Structural Basis for the Recognition and Cross-linking of Amyloid Fibrils by Human Apolipoprotein E*

Menachem J. Gunzburg, Matthew A. Perugini, and Geoffrey J. Howlett

From the Department of Biochemistry and Molecular Biology and Bio21 Molecular Science and Biotechnology Institute, the University of Melbourne, Parkville, Victoria 3010, Australia

Apolipoprotein (apo) E is a well characterized lipid-binding protein in plasma that also exists as a common nonfibrillar component of both cerebral and systemic amyloid deposits. A genetic link between a common isoform of apoE, apoE4, and the incidence of late onset Alzheimer disease has drawn considerable attention to the potential roles of apoE in amyloid-related disease. We examined the interactions of apoE with amyloid fibrils composed of apoC-II and the amyloid-β (Aβ) peptide. Aggregates of apoE with Aβ and apoC-II are found in Alzheimer and atherosclerotic plaques, respectively. Sedimentation velocity and fibril size distribution analysis showed that apoE3 and E4 isoforms bind and noncovalently cross-link apoC-II fibrils in a similar manner. This ability to cross-link apoC-II fibrils was abolished by the dissociation of the apoE tetramer to monomers or by thrombin cleavage to yield separate N- and C-terminal domains. Preparative ultracentrifuge binding studies indicated that apoE and the isolated N- and C-terminal domains of apoE bind with submicromolar affinities to both apoC-II and Aβ fibrils. Fluorescence quenching and resonance energy transfer experiments confirmed that both domains of apoE interact with apoC-II fibrils and demonstrated that the binding of the isolated N-terminal domain of apoE to apoC-II or Aβ fibrils is accompanied by a significant conformational change with helix three of the domain moving relative to helix one. We propose a model involving the interaction of apoE with patterns of aligned residues that could explain the general ability of apoE to bind to a diverse range of amyloid fibrils.

Apolipoprotein E (apoE) is a component of many cerebral and systemic amyloid deposits, including Aβ deposits in Alzheimer disease, prion protein deposits in Creutzfeldt-Jakob disease, and light chain deposits in immunoglobulin-related primary amyloidosis (1–4). Interest in the role of apoE in amyloid diseases has arisen from the link between the apoE4 isoform and the occurrence of Alzheimer disease, with APOE4 conveying a higher risk and earlier age of onset of Alzheimer disease than APOE3 (5). The three major isoforms of apoE differ by single arginine/cysteine substitutions at two positions. ApoE3 (112C/158R) is the most common isoform, whereas apoE4 (112R/158R) is the second most common isoform (6). There is currently no clear understanding of the molecular mechanisms of isoform-specific differences of apoE and how they are related to Alzheimer disease risk. Although recent studies suggest that apoE may form amyloid fibrils directly (7), the observation that apoE co-localizes in all amyloid deposits (2, 4) and interacts specifically with amyloid fibrils in vitro, including fibrils composed of Aβ-(1–40), Aβ-(1–42), β2-microglobulin, gelsolin, and apoC-II (8–11), suggest that apoE plays a general role as a nonfibrillar amyloid component. Other nonfibrillar components of amyloid deposits include proteoglycans and serum amyloid P (SAP) (3, 4).

Apoe is a 299-residue plasma apolipoprotein that exists primarily as a tetramer in the absence of lipid (12, 13). The presence of micellar concentrations of the short-chain phospholipid dihexanoylphosphatidylcholine (DHPC) dissociates the apoE tetramer to monomer (12, 13). ApoE consists of two independently folded domains, separated by a loop region containing a thrombin cleavage site (12, 14). Thrombolytic cleavage of apoE produces two primary fragments, an N-terminal fragment consisting of residues 1–191 and a C-terminal fragment consisting of residues 216–299. The 22-kDa C-terminal domain (residues 20–165) consists of a four-helix bundle that contains the low density lipoprotein receptor binding region (15, 16). The 10-kDa C-terminal domain (residues 225–299) consists of three putative amphipathic α-helices. This C-terminal domain contains the major lipid binding region and in the absence of lipid mediates the self-association of apoE (12, 17, 18). In the absence of lipid, apoE and the isolated C-terminal domain exist primarily as tetramers, whereas the isolated N-terminal domain of apoE is monomeric (12, 13, 18). The N-terminal domain undergoes a conformational change in the presence of lipid where the four-helix bundle opens and helix one moves with respect to helix three (19–21). Binding of apoE to lipid also has consequences for the positioning of the two domains with respect to each other, with the two domains moving farther apart when bound to lipid (22, 23).

There is growing evidence of a role for amyloid deposition in atherosclerosis (24). Many apolipoproteins are prone to amyloid fibril formation and several, including apoA-I, apoB, apoC-II, and apoE, accumulate as deposits in atherosclerotic plaques (25, 26). ApoC-II is a 79-residue apolipoprotein which, in the
Absence of lipid, self-associates to form fibrils with all the hallmark features of amyloid, including thioflavin T and Congo red binding, increased β-structure, and cross-β-structure indicated by X-ray diffraction (27, 28). ApoC-II in atherosclerotic plaques co-localizes with apoE and other apolipoproteins and with serum amyloid P (SAP), a marker for in vivo amyloid deposits (29). Furthermore, apoC-II and Ap β fibrils activate macrophage inflammatory responses via the CD36 scavenger receptor, implicating a role for amyloid fibrils in the development of atherosclerosis (29). In addition to disease relevance, the ready availability of apoC-II to form homogeneous fibrils in vitro provides a convenient model system to examine the solution properties of amyloid fibrils. Size distribution and rheology analysis of apoC-II amyloid fibrils demonstrated that both SAP and apoE noncovalently cross-link apoC-II fibrils increasing their network structure and condensing them into more localized fibrillar aggregates (11, 30). These results suggest that nonfibrillar components, such as SAP and apoE, may act as mediators of amyloid fibril deposition and stability. However, structural details of the interaction between these nonfibrillar components and amyloid fibrils are lacking.

Here we use apoC-II fibrils as a model system to explore the structural requirements for apoE binding and cross-linking of amyloid fibrils. These studies reveal that binding and cross-linking of amyloid fibrils is reliant upon independent interaction sites in the N- and C-terminal domains of apoE and provide insight into the nature of the recognition of amyloid fibrils by these binding sites.

**Experimental Procedures**

**Materials**—The pET32a expression vectors for apoE3, apoE4, apoE3 N-terminal domain, apoE C-terminal domain, apoE4S53C, apoE4S76C, apoE4A241C, apoE4S263C, and apoE4T289C were kindly provided by Karl Weisgraber and Danny Hatters (Gladdstone Institute, San Francisco) (22). apoE4T89C, apoE4Q98C, and the apoE4S139C pET32a expression plasmids were generated from pET32a-apoE4 using QuikChange® mutisite-directed mutagenesis kit with oligonucleotide primers CGGTGGCGGAGGAGTGGCCGGAGGCTGC for T89C, GTCCAAGAGGCGTGCCGGCCGGACGCGCCG for Q98C, and GTTGGCCCTCCTGCGACACTGCGCAAAG for S139C.

ApoE3, apoE4, apoE3 N-terminal domain, apoE C-terminal domain, apoE4S53C, apoE4S76C, apoE4T89C, apoE4Q98C, apoE4S139C, apoE4A241C, apoE4S263C, and apoE4T289C were all expressed and purified as described previously (21, 31, 32). Sedimentation velocity analysis and circular dichroism spectroscopy were used to relate sedimentation coefficient to molecular mass (30). Data were fitted with time-independent noise, using second derivative regularization with a regularization parameter of 0.95 and a resolution of 100 with a log spaced sedimentation coefficient grid, a persistence length of 36 nm, a dehydrated cross-sectional area of 23 nm^2, a monomer molecular weight of 8914 Da, a hydration of 0.4 g/g, and a partial specific volume of 0.73 ml/g (30).

**Preparative Ultracentrifugation and Gel Electrophoresis**—ApoC-II preformed fibrils were equilibrated with apoE3 (0.1 mg/ml) for 15 min in the presence or absence of 20 mM DHPC. Samples (150 μl) were centrifuged at 100,000 rpm (390,000 × g) for 15 min at 20 °C (TLA-100 rotor, Optima™ MAX, Beckman-Coulter) to sediment the apoC-II fibrils but not unbound apoE3 (35). Control experiments confirmed that apoE alone did not sediment under these conditions. The supernatant was removed, and the pellet was resuspended in the original volume of buffer. The original pre-centrifugation sample, supernatant fraction, and pellet fraction were subjected to SDS-PAGE. Gels were stained with Coomassie Blue R-250.

**Binding Studies**—ApoC-II fibrils were preformed at 112 μM, and Ap β fibrils were formed at 50 μM, and then both fibrils were diluted to a final concentration of 5 μM and incubated with different concentrations of apoE derivatives (0–9 μM) in the presence or absence of 20 mM DHPC in TBS with 2 mg/ml dextran, in a total volume of 200 μl. The presence of dextran provided density stabilization during centrifugation.
and avoided stirring effects upon braking. The samples were equilibrated for 15 min at 25 °C and then centrifuged at 100,000 rpm (390,000 × g) at 25 °C for 7 min (TLA-100 rotor, Optima MAX, Beckman-Coulter). Control experiments with fibrils or apoE alone confirmed that these conditions sediment apoC-II and Aβ-(1–40) fibrils but not unbound apoE (35). After centrifugation the top 150 μl of supernatant solution was removed for analysis of the unbound protein concentration. Unbound protein concentration was determined by fluorescence spectroscopy. Samples were read using a SPEX fluorolog τ-2. The sample was excited at 295 nm, and emission was collected at 350 nm. A standard curve was prepared using the appropriate ligand as the standard in the appropriate buffer. The concentration of unbound ligand was calculated and corrected for the presence of a small amount of free monomeric apoC-II or Aβ-(1–40) measured for control samples of apoC-II or Aβ-(1–40) fibrils in the absence of apoE. The free monomer concentration was determined to include <5% of the total apoC-II or Aβ-(1–40) concentration. SDS-PAGE was used to confirm that the unbound pools of Aβ and apoC-II were not affected by the addition of apoE. Samples were prepared in triplicate. Bound ligand concentrations were calculated by subtraction of the free ligand concentration from the total ligand concentration. The binding curves were fitted to a single site binding model as shown in Equation 1,

\[
\frac{[LR]}{[R]_b} = \frac{n[L]}{K_D + [L]}
\]  
(Eq. 1)

where \([R]_b\) is the total receptor concentration; \(n\) is the binding capacity; \([LR]\) is the bound ligand concentration; \([L]\) is the free ligand concentration, and \(K_D\) is the dissociation equilibrium constant.

**AEDANS Labeling**—Cysteine containing apoE derivatives were all labeled with AEDANS as described previously (22). The labeling efficiency was determined using absorbance at 280 and 337 nm and extinction coefficients for AEDANS of 6100 M⁻¹ cm⁻¹ at 337 nm and 1060 M⁻¹ cm⁻¹ at 280 nm, an extinction coefficient of 44,400 M⁻¹ cm⁻¹ at 280 nm for full-length apoE variants, and an extinction coefficient of 27,960 M⁻¹ cm⁻¹ at 280 nm for apoE3 N-terminal domain (37, 38). Labeling efficiency ranged from 60 to 100%, whereas the apoE3 N-terminal domain used in resonance energy transfer experiments was 100% labeled.

**Fluorescence Emission Spectra**—The emission spectra of AEDANS-labeled derivatives were measured in the presence and absence of preformed apoC-II fibrils in TBS. The AEDANS probe was excited at 336 nm, and emission was collected between 400 and 600 nm. The emission spectra were corrected for the light-scattering contributions of apoC-II fibrils and the buffer alone.

**Acrylamide Quenching**—AEDANS-labeled apoE derivatives in the presence and absence of preformed apoC-II fibrils were incubated in the presence of varying concentrations of acrylamide (0–0.5 M) in TBS. Emission scans of these samples were taken. Stern-Volmer plots were constructed by plotting the fluorescence at the wavelength of the emission maximum, relative to the unquenched fluorescence emission maximum, as a function of acrylamide concentration. Data were fitted to the Stern-Volmer Equation 2,

\[
\frac{F_0}{F} = 1 + K\left(Q\right)
\]  
(Eq. 2)

where \(F_0\) and \(F\) are the fluorescence intensities of the unquenched and quenched fluorophore; \([Q]\) is the concentration of quencher, and \(K\) is the Stern-Volmer coefficient (39).

**Fluorescence Resonance Energy Transfer**—The emission spectra of AEDANS-labeled and unlabeled apoE3 N-terminal domain was measured in the presence and absence of preformed apoC-II or Aβ-(1–40) fibrils in TBS. The tryptophan residues were excited at a wavelength of 295 nm, and emission was collected between 300 and 600 nm. The emission spectra were corrected for the light-scattering contributions of apoC-II or Aβ-(1–40) fibrils and buffer alone. The fluorescence resonance energy transfer (FRET) efficiency (\(E\)) was calculated according to Equation 3,

\[
E = 1 - \frac{F_{DA}}{F_D}
\]  
(Eq. 3)

where \(F_{DA}\) is the fluorescence intensity of the molecule with the donor and acceptor attached, whereas \(F_D\) is the fluorescence intensity of the molecule with only the donor fluorophore attached (39). The FRET distance \(r\) was calculated according to Equation 4,

\[
E = \frac{R_0^6}{R_0^6 + r^6}
\]  
(Eq. 4)

where \(R_0\) is the Förster radius (39).

**Rigid Body Docking**—The apoE3 N-terminal domain crystal structure (PDB code 1LPE) was used for rigid body docking (16). A PDB file of a model of Aβ-(1–42) fibrils composed of 15 peptides was constructed using the best model from the Aβ-(1–42) model structure (PDB code 2BEG) and extending the fibril along the c axis (40). The fully automated online docking program ClusPro was used to dock the structures (41–43). The PDB file for the apoE3 N-terminal domain crystal structure was submitted as the ligand structure, whereas the PDB file of a Aβ-(1–42) fibril composed of 15 peptides was submitted as the receptor structure. The docking program used was ZDOCK, and a clustering radius of 9 Å was used, whereas 2000 structures were selected for clustering consisting of the top 1500 electrostatic hits and the top 500 desolvation hits (41, 42, 44, 45). The top 30 structures were returned. Images were generated using PyMOL (46).

**RESULTS**

**The Interaction of ApoE with ApoC-II Fibrils**—We used analytical ultracentrifugation to examine the isofrom-specific effects of apoE on apoC-II amyloid fibril cross-linking. The sedimentation velocity data for preformed apoC-II fibrils in the presence and absence of apoE3 and apoE4 are presented in Fig. 1. The optical density profiles show that both apoE3 and apoE4 increase the sedimentation rate of apoC-II fibrils. We have shown previously that the increase in the average sedimenta-
tion coefficient of apoC-II fibrils induced by nonfibrillar components such as SAP and apoE is much greater than can be accounted for on the basis of the molecular weight of the bound component. Together with the results of rheological and electron microscopy analysis, the increase in the rate of sedimentations is attributed to a change in the network structure of the fibrils with increased cross-linking and condensation forming more localized fibrillar aggregates (11). Under the conditions used, unbound apoE3 and apoE4 do not sediment to any extent and remain in the supernatant, contributing to the increase in the nonsedimenting optical density observed in Fig. 1, b and c. Analysis of the sedimentation velocity profiles using a \( c(s) \) continuous size distribution model gave excellent fits (Fig. 1) and yielded the sedimentation coefficient distributions shown in Fig. 2a. The data are consistent with our previous studies showing apoE cross-linking of apoC-II fibrils (11). The data also show that apoE3 and apoE4 cross-link the fibrils to the same extent.

To determine whether apoE tetramers are required for cross-linking of apoC-II fibrils, we examined the effect of the short chain phospholipid DHPC on the process. Sedimentation velocity studies confirmed that apoE3 and apoE4 were primarily tetrameric and monomeric in the presence of sub-micellar DHPC (4 mM) and micellar DHPC (20 mM), respectively (data not shown) (13, 18). Fig. 2b shows that the presence of 4 mM DHPC has little effect on the ability of apoE3 and apoE4 to cross-link apoC-II fibrils. However, the addition of micellar DHPC reduced the ability of apoE3 and apoE4 to increase the rate of fibril sedimentation (Fig. 2c). Under these conditions, apoE3 had a greater residual effect on cross-linking than apoE4. A trivial explanation for the reduced ability of apoE to cross-link fibrils in the presence of micellar DHPC is that micelle-bound apoE does not bind to apoC-II fibrils. Preparative ultracentrifugation coupled with SDS-PAGE was used to show that apoE bound to apoC-II fibrils, appearing in the pellet in both the presence and absence of micellar DHPC (results not shown). Together with the results of the sedimentation velocity analysis (Fig. 2), these results demonstrate that although DHPC-solubilized monomers bind to apoC-II fibrils, they do not promote cross-linking.
The salt dependence of apoE-induced cross-linking of apoC-II fibrils was also examined. Fig. 3b shows that 1.5 M NaCl reduces the increase in the modal and weight average sedimentation coefficient of apoC-II fibrils induced by the addition of apoE3. Preparative ultracentrifugation analysis of apoE3 binding to apoC-II fibrils in the presence of 1.5 M NaCl revealed that apoE3 still binds strongly to the fibrils (Fig. 3b, inset). These results demonstrate that the ability of apoE to cross-link apoC-II fibrils is reduced in the presence of high salt concentrations.

Thrombin was used to cleave apoE into its separate N- and C-terminal domains. Complete cleavage and lack of degradation of the domains were confirmed by Western analysis (Fig. 3a, inset). Sedimentation velocity analysis of apoC-II fibrils incubated in the absence or presence of intact apoE3 or thrombin-treated apoE3 are presented in Fig. 3a. The incubation of apoC-II fibrils with thrombin-cleaved apoE3 had much less effect on the sedimentation coefficient distribution of the apoC-II fibrils, compared with intact apoE3 (Fig. 3a). This shows that thrombin cleavage of apoE essentially abolishes the ability of apoE to cross-link apoC-II fibrils and that both domains need to be joined to promote cross-linking.

Fig. 4a compares the effect of 1 μM apoE3 on the rate of sedimentation of apoC-II fibrils with the effects of 10 μM apoE3 N-terminal domain and 10 μM apoE C-terminal domain. The results confirm that the individual domains of apoE lack the ability to cross-link apoC-II fibrils. The domains were tested to determine whether they compete for binding with full-length apoE and inhibit cross-linking. ApoE3 domains (10 μM) were incubated in the presence of 10 μM apoC-II fibrils and 1 μM full-length apoE3 (Fig. 4b). Analysis of the sedimentation distributions shows that apoC-II fibrils incubated with 1 μM full-length apoE3 alone had a modal sedimentation coefficient of 232 S, whereas fibrils incubated with 10 μM N-terminal domain of apoE3 and 1 μM full-length apoE3 had a modal sedimentation coefficient of 105 S, and fibrils incubated with 10 μM C-terminal domain of apoE3 and 1 μM full-length apoE3 had a modal sedimentation coefficient of 119 S. These modal sedimentation coefficients in the presence of the full-length apoE (1 μM) and apoE domains (10 μM) are similar to those in the absence of the full-length apoE3 (Fig. 4a). These findings show that the N-terminal and C-terminal domains of apoE compete with full-length apoE for fibril binding and inhibit fibril cross-linking, suggesting that the binding of both domains to apoC-II fibrils is required for cross-linking to occur.

Affinity and Stoichiometry of ApoE and Its Domains for Amyloid Fibrils—Quantitative binding studies were performed to determine the affinity and stoichiometry of the interaction of apoE and the apoE domains for both apoC-II and Aβ fibrils. The binding curves show that binding of apoE to apoC-II and Aβ fibrils is saturable (Fig. 5a). Analysis of binding data such as that shown in Fig. 5 is complex because of the potential for fibril heterogeneity. In addition, the binding of a ligand to a one-dimensional lattice with overlapping binding sites restricts the ability of subsequent ligands to bind at the overlapping sites (47). Furthermore, in the case of intact apoE significant cross-linking of fibrils occurs (Figs. 1–4). In view of these complications, analysis of the data in Fig. 5, assuming a single class of
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FIGURE 5. Binding curve of apoE3 and the N- and C-terminal domains binding to apoC-II fibrils and Aβ-(1–40) fibrils. apoE3 (a), apoE3 N-terminal domain (b), and apoE C-terminal domain (c) in TBS, pH 7.4, with apoC-II fibrils (filled circles, solid line) or Aβ-(1–40) fibrils (open circles, dashed line). d, binding curves for apoE3 (circles, solid line), apoE3 N-terminal domain (squares, dashed line), and apoE C-terminal domain (triangles, dotted line) binding to apoC-II fibrils in the presence of 20 mM DHPC. Lines show best fits to a one-site saturation model. Error bars show standard deviation of triplicate samples.

TABLE 1

| Variant | Amyloid fibril | $K_d$ | $n$ |
|---------|----------------|------|-----|
| apoE3   | ApoC-II        | 0.31 ± 0.1 | 0.36 ± 0.03 |
| apoE4   | ApoC-II        | 0.22 ± 0.04 | 0.36 ± 0.02 |
| apoE3 N-terminal domain | ApoC-II | 3.4 ± 0.3 | 0.31 ± 0.07 |
| apoE C-terminal domain | ApoC-II | 0.51 ± 0.08 | 0.44 ± 0.02 |
| apoE3 + 20 mM DHPC | ApoC-II | 0.11 ± 0.02 | 0.06 ± 0.003 |
| apoE3 N-terminal domain + 20 mM DHPC | ApoC-II | 2.0 ± 0.8 | 0.24 ± 0.05 |
| apoE C-terminal domain + 20 mM DHPC | ApoC-II | 0.29 ± 0.1 | 0.30 ± 0.02 |
| Aβ     | Aβ             | 0.96 ± 0.5 | 0.26 ± 0.04 |
| apoE4   | Aβ             | 0.51 ± 0.2 | 0.18 ± 0.03 |
| apoE3 N-terminal domain | Aβ | 0.40 ± 0.2 | 0.11 ± 0.02 |
| apoE C-terminal domain | Aβ | 0.16 ± 0.04 | 0.20 ± 0.01 |

$K_d$ and $n$ (binding capacity) from least squares fit assuming a one-site saturation model (Equation 1). Binding data were obtained using 5 μM apoC-II or Aβ fibrils and varying the concentrations of the apoE variants. Errors are S.E. arising from least squares fit.

sites, must be considered an approximation yielding apparent values for the binding constants and stoichiometry. Analysis of apoE3 binding to apoC-II fibrils gave reasonable fits to a one-site saturation model ($R^2 = 0.954$) (Fig. 5a), whereas the binding of apoE3 to Aβ fibrils gave a poorer fit ($R^2 = 0.892$) with evidence of multiephasic behavior, suggesting the presence of a number of classes of binding sites arising from heterogeneity of the Aβ fibrils. The apparent dissociation constants obtained from nonlinear regression analysis, assuming a single class of sites (Table 1), indicates similar affinities of apoE3 for both apoC-II and Aβ fibrils. apoE4 binding to both apoC-II and Aβ fibrils was similar to apoE3 (Table 1). The N-terminal domain of apoE3 bound apoC-II fibrils more weakly than either full-length apoE or the C-terminal domain (Fig. 5, a–c, and Table 1). In contrast, the N-terminal domain had a similar apparent affinity for Aβ fibrils as full-length apoE3 and the C-terminal domain (Fig. 5, a–c, and Table 1).

Fig. 5d shows the binding properties of apoE3 and its N- and C-terminal domains to apoC-II fibrils in the presence of micellar DHPC. The data fit well to a single-site model ($R^2 = 0.984$ for apoE3). The apparent dissociation constants calculated from this model (Table 1) indicate that the DHPC-bound apoE3, N-terminal domain, and C-terminal domain all have a higher affinity for apoC-II fibrils than in the absence of lipid. The apparent stoichiometries indicate that in the absence of lipid, one apoE subunit binds two to three apoC-II monomers, whereas in the presence of micellar DHPC, one apoE subunit binds 10–11 apoC-II monomers (Table 1). The reduction in the amount apoE bound per apoC-II in the presence of lipid is attributed to the larger footprint of lipid-bound monomeric apoE compared with lipid-free apoE tetramer. Taken together, these results show that full-length apoE binds with sub-micromolar affinity to apoC-II and Aβ fibrils via both domains. Lipidation of the apoE increases its affinity for apoC-II fibrils and increases the affinity of the individual domains.

Identification of ApoE Interaction Sites with Amyloid Fibrils—

Fluorescence spectroscopy was used to further characterize the interaction of apoE with amyloid fibrils. apoE3 and eight cysteine variants of apoE4 were labeled with AEDANS and fluorescence emission spectra of AEDANS obtained in the presence and absence of apoC-II fibrils. Fig. 6, a and b, shows typical results for AEDANS-labeled apoE3 and apoE4T89C. Table 2 shows that addition of apoC-II fibrils to AEDANS-labeled apoE4S53C, apoE4S76C, apoE4A241C, apoE4S263C, apoE4S76C, apoE4S139C, apoE4T289C, and apoE3 resulted in a large increases in fluorescence intensity, accompanied by blue shifts in the emission maximum. These changes indicate that the binding of apoE to apoC-II fibrils places the probes in a more hydrophobic environment. Fluorescence quenching by acrylamide was used to further characterize this environmental change. Fig. 7, a and b, showed Stern-Volmer plots of AEDANS-labeled apoE3 and apoE4T89C. Table 2 shows that addition of apoC-II fibrils to AEDANS-labeled apoE4T89C, apoE4S53C, apoE4S76C, apoE4A241C, apoE4S263C, apoE4T289C, and apoE3 resulted in a large increases in fluorescence intensity, accompanied by blue shifts in the emission maximum. These changes indicate that the binding of apoE to apoC-II fibrils places the probes in a more hydrophobic environment. Fluorescence quenching by acrylamide was used to further characterize this environmental change. Fig. 7, a and b, showed Stern-Volmer plots of AEDANS-labeled apoE3 and apoE4T89C. Table 2 shows that addition of apoC-II fibrils to AEDANS-labeled apoE4T89C, apoE4S53C, apoE4S76C, apoE4A241C, apoE4S263C, apoE4T289C, and apoE3 resulted in a large increases in fluorescence intensity, accompanied by blue shifts in the emission maximum. These changes indicate that the binding of apoE to apoC-II fibrils places the probes in a more hydrophobic environment.
resulted in decreases in the Stern-Volmer coefficient. This suggests that the AEDANS on all positions of apoE are in a more hydrophobic environment when bound to apoC-II fibrils. The most notable change in fluorescence intensity upon apoC-II binding was seen for apoE4T89C-AEDANS (Fig. 6b and Table 2). The probe at this position also showed the greatest blue shift upon addition of apoC-II fibrils, suggesting position 89 undergoes the greatest change in environment upon binding to apoC-II fibrils.

**TABLE 2**

| ApoE variant | \( \lambda_{\text{max}} \) in the presence of apoC-II fibrils | \( \Delta \lambda_{\text{max}} \) | Relative change in emission intensity |
|--------------|--------------------------------|----------------|-----------------------------------|
| ApoE4S53C    | 477.0                         | -3.7           | 1.68                              |
| ApoE4S76C    | 482.0                         | -5.9           | 1.61                              |
| ApoE4S76C    | 478.6                         | -8.3           | 2.03                              |
| ApoE4S97C    | 481.9                         | -5.0           | 1.76                              |
| ApoE4A98C    | 479.2                         | -6.9           | 1.59                              |
| ApoE4A98C    | 481.9                         | -4.1           | 1.46                              |
| ApoE4A98C    | 484.8                         | -2.4           | 1.38                              |
| ApoE4A98C    | 483.9                         | -5.8           | 1.57                              |
| ApoE4A98C    | 481.9                         | -4.7           | 1.50                              |

\( \lambda_{\text{max}} \) at emission maximum of AEDANS-labeled apoE cysteine mutants (0.5 \( \mu \)M) in the presence of 20 \( \mu \)M apoC-II fibrils or the absence of apoC-II fibrils is shown. Excitation wavelength was 336 nm. Fluorescence was corrected for the effect of apoC-II fibrils alone, and maximum emission wavelength was derived by fitting spectrum to 4 or 5 Gaussian curves.

**FIGURE 6.** Fluorescence spectra of AEDANS-labeled apoE cysteine variants (0.5 \( \mu \)M) in the absence (solid line) and presence (dotted line) of apoC-II fibrils (20 \( \mu \)M). Samples were excited at 336 nm, and the fluorescence relative to maximum fluorescence of the AEDANS-labeled protein alone is plotted as a function of wavelength.

**FIGURE 7.** Acrylamide quenching of AEDANS-labeled apoE cysteine variants in the presence and absence of apoC-II fibrils. Stern-Volmer plot of AEDANS-labeled apoE cysteine variants (0.5 \( \mu \)M) in the absence (open circles) and presence (closed circles) of apoC-II fibrils (20 \( \mu \)M). Representative data for apoE3 (a) and apoE4T89C (b) are shown. Ratio of unquenched fluorescence to quenched fluorescence at the emission maximum plotted as a function of acrylamide concentration. Samples were excited at 336 nm. Linear best fits to the Stern-Volmer equation are shown. c, calculated Stern-Volmer coefficients of AEDANS-labeled apoE cysteine mutants in the presence (black bars) and absence (white bars) of apoC-II fibrils.
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**FIGURE 8.** Fluorescence emission spectra of apoE3 N-terminal domain in the presence and absence of apoC-II fibrils. The excitation wavelength was 295 nm. Unlabeled apoE3 N-terminal domain (solid line) and apoE3 N-terminal domain labeled with AEDANS (dotted line), at a concentration of 0.5 μM, were analyzed in the absence (a) and presence (b) of 20 μM apoC-II fibrils in TBS, pH 7.4, or in the absence (c) and presence (d) of 20 μM Aβ (1–40) fibrils in TBS, pH 6.7. Fluorescence relative to the maximum fluorescence of unlabeled apoE3 alone is plotted as a function of emission wavelength.

**TABLE 3**

| Fibrils   | pH  | FRET efficiency* | FRET distance* |
|-----------|-----|------------------|----------------|
| ApoC-II | 7.4 | 0.42             | 23.2 Å         |
|          | 7.4 | 0.28             | 25.8 Å         |
| Aβ       | 6.7 | 0.46             | 22.6 Å         |
|          | 6.7 | 0.22             | 27.2 Å         |

*FRET efficiency was calculated according to Equation 3.

**DISCUSSION**

The observation that both the N- and C-terminal domains of apoE are able to compete with full-length apoE and thereby inhibit cross-linking of apoC-II amyloid fibrils by full-length apoE shows that the two domains of apoE interact with the fibrils via independent binding sites (Fig. 4). Further evidence to calculate the average distance between the tryptophans and the AEDANS attached at position 112 (Table 3). FRET studies with apoE and apoC-II fibrils were performed with the tryptophan-deficient apoC-II fibrils, because they avoid intermolecular FRET. Fig. 8, a and b, and Table 3 show that addition of apoC-II fibrils resulted in a reduction in the FRET efficiency, corresponding to an average increase in distance of 2.6 Å between the four tryptophans on helix one and the AEDANS on position 112 of helix three. These results indicate that the apoE N-terminal domain undergoes a conformational change upon binding to apoC-II fibrils with helix three moving an average distance of ~2.6 Å with respect to helix one. FRET experiments were also performed with the AEDANS-labeled apoE3 N-terminal domain in the presence and absence of Aβ fibrils. Similar to the case with apoC-II fibrils, the addition of Aβ fibrils to the apoE3 N-terminal domain resulted in a decrease in the FRET efficiency (Fig. 8, c and d, and Table 3) corresponding to an increase of 4.6 Å in the average distance between the tryptophans and the AEDANS on position 112. Taken together these results indicate that binding of apoE to either apoC-II fibrils or Aβ fibrils results in a conformational change, in which helix three moves with respect to helix one.

apoC-II fibrils (Table 2). This finding is supported by the results in Fig. 7c that show that the AEDANS on position 89 has a larger change in Stern-Volmer coefficient compared with other AEDANS-labeled apoE variants.

In the C-terminal domain, AEDANS-labeled cysteine 289 showed a larger decrease in Stern-Volmer coefficient upon addition of apoC-II fibrils, compared with residues 241 and 263 (Fig. 7c). This suggests that residue 289 interacts directly with apoC-II fibrils or a conformational change occurs close to the C terminus resulting in residue 289 moving to a more hydrophobic environment. In both cases the region at the end of the C terminus is implicated in interacting directly with apoC-II fibrils.

Conformational changes in the apoE N-terminal domain were examined using FRET measurements with purified apoE3 N-terminal domain labeled with AEDANS at position 112. Because the N-terminal domain has all four of its tryptophan residues within the first helix, one can interpret FRET efficiency changes in terms of an average distance of the helix to the single AEDANS acceptor. Previous studies using this approach reveal that FRET distance between the tryptophans and the AEDANS on position 112 is consistent with the average distance of 21 Å from the crystal structure of the apoE N-terminal domain (16, 19). The emission spectrum of the AEDANS-labeled apoE3 N-terminal domain (Fig. 8a) shows a decrease in tryptophan emission compared with the spectrum of unlabeled apoE3 N-terminal domain, consistent with FRET between the tryptophan residues and AEDANS. The Förster radius for FRET between tryptophan residues of the apoE-N-terminal domain and AEDANS on position 112 has been calculated previously as 22 Å (19) and was used to calculate the average distance between the tryptophans and the AEDANS attached at residue 112 (Table 3). FRET studies with apoE and apoC-II fibrils were performed with the tryptophan-deficient apoC-II fibrils to avoid intermolecular FRET. Fig. 8, a and b, and Table 3 show that addition of apoC-II fibrils resulted in a reduction in the FRET efficiency, corresponding to an average increase in distance of 2.6 Å between the four tryptophans on helix one and the AEDANS on position 112 of helix three. These results indicate that the apoE N-terminal domain undergoes a conformational change upon binding to apoC-II fibrils with helix three moving an average distance of ~2.6 Å with respect to helix one. FRET experiments were also performed with the AEDANS-labeled apoE3 N-terminal domain in the presence and absence of Aβ fibrils. Similar to the case with apoC-II fibrils, the addition of Aβ fibrils to the apoE3 N-terminal domain resulted in a decrease in the FRET efficiency (Fig. 8, c and d, and Table 3) corresponding to an increase of 4.6 Å in the average distance between the tryptophans and the AEDANS on position 112. Taken together these results indicate that binding of apoE to either apoC-II fibrils or Aβ fibrils results in a conformational change, in which helix three moves with respect to helix one.
for this was seen in the binding studies showing that the isolated domains of apoE had sub-micromolar affinity for both apoC-II and Aβ fibrils indicating that the interaction of apoE with amyloid fibrils is mediated through both domains (Fig. 5). Fluorescence spectroscopy of AEDANS-labeled cysteine variants of apoE revealed that of the residues examined, residues 89 and 112 within helix three of the N-terminal domain underwent the greatest change in hydrophobicity upon binding to apoC-II fibrils, whereas residue 289 underwent the greatest change in hydrophobicity of the residues examined in the C-terminal domain (Figs. 6 and 7 and Table 2). Among the potential binding sites tested, these observations identify residues 89–112 within helix three of the N-terminal domain and the region near the C terminus of the C-terminal domain as the two independent amyloid-binding sites on apoE. The FRET studies of apoE illustrated that similar conformational changes occur in the apoE N-terminal domain upon binding to either apoC-II fibrils or Aβ fibrils (Fig. 8 and Table 3), implying that apoE interacts with these different fibrils in a similar manner.

**Modeling the ApoE N-terminal Domain-Amyloid Fibril Interaction**—Using the insights provided by these experimental observations, it is possible to model the interaction between the N-terminal domain of apoE and amyloid fibrils. The apoE3 N-terminal domain crystal structure serves as an appropriate model of apoE within the apoE-amyloid fibril complex, whereas a number of high resolution models of amyloid structure have become available, which are useful for modeling the apoE amyloid fibril complex (16, 40, 48–50). Molecular models determined for Aβ-(1–42) and Aβ-(1–40) fibrils are similar (40). We used the rigid-body docking program ZDOCK and ClusPro to generate models of the apoE N-terminal domain-Aβ-(1–42) complex (16, 41, 42, 44, 45, 48). Fig. 9a shows one of the 30 highest ranking structures calculated by ClusPro. This model most closely agreed with the experimental data linking apoE residues 89 and 112 to the interaction. Although atomic detail on this model cannot be achieved, because of the inability to account for small conformational changes and because of the limitations in the Aβ structural model, important features of this model can be identified that are consistent with and give insight into our experimental observations. The model shows close contact between a large section of helix three with evenly spaced negatively charged residues on the fibril (Fig. 9a). The alignment of basic residues in helix three shown in the crystal structure (Fig. 9b), with a distance of 15 Å between Arg-92 and Arg-103 and 23 Å between Arg-103 and Arg-114, illustrates the potential to interact with aligned negative charges on the amyloid fibril. These regularly spaced and negatively charged residues arise from the sole glutamate residue (Glu-22) in the Aβ fibril model. Such negatively charged aligned residues would be a feature of all amyloid fibrils with a parallel in-register conformation, which naturally aligns identical amino acid residues in adjacent subunits of the fibrils (50). Similar patterns of aligned residues would also be a feature of other amyloid fibril subunit arrangements (50). This model illustrates a mechanism by which apoE may interact generally with amyloid fibrils. The interaction of basic residues on apoE helix three with negative charges on the fibril is consistent with the cross-linking and binding study under high salt (Fig. 3b), which suggest that the apoE interaction with amyloid fibrils is partially dependent upon ionic interactions (Fig. 3b). The C-terminal domain of apoE may interact with amyloid fibrils via a similar mechanism. The region identified as the C-terminal domain binding site, involving residue 289, contains a high number of hydrophobic residues. We hypothesize that the C-terminal domain interacts with amyloid fibrils via a pattern of hydrophobic residues in a parallel in-register alignment. SAP is a pattern recognition binding protein and may bind to amyloid fibrils by a similar recognition process (51). This method of recognition could provide a generic basis used by other ligands that interact generally with amyloid fibrils regardless of sequence, such as glycosaminoglycans and monoclonal antibodies specific for amyloid fibrils (3, 4, 52, 53).

**FIGURE 9. Model of apoE N-terminal domain binding to Aβ-(1–42) peptide fibril.** a, model was calculated using the rigid-body docking program ZDOCK program incorporated into ClusPro docking algorithm (41, 42, 44, 45), with the crystal structure of apoE3 N-terminal domain and a fibril constructed from 15 peptides using the high resolution model structure of Aβ-(1–42) peptide (16, 40). The model is viewed perpendicular to the fibril, and β-strand axes with apoE residues outside helix 3 are hidden for clarity. apoE3 N-terminal domain helix three and the Aβ-(1–42) fibril are shown in schematic representation. Residues Thr-89, Arg-90, Arg-92, Arg-95, Arg-103, Cys-112, Arg-114, and Arg-119 of apoE3 and E22 of Aβ are shown in space-filling representation. b, crystal structure of helix three of apoE3 N-terminal domain, with distances between basic residues indicated. Images were generated using PyMOL (46).
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free x-ray structure, the model and results are relevant to the lipoprotein-bound form of apoE. Lipidation of apoE and its domains has only a small effect on the affinity for apoC-II fibrils, suggesting that there is limited structural change in apoE at the sites of interaction. This is consistent with models of lipoprotein-bound apoE, which suggest that helix three is curved around the lipoprotein surface, with the hydrophobic face interacting with the lipid surface (54, 55). This conformation would still allow basic residues on helix three of apoE to interact with alignments of negative charges on amyloid fibrils as shown in Fig. 9.

Implication of Amyloid Fibril Cross-linking in Vivo—The observation that apoE cross-linking of apoC-II fibrils is significantly diminished in the presence of picellar concentrations of DHPC (Fig. 2), yet full-length apoE and the individual domains of apoE are able to bind with similar affinities in the presence of picellar DHPC (Fig. 5d), indicates that tetrameric apoE is required for cross-linking, whereas DHPC-solubilized monomeric apoE lacks this ability. Although apoE has various proposed roles in Alzheimer disease, including effects on Aβ (1–40) fibrillogenesis, Aβ production, Aβ toxicity, tau phosphorylation, and neurite outgrowth (56), it is possible that cross-linking of amyloid fibrils contributes to the pathology of the disease. It is of interest that apoE4 is more prone to proteolytic cleavage than apoE3 (57). Such cleavage would inhibit the cross-linking of fibrils and present a potentially important mechanism for isoform-specific differences in apoE cross-linking in vivo.

The observation that apoE binds to both apoC-II fibrils and Aβ(1–40) fibrils with similar affinities, via both domains, and undergoes similar conformational changes upon binding suggests a common mechanism for the ability of apoE to cross-link amyloid fibrils. The findings that cross-linking is dependent upon apoE tetramer, connectivity of the two domains, the binding of both domains to the fibrils, and the finding that neither the N-terminal nor C-terminal domain alone can cross-link fibrils provides a great deal of insight into the mechanism by which apoE cross-links fibrils. The fact that the C-terminal domain alone does not cross-link fibrils, yet is tetrameric and is able to bind to fibrils, is explained by one of two possibilities. Either the tetrameric apoE C-terminal domain has only one binding site and therefore can only bind to one fibril or this domain has a number of binding sites and steric hindrance prevents binding to more than one fibril at any one time. Monomeric apoE possesses at least two binding sites, an N-terminal domain binding site and a C-terminal domain binding site, yet it lacks the ability to cross-link amyloid fibrils. This suggests that steric hindrance or lipid-induced conformational changes prevent monomeric apoE binding to two fibrils simultaneously. The formation of an apoE tetramer must therefore produce a conformation that allows both the N-terminal domain to bind to one fibril and the C-terminal domain to bind to a different fibril, thereby increasing network structure. The observation that the C-terminal domain inhibits the ability of full-length apoE to cross-link fibrils indicates that in the tetrameric form the N-terminal domains can only bind to a single fibril, again possibly because of steric hindrance.

There is growing evidence to support a physiological role for amyloid deposits within atherosclerotic plaques (26, 29, 58–63). In this regard, the ability of apoE to cross-link different types of fibrils and condense them into more compact aggregates has several implications. More condensed deposits may have altered permeability to nutrients, and their reduced bulk could affect vessel cross-section and elasticity. Cross-linking of amyloid fibrils may affect the steady-state levels of toxic amyloid intermediates that have been postulated to mediate the disease-related effects of amyloid fibrils (64, 65). ApoE- and SAP-mediated cross-linking of amyloid fibrils may also affect the ability of amyloid fibrils to activate macrophages (25). ApoE knock-out mice are widely used as models for the accelerated development of atherosclerosis (66). The ability of apoE to cross-link amyloid fibrils may contribute to the atheroprotective effects of apoE. Additionally, the ability of apoE to cross-link amyloid fibrils may explain the requirement for apoE in the deposition of Aβ, in mouse models of Alzheimer disease (67, 68). It is currently not clear whether these proposed cross-linking functions of apoE play an overall protective or detrimental role in amyloid diseases. The results presented in this study suggest that if fibril cross-linking does play a role in the progression of amyloid diseases, then inhibition of either the N-terminal or C-terminal domain interactions could become therapeutic targets.

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REFERENCES

1. Namba, Y., Tomonaga, M., Kawasaki, H., Otomo, E., and Ikeda, K. (1991) Brain Res. 541, 163–166
2. Wisniewski, T., and Frangione, B. (1992) Neurosci. Lett. 135, 235–238
3. Sipe, J. D., and Cohen, A. S. (2000) J. Struct. Biol. 130, 88–98
4. Kisilevsky, R. (2000) Amyloid 7, 23–25
5. Corder, E. H., Saunders, A. M., Strittmatter, W. J., Schmechel, D. E., Gaskell, P. C., Small, G. W., Roses, A. D., Haines, J. L., and Pericak-Vance, M. A. (1993) Science 261, 921–923
6. Mahley, R. W., and Rall, S. C., Jr. (2000) Annu. Rev. Genomics Hum. Genet. 1, 507–537
7. Hatters, D. M., Zhong, N., Rutenber, E., and Weisgraber, K. H. (2006) J. Mol. Biol. 361, 932–944
8. Fadila, G. O., and Baumann, M. (2002) Amyloid 9, 75–82
9. LeVine, H. J. (2000) Amyloid 7, 83–89
10. Yamaguchi, I., Hasegawa, K., Takahashi, N., Geiyo, F., and Naiki, H. (2001) Biochemistry 40, 8499–8507
11. MacRaid, C. A., Stewart, C. R., Mok, Y. F., Gunzburg, M. I., Perugini, M. A., Lawrence, L. J., Tirta, Madja, V., Cooper-White, J. J., and Howlett, G. J. (2004) J. Biol. Chem. 279, 21038–21045
12. Aggerbeck, L. P., Wetterau, J. R., Weisgraber, K. H., Wu, C. S., and Lindgren, F. T. (1988) J. Biol. Chem. 263, 6249–6258
13. Perugini, M. A., Schuck, P., and Howlett, G. J. (2000) J. Biol. Chem. 275, 36758–36765
14. Wetterau, J. R., Aggerbeck, L. P., Rall, S. C., Jr., and Weisgraber, K. H. (1988) J. Biol. Chem. 263, 6240–6248
15. Innerarity, T. L., Friedlander, E. J., Rall, S. C., Jr., Weisgraber, K. H., and Mahley, R. W. (1983) J. Biol. Chem. 258, 12341–12347
