, and Table 1 show that both the N- and C-terminal domains are less stable in apoE4 than in apoE3. It is well known that human apoE3 and apoE4 exhibit biphasic denaturation by GdnHCl or urea, in which the C-terminal domain unfolds first followed by the unfolding of the N-terminal domain [20, 26, 42]. Although thermodynamic parameters of denaturation for each domain can be derived from a three-state treatment of the denaturation curves, they appear to contain some ambiguities. In contrast, our denaturation studies using Trp-substituted mutants clearly demonstrate that not only the N-terminal domain but also the C-terminal domain in apoE4 has less stable structure compared to apoE3. Importantly, we found that segment spanning residues 261–272 which is folded differently in apoE3 and apoE4 as monitored by the fluorescence of W264 [20] has a great stabilizing effect on the N-terminal helix bundle in apoE3 (Supplementary Fig. 2 and Table 1). This agrees with the NMR structure of the monomeric apoE3 demonstrating that several residues in this region are involved in the formation of hydrogen bonds and salt-bridges with the N-terminal domain [25, 27]. In addition, ^{19}F NMR spectra of 5- ^{19}F -Trp incorporated into apoE3 and apoE4 demonstrated the structural differences in the N-terminal domain as a consequence of interaction with the C-terminal

domain [52]. Therefore, the N-terminal domain serves as a folding template for the C-terminal domain [27], and thus, the less pronounced stabilizing domain-domain interactions in apoE4 would lead to the less organized conformation of the C-terminal domain, resulting in the different self-association behaviors through the C-terminal domain between apoE3 and apoE4 in solution [20]. Overall, our results are consistent with the common structural model of apoE isoforms in which apoE4 has a less stable N-terminal helix bundle structure than apoE3. However, the finding that the stabilizing interaction of the C-terminal segment with the N-terminal helix bundle in apoE3 is less pronounced in apoE4 does not support the proposed model in which the salt-bridge between the N- and C-terminal domains in apoE4 leads to more pronounced domain-domain interaction [50].

A major question in this study is whether or not the different conformational organization of the N- and C-terminal domains in apoE3 and apoE4 affects their lipid binding functionalities, given that the preferential binding of apoE4 to very low-density lipoprotein is a consequence of the greater lipid-binding ability of this isoform [18, 28]? The similar changes in WMF of Trp fluorescence for the N- and C-terminal domains upon SUV binding of apoE3 and apoE4 (Fig. 5A) indicate that the N- and C-terminal domains of apoE isoforms bind similarly to the SUV surface. In addition, FRET from intrinsic Trp residues to acrylodan attached to the N-terminal helix changed similarly for apoE3 and apoE4 upon binding to SUV (Fig. 5C), indicating that the N-terminal helix bundle in apoE3 and apoE4 has a similar tendency to open upon lipid binding [34]. Based on these results, it seems that the different conformational organization of the N- and C-terminal domains has little effect on the steady-state binding behavior of apoE isoforms to stable phospholipid vesicles whereas conformational differences between the isoforms are responsible for the enhanced binding of apoE4 to lipid emulsions and lipoproteins [33, 47]. This discrepancy may be a consequence of the insensitivity of the Trp fluorescence or FRET used in this study to small differences in steady-state surface concentration and conformation of apoE isoforms.

The self-association behavior of apoE has been suggested to play a major role in the lipidation kinetics: dissociation of oligomers to monomers is the rate-determining step for interaction of the C-terminal domain of apoE with a lipid surface [26]. Comparison of increases in pyrene fluorescence intensity upon binding of pyrene-labeled apoE3 and apoE4 to SUV (Figs. 7A and 7B) demonstrates that while the N-terminal helices of apoE3 and apoE4 bind to a SUV surface with similar kinetics, the binding of the C-terminal helices is much slower in apoE3 than in apoE4 perhaps because of a greater degree of oligomerization in apoE3 than in apoE4 (Supplementary Figs. 4A and 4B). Indeed, the monomeric variants of pyrene-labeled apoE3 and apoE4 exhibited similar kinetics of binding of the C-terminal helices at greatly increased rates (Figs. 7C and 7D), indicating that dissociation of oligomers to monomers is the rate-limiting step for lipid binding of apoE through the C-terminal domain. In contrast, the fact that the monomer-inducing mutations have negligible effects on the binding kinetics of the N-terminal helices of apoE3 and apoE4 suggests that opening of the helix bundle is the rate-determining step in binding of the N-terminal domain to a lipid surface; the different unfolding stabilities of the N-terminal helix bundle between apoE3 and apoE4 have a minor effect.

In summary, the results presented in this study demonstrate that apoE4 adopts less organized conformations in both the N- and C-terminal domains compared to apoE3, leading to the different self-association behaviors of apoE3 and apoE4 in solution. However, such different conformational organizations of the N- and C-terminal domains themselves appear to have a minor effect on the steady-state lipid-binding behavior of apoE3 and apoE4. Rather, the degree of self-association is a critical determinant in the kinetics of lipid binding mediated through the C-terminal helices of apoE isoforms, and this oligomerization likely impacts on the pathological properties of apoE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Ac	acrylodan
apoE	apolipoprotein E
FRET	fluorescence resonance energy transfer
GdnHCl	guanidine hydrochloride
LDL	low-density lipoprotein
PC	phosphatidylcholine
PL	phospholipid
SUV	small unilamellar vesicle
TBS	Tris-buffered saline
WMF	wavelength of maximum fluorescence

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Highlights

- Human apoE isoforms exhibit different stabilities and lipid-binding properties.
- ApoE4 adopts less stable conformations in the N- and C-terminal domains than apoE3.
- Conformational difference has a minor effect on lipid binding of apoE isoforms.
- Self-association property is critical to the lipid-binding kinetics of apoE isoforms.

Fig. 1. Schematic representation of the location of Trp residues in the N- and C-terminal domains of human apoE. The isoform-specific differences in amino acid sequence are also shown in the linear diagram. The α -helical segments are depicted as boxes based on the NMR structure of monomeric apoE3 [27].

Fig. 2. GdnHCl-induced denaturation of the Trp variants of apoE isoforms monitored by the change in Trp fluorescence intensity. (A) Denaturation curves for apoE3 W-NT (W20F/W26F/W34F/W39F) ($\blacktriangle$) and apoE3 W-CT (W210F/W264F/W276F) ($\blacksquare$). The results for apoE3 12-kDa (residues 192–299) ($\triangle$) and intact apoE3 ($\square$) are also shown for comparison [20]. (B) Denaturation curves for the W-CT variants of apoE isoforms monitoring the N-terminal domain. ApoE3 W210F/W264F/W276F ($\bullet$), apoE4 W210F/W264F/W276F ($\triangle$), apoE3 261–272 W210F/W276F ($\circ$), and apoE4 261–272 W210F/W276F ($\blacktriangledown$). The inset shows the linear plots of ΔG_D for apoE3 W210F/W264F/W276F ($\bullet$) and apoE4 W210F/

W264F/W276F (Δ) as a function of GdnHCl concentration. (C) Denaturation curves for the W-NT variants of apoE isoforms monitoring the C-terminal domain. ApoE3 W20F/W26F/W34F/W39F ($\bullet$) and apoE4 W20F/W26F/W34F/W39F (Δ).

Fig. 3.

KI quenching of the Trp variants of apoE isoforms. (A) Stern-Volmer plot for quenching of the W-CT variants of apoE isoforms monitoring the N-terminal domain. ApoE3 W210F/W264F/W276F (●) and apoE4 W210F/W264F/W276F (△). The inset shows a modified Stern-Volmer plot. (B) Stern-Volmer plot for quenching of the W-NT variants of apoE isoforms monitoring the C-terminal domain. ApoE3 W20F/W26F/W34F/W39F (●) and apoE4 W20F/W26F/W34F/W39F (△).

Fig. 4. FRET between Trp residues and acrylodan in apoE3 S94C-acrylodan/C112S. Fluorescence emission spectra excited at 290 nm for unlabeled (solid line) and acrylodan-labeled (dashed line) apoE3 S94C/C112S were recorded in the absence (A) or presence (B) of 4.6 M GdnHCl. (C) GdnHCl denaturation curves of apoE3 S94C-acrylodan/C112S (●) and apoE4 S94C-acrylodan (△) monitored by FRET between Trp residues and acrylodan.

Fig. 5.

(A) Change in WMF of Trp fluorescence spectra for apoE3 W20F/W26F/W34F/W39F (●), apoE4 W20F/W26F/W34F/W39F (△), apoE3 W210F/W264F/W276F (○), and apoE4 W210F/W264F/W276F (▼) as a function of the weight ratio of PC to apoE variants. (B) Binding isotherms of binding of the W20F/W26F/W34F/W39F variants for apoE3 (●) and apoE4 (△) to egg PC SUV. The inset shows the linearized plots according to the Hanes-Woolf equation. (C) Relative changes in FRET efficiency between Trp residues and

acrylodan for apoE3 S94C-acrylodan/ C112S (○) and apoE4 S94C-acrylodan (▼). The inset shows the linearized plots according to the Hanes-Woolf equation.

Fig. 6. (A) Pyrene fluorescence emission spectra of apoE3 C112S/S290C-pyrene at protein concentrations of 5–75 µg/ml. (B) The pyrene excimer (at 470 nm)/monomer (at 375 nm) intensity ratio for pyrene-labeled apoE3 and apoE4 variants.

Fig. 7.

Time courses of increases in fluorescence intensity upon binding to egg PC SUV for pyrene-labeled apoE3 (A, C) and apoE4 (B, D) variants. SUV was added to apoE variants at final concentrations of 10 $\mu\text{g/ml}$ protein and 0.4 mg/ml PC.

Table 1

GdnHCl denaturation parameters for Trp variants of apoE3 and apoE4

apoE Trp variant	thermodynamic parameters of denaturation		
	G_D°	m	$D_{1/2}$
	<i>kcal/mol</i>	<i>kcal/mol apoE/mol GdnHCl</i>	<i>M</i>
apoE W-CT			
apoE3 W210F/W264F/W276F	2.9 ± 0.2	1.3 ± 0.1	2.3 ± 0.1
apoE4 W210F/W264F/W276F	3.2 ± 0.2	1.6 ± 0.1	2.0 ± 0.1
apoE3 261-272 W210F/W276F	2.4 ± 0.1	1.2 ± 0.1	2.0 ± 0.1
apoE4 261-272 W210F/W276F	2.3 ± 0.2	1.3 ± 0.1	1.8 ± 0.2
apoE W-NT			
apoE3 W20F/W26F/W34F/W39F	1.6 ± 0.1	2.1 ± 0.1	0.74 ± 0.11
apoE4 W20F/W26F/W34F/W39F	1.2 ± 0.2	2.0 ± 0.2	0.60 ± 0.15

Table 2

KI quenching parameters for Trp fluorescence of apoE Trp variants

apoE Trp variant	<u>KI quenching parameters</u>	
	f_a	K_{SV} M^{-1}
apoE W-CT		
apoE3 W210F/W264F/W276F	0.59 ± 0.04	4.0 ± 0.3
apoE4 W210F/W264F/W276F	0.65 ± 0.02	4.4 ± 0.2
apoE3 261-272 W210F/W276F	0.58 ± 0.01	5.7 ± 0.2
apoE4 261-272 W210F/W276F	0.61 ± 0.02	6.6 ± 0.3
apoE W-NT		
apoE3 W20F/W26F/W34F/W39F	0.58 ± 0.02	7.2 ± 0.4
apoE4 W20F/W26F/W34F/W39F	0.62 ± 0.02	6.5 ± 0.4

Table 3

Parameters of binding of apoE variants to egg PC SUV

apoE variant	K_d	B_{max}
	$\mu\text{g/ml}$	<i>amino acids/mol PC</i>
apoE3 W-NT (W20F/W26F/W34F/W39F)	2.1 ± 0.4	0.94 ± 0.04
apoE4 W-NT (W20F/W26F/W34F/W39F)	1.2 ± 0.2	0.95 ± 0.02
apoE3 S94C-acrylodan/C112S	3.4 ± 0.6	0.85 ± 0.04
apoE4 S94C-acrylodan	3.2 ± 1.7	0.89 ± 0.11

Table 4

Amplitude and rate constants for binding of pyrene-apoE variants to egg PC SUV

pyrene-apoE variant	A^*	$k_{\text{fast}} (\text{s}^{-1})^*$	$k_{\text{slow}} (\text{s}^{-1})^*$
apoE3 S94C-pyrene/C112S	0.37	$(0.42 \pm 0.05) \times 10^{-1}$	$(2.8 \pm 0.2) \times 10^{-3}$
apoE3 C112S/S290C-pyrene	0.37	$(0.50 \pm 0.03) \times 10^{-1}$	$(2.9 \pm 0.1) \times 10^{-3}$
apoE4 S94C-pyrene	0.24	$(0.40 \pm 0.02) \times 10^{-1}$	$(2.7 \pm 0.1) \times 10^{-3}$
apoE4 S290C-pyrene	0.46	$(0.43 \pm 0.06) \times 10^{-1}$	$(4.5 \pm 0.3) \times 10^{-3}$
monomeric apoE3 S94C-pyrene/C112S	0.38	$(0.38 \pm 0.07) \times 10^{-1}$	$(2.6 \pm 0.3) \times 10^{-3}$
monomeric apoE3 C112S/S290C-pyrene	0.60	$(2.9 \pm 0.3) \times 10^{-1}$	$(5.3 \pm 0.2) \times 10^{-3}$
monomeric apoE4 S94C-pyrene	0.31	$(0.28 \pm 0.05) \times 10^{-1}$	$(2.0 \pm 0.3) \times 10^{-3}$
monomeric apoE4 S290C-pyrene	0.58	$(4.3 \pm 1.0) \times 10^{-1}$	$(5.4 \pm 0.4) \times 10^{-3}$

* The increase in pyrene fluorescence were analyzed according to $F = A[1 - \exp(-k_{\text{fast}} \cdot t)] + (1 - A)[1 - \exp(-k_{\text{slow}} \cdot t)]$.