Examination of Lipid-bound Conformation of Apolipoprotein E4 by Pyrene Excimer Fluorescence*

Received for publication, December 14, 2004, and in revised form, February 11, 2005
Published, JBC Papers in Press, February 11, 2005, DOI 10.1074/jbc.M414019200

Jessica Drury and Vasanthy Narayanaswami‡
From the Lipid Biology in Health and Disease Research Group, Children's Hospital Oakland Research Institute, Oakland, California 94609

Apolipoprotein E (apoE) is a 34-kDa resident of lipoproteins that plays a key role in cholesterol homeostasis in plasma and in brain. It is composed of an N-terminal (NT) domain (residues 1–191) and a C-terminal (CT) domain (residues 201–299). Of the three major isoforms (apoE2, -E3, and -E4), apoE4 is considered a risk factor for both cardiovascular and Alzheimer disease. Compared with apoE3, domain interaction between NT and CT domains is believed to direct the lipoprotein distribution preference of apoE4 for very low density lipoprotein-sized particles. We examined the relative disposition of apoE4 NT and CT domains in lipid-free and lipid-bound forms by monitoring pyrene excimer fluorescence emission as a direct indicator of spatial proximity. Site-specific labeling of apoE4 by N-(1-pyrene)maleimide was accomplished after substitution of Cys residues for Arg-61 in NT domain and Glu-255 in CT domain. Pyrene labeling did not alter the lipoprotein distribution pattern of apoE4 in plasma. Pyrene excimer fluorescence was noted in lipid-free pyrene-R61C/E255C/apoE4 in mixtures containing excess wild-type apoE4, which was attributed to intramolecular spatial proximity between these specified sites. Upon disruption of tertiary interaction, a large decrease in excimer fluorescence emission was noted in pyrene-R61C/E255C/apoE4 in dimyristoylphosphatidylcholine/pyrene-R61C/E255C/apoE4 discoidal complexes, pyrene excimer fluorescence emission was retained. Taken together with fluorescence quenching and cross-linking analysis, a looped-back model of apoE4 is proposed in lipid-bound state, including spherical lipoprotein particles, wherein residues Arg-61 and Glu-255 are proximal to one another.

Apolipoprotein E (apoE)1 belongs to the super family of exchangeable apolipoproteins that are major constituents of lipoproteins (1). It plays a crucial role in lipid homeostasis, not only in the plasma but also in the brain. ApoE exerts a protective effect against development of atherosclerosis (2, 3) by promoting cellular uptake (4) and clearance of triglyceride-rich lipoproteins from blood (5, 6). In addition, apoE in macrophages has a direct antiatherogenic effect independent of alterations in plasma lipoproteins (7) by promoting cholesterol efflux (8) from cell lining of arteries by a process termed reverse cholesterol transport (9–11). The role of apoE in cholesterol homeostasis in the brain is emerging with evidence of its protective role against neuronal injury and neurodegeneration (12).

In humans, apoE displays polymorphism with three major genetic variants identified in the population, apoE2, -E3, and -E4, with allelic frequencies of 8%, 77%, and 14%, respectively, determining six apoE phenotypes, apoE2/E2, apoE3/E3, apoE4/E4, apoE2/E3, apoE2/E4, and apoE3/E4 (13). The apoE isoforms determine the atherogenic fate of the lipoproteins on which they reside. Although the apoE2 isoform is associated with type III hyperlipoproteinemia, peripheral atherosclerosis, and accumulation of remnant lipoproteins, apoE4 is consistently associated with higher levels of low density lipoprotein (LDL) and cholesterol in plasma, and persons bearing the apoE4 isoform are more prone to develop atherosclerosis than those with the apoE3 isoform, which is generally considered antiatherogenic (5, 14). In addition, the apoE4 allele is strongly linked to both sporadic and familial late-onset Alzheimer disease, although the precise mechanistic details of this association are unclear (15, 16).

Understanding the structural basis of apoE isoforms provides insights into its physiological role in lipoprotein metabolism. ApoE is a 34-kDa, 299-residue protein composed of a 22-kDa N-terminal (NT) domain (residues 1–191) folded as a four-helix bundle (17) and a 10-kDa C-terminal (CT) domain (residues 201–299) that promotes apoE oligomerization (18–22). The two domains are linked via a protease-sensitive linker loop region. The three isoforms differ at positions 112 and 158: apoE2 bears cysteines and apoE4 bears arginines at both sites, whereas apoE3 contains a cysteine and an arginine at positions 112 and 158, respectively. Thus, the differences between the three isoforms lie in the NT domain, which houses receptor-binding sites (residues 130–150) for the LDL receptor family. Whereas apoE3 and -E4 display identical receptor binding activities, apoE2 seems to bear 1–60% activity compared with apoE3 (13). In turn, subtle differences exist in the lipoprotein binding preference of apoE3 and -E4; apoE3 displays a preference for high density lipoprotein (HDL)-sized particles, whereas apoE4 displays a preference for the larger very low density lipoprotein (VLDL)-sized particles compared with apoE3 (23–27).

Employing a combination of x-ray analysis of isolated apoE3 and E4 NT domains, site-directed mutagenesis, and plasma lipoprotein binding analysis of intact apoE, the presence of Arg at position 112 in apoE4 has been suggested to lead to salt bridge formation with Glu-109, which in turn displaces Arg-61 to potentially form a salt bridge with Glu-255 in the CT domain. ApoE3, however, bearing a Cys at position 112, accom-
modates Arg-61 in a locale where it is unable to form a salt bridge with Glu-255. The salt bridge between Arg-61 and Glu-255 has been postulated to contribute to domain-domain interaction, which in turn directs the lipoprotein binding preference of apoE4 for VLDL (23, 24). Indeed, replacement of Arg-61 in apoE4 with threonine (as noted in mouse apoE) shifts the lipoprotein binding preference from VLDL to HDL (28).

In the present study, we examined the relative disposition between apoE4 NT and CT domains in lipid-free and bound states, taking advantage of the unique fluorescence properties of N-(1-pyrene)maleimide as a probe of spatial proximity. We monitored the location of Arg-61 and Glu-255 with respect to each other in different environments and concluded that they remained proximal to each other after lipid interaction.

**EXPERIMENTAL PROCEDURES**

**Materials**—N-(1-Pyrene)maleimide and tris(2-cyanoethyl)-phosphine were obtained from Molecular Probes, Inc. (Eugene, OR). Di-methyl suberimidate (DMS) was from Pierce Biotechnology. Dimyristoylphosphatidylcholine (DMPC) was obtained from Sigma. All other chemicals and solvents were of analytical grade.

**Site-directed Mutagenesis and Protein Expression**—Cysteine residues were introduced into human apoE4 by site-directed mutagenesis as described previously, and the DNA sequences were verified (29). Arg-61 and Glu-255 were replaced by Cys to create a double-cysteine-containing apoE4 variant R61C/E255C/apoE4. Recombinant human wild-type (WT) and R61C/E255C/apoE4 were expressed in *Escherichia coli* using the pTYB2 vector and purified using the IMPACT-CN system (New England BioLabs, Beverly, MA) as described earlier (30). The purified protein was present in 25 mM Tris-HCl, pH 7.4, containing 140 mM NaCl and 3 mM KCl (TBS), unless otherwise specified.

**Pyrene Modification**—R61C/E255C/apoE4 was initially incubated with 5-fold molar excess tris(2-cyanethyl)-phosphine for 2 h at 37 °C to maintain all cysteine groups in a reduced state, followed by incubation with a 5-fold molar excess of N-(1-pyrene)maleimide at 37 °C for 16 h (31). Unbound N-(1-pyrene)maleimide was removed by gel filtration chromatography. The stoichiometry of labeling was calculated to be 1.9 pyrene/apoE4 molecule in the pyrene-labeled variant (pyr-R61C/E255C/apoE4), using the extinction coefficient for pyrene ε = 40,000 M⁻¹ cm⁻¹. Circular dichroism spectroscopy of the modified variant revealed that the labeling did not affect the overall secondary structural characteristics of apoE4.

**Fluorescence Measurements**—Fluorescence spectra were recorded on a Perkin-Elmer MFP-44B spectrofluorometer. A slit width of 5 nm was used for both excitation and emission monochromators. Emission spectra were recorded from 370 to 410 nm, whereas the area under the excitation peak was calculated from 440 to 510 nm. To monitor excimer formation, the excimer emission peak was calculated from 370 to 410 nm, whereas the area under the excitation peak was calculated from 440 to 510 nm.

**Fluorescence Emission Analysis of Lipid-associated pyr-R61C/E255C/apoE4 Fluorescence**—Fluorescence emission spectra of DMPC/pyr-R61C/E255C/apoE4 complexes were recorded after equilibrating samples of pyr-R61C/E255C/apoE4 with 50 mM sodium phosphate (pH 6.0, 7.0, and 8.0) and 50 mM sodium acetate (pH 3.0, 4.0, and 5.0) for 16 h, followed by fluorescence analysis. The effect of micelle formation was followed by incubating 12 µg of pyr-R61C/E255C/apoE4 with 0.4% lysophosphatidylcholine (lyso-PC).

**Preparation and Characterization of DMPC/pyr-R61C/E255C/apoE4 Complexes**—Discoidal complexes were prepared as described previously (30). After disc formation, pyr-R61C/E255C/apoE4 was mixed with a 10-fold molar excess of WT apoE to enhance the possibility of the presence of one pyr-R61C/E255C/apoE4 molecule surrounded by several WT apoE on a lipoprotein complex. Discoidal complexes were separated from unbound protein by density gradient ultracentrifugation, followed by extensive dialysis. The phospholipid and protein content were estimated using the phospholipids assay kit (Wako Chemicals GmbH, Neuss, Germany) and the bicinchoninic acid kit (Pierce Biologicals, respectively, to calculate lipid/protein ratio of the constituted lipoprotein particles. Non-denaturing PAGE of the isolated lipoprotein complexes was carried out to evaluate the molecular mass and size of the particle on a 4–20% gradient gel for 2 h at 150 V and stained with Amido Black. The particle sizes were calculated from a calibration curve using the following standards and their corresponding Stokes diameters: thyroglobulin, 17 nm; ferritin, 12.2 nm; catalase, 8.2 nm; and lactate dehydrogenase (LDH), 4.2 nm. Microscopic analysis, DMPC/pyr-R61C/E255C/apoE4 complexes were dialyzed against ammonium acetate buffer followed by negative staining with 2% sodium phosphotungstate as described previously (Zeis 10; 80 kV) (32).

**Cross-linking Studies**—Cross-linking of DMPC/pyr-R61C/E255C/apoE4 (10 µg of protein) was carried out at DMPC concentrations of 0.46, 4, 40, and 230 nm for 2 h at 37 °C (33). DMPC was prepared in 1 µL triethanolamine HCl, pH 9.7. The volume of 1 µL triethanolamine HCl was maintained at one tenth of the final volume of the reaction mixture. DMPC is a non-cleavable homobifunctional, amine-specific cross-linker with a spacer arm of 11.4 Å. The reaction was stopped by the addition of SDS-PAGE sample treatment buffer followed by electrophoresis on 4–20% acrylamide gradient gel.

**Fluorescence Emission Analysis of Lipid-associated pyr-R61C/E255C/apoE4—Fluorescence emission analysis of lipid-protein-associated pyr-R61C/E255C/apoE4 was carried out to evaluate lipid-triggered spatial repositioning of apoE4 domains on reconstituted discoidal lipoprotein particles and on large and small spherical lipoprotein particles. Fluorescence emission spectrum of DMPC/pyr-R61C/E255C/apoE4 complexes (12 µg of protein) was recorded after excitation at 340 nm. Pyr-R61C/E255C/apoE4 was incubated with various lipoprotein classes and the various lipoprotein classes and the various lipoproteins were used as described above. Fluorescence emission spectra of VLDL- and HDL-associated pyr-R61C/E255C/apoE4 were recorded (fractions 7 and 21, respectively). Spectra of corresponding lipoprotein factions collected from plasma incubations without any added pyr-R61C/E255C/apoE4 were subtracted to account for background fluorescence contribution.

**Quenching Studies**—Quenching of fluorescence emission of lipid-free pyr-R61C/E255C/apoE4 and DMPC/pyr-R61C/E255C/apoE4 complexes was carried out as described earlier (31, 35) using aqueous quenchers such as potassium iodide (KI) to evaluate the degree of solvent exposure of the fluorophores. Increasing concentrations of KI were added in 50 mM potassium phosphate, pH 7.4, with 1 mM sodium thiosulfate to suppress free iodine formation. To assess the depth of location of the fluorophores with respect to the phospholipid bilayer in DMPC/pyr-R61C/E255C/apoE4 discoidal complexes, 5-DOXYL stearic acid (5-DSA) and 12-DOXYL stearic acid (12-DSA) were employed as extrinsic fluorophores. The 5-DSA (12-DSA) was excited at 370 nm (400 nm), and fluorescence intensities were measured at 375 nm after equilibration for 5 min. Effective quenching constants were calculated employing the Stern-Volmer equation, 

\[
F/F_0 = 1 + K_{SV}[Q],
\]

where \(F\) and \(F_0\) are the fluorescence intensities in the absence and the presence of varying quencher concentrations, respectively, \(K_{SV}\) is the Stern-Volmer quenching constant, and \(Q\) is the quencher concentration (36). Quenching by KI and DSA cannot be compared directly by the classic Stern-Volmer equation because, in the case of DSA, the quencher is limited to the context of the bilayer and is not freely diffusing in solution. Therefore, apparent quenching constants were calculated for purposes of comparison between 5-DSA and 12-DSA.
Pyrene Excimer Fluorescence: An Indicator of Spatial Proximity—When attached specifically to a single free sulfhydryl group, pyrene typically exhibits fluorescence emission maxima at 375 and 395 nm (excitation at 340 nm), attributed to a monomeric moiety. However, when there is a second bound pyrene within 10 Å in spatial proximity, it displays an additional broad and red-shifted fluorescence emission peak (~460 nm), attributed to formation of an excited state dimer or ‘excimer’ (37). We exploit this unique spectral feature of pyrene to assess lipid-triggered repositioning of two specific sites, one in the NT and the other in the CT domain of apoE4, in an approach successfully employed to evaluate spatial proximity in other proteins (37–40) including apolipoproteins such as apolipoporphin III (31, 41). Furthermore, when two pyrene molecules giving rise to excimer fluorescence move away from each other, the excimer fluorescence emission intensity is decreased or lost entirely. We employed site-directed mutagenesis to substitute cysteines in WT apoE4 (lacks endogenous cysteine residues) at positions 61 and 255, located in the NT and CT domains, respectively, yielding R61C/E255C/apoE4. R61C/E255C/apoE4 was labeled with N-(1-pyrene)maleimide followed by secondary structural analysis using circular dichroism spectroscopy, which indicated that the global fold of the labeled variant was similar to that of WT apoE4 (data not shown).

Pyrene Excimer Fluorescence in Lipid-free pr-R61C/E255C/apoE4—Fluorescence emission spectrum of pr-R61C/E255C/apoE4 alone (in the absence of excess WT apoE4) in aqueous buffer (Fig. 1) reveals the presence of a strong unstructured excimer peak centered at ~460 nm, in addition to the characteristic emission at 375, 385, and 395 nm attributed to vibronic transitions. Because excimer formation is an excited state event, the emission spectrum of pr-R61C/E255C/apoE4 is not a mirror image of its excitation spectrum (data not shown) (42). Excimer emission formation is indicative of spatial proximity between positions 61 and 255 in lipid-free apoE4. However, apoE has been indicated to exist in solution as a tetramer by protein-protein interactions via its CT domain (18, 21, 22, 43), leading us to examine whether the excimer emission originates from intermolecular spatial proximity between these specified sites. Pr-R61C/E255C/apoE4 was mixed with a 10-fold molar excess of WT apoE4 under denaturing conditions, followed by slow refolding as described under “Experimental Procedures.” This increases the likelihood that a given molecule of pr-R61C/E255C/apoE4 is surrounded by unlabeled WT apoE4 in the native tetrameric organization. Fluorescence measurements reveal that excimer fluorescence emission was retained in these mixtures, indicative of intramolecular rather than intermolecular spatial proximity between pyrene moieties residing on R61C and E255C in apoE4.

Lipoprotein Binding Behavior of pr-R61C/E255C/apoE4—To ascertain that modification of apoE4 did not alter its lipoprotein-binding behavior, pr-R61C/E255C/apoE4 was incubated with human plasma, followed by examination of its distribution profile among the various lipoprotein fractions by size-exclusion chromatography. The location of pr-R61C/E255C/apoE4 was monitored by fluorescence analysis of each fraction (Fig. 2). Taking the sum of fluorescence intensities of all lipoprotein-bound fractions as 100%, the percentage distributions of pr-R61C/E255C/apoE4 among the various lipoprotein fractions were calculated to be as follows: 38% in VLDL (fractions 5–9); 25% in intermediate density lipoprotein (IDL) (fractions 17–21); 37% in HDL (fractions 17–21). This distribution profile is largely consistent with that reported previously (24), indicating that the distinctive binding preference of apoE4 is retained in pr-R61C/E255C/apoE4. A minor difference observed in the present case is the larger percentage of unbound pr-R61C/E255C/apoE4 that is probably a result of the excess apoE4 total protein ratio used under our experimental conditions.

Effect of Alteration in Tertiary Contacts on Pyrene Excimer Formation—To validate the use of pyrene excimer fluorescence as a sensitive monitor of spatial proximity in apoE4, we employed three independent tools with predictable features: effect of TFE, effect of altering pH, and effect of micelle formation with lyso-PC on pyrene excimer fluorescence. TFE is a solvent that destabilizes tertiary interactions in proteins while stabilizing and increasing α-helical structures. The effect of increasing TFE concentration on excimer fluorescence in pr-R61C/E255C/apoE4 was evaluated (Fig. 3A). A gradual decrease in excimer fluorescence intensity was noted with incremental increase in TFE (0–50% v/v), with maximal decrease noted at 50% TFE. The decrease in excimer emission intensity in 50% TFE was accompanied by a decrease in the fluorescence intensity ratio of 395 nm (band III) to 375 nm (band I), indicative of an increase in local environment polarity (44–46).
that the decrease in excimer formation is the result of disruption of tertiary interaction between specified sites on NT and CT domains, leading to movement of R61C away from E255C. Next, the effect of altering pH on excimer fluorescence in pyr-R61C/E255C/apoE4 was examined (Fig. 3B). No alteration in excimer fluorescence occurred at pH 8.0 and pH 6.0, compared with that noted at pH 7.0. However, a substantial increase in excimer was observed at pH 5.0, followed by a trend of decrease at pH 4.0 and pH 3.0. The increase in excimer fluorescence emission may be attributed to: 1) movement of the pyrene moieties closer with respect to each other in a given population of pyr-R61C/E255C/apoE4 (pI 5.6 for R61C/E255C/apoE4) or 2) an increase in the fraction of apoE4 variant bearing proximal pyrene moieties. A similar movement of the two domains toward each other around pH 5 was also noted in earlier studies for apoE3 (pI 5.5), wherein sites Cys-112 and Trp-264 at the NT and CT domains, respectively, repositioned closer by 5 Å (30). It is conceivable that gradual protonation of the negatively charged residues draws the two domains toward each other (47, 48). Finally, the effect of micelle formation on excimer fluorescence in pyr-R61C/E255C/apoE4 was examined (Fig. 3C). In the presence of 0.4% lyso-PC, a loss in excimer fluorescence was noted, consistent with movement of the two domains away from each other on a micellar surface. Taken together, these results validate the use of pyrene excimer fluorescence to monitor alterations in tertiary contacts in apoE4.

Effect of Lipid Interaction of pyr-R61C/E255C/apoE4 on Pyrene Excimer Formation—The relative spatial positioning of R61C and E255C in pyr-R61C/E255C/apoE4 was examined on three different lipid surfaces: reconstituted discoidal particles, plasma HDL, and VLDL particles. Because several apoE molecules are expected to be present on lipid particles, the presence of excess WT apoE4 reduced the likelihood of intermolecular excimer fluorescence formation. In the first case, DMPC-pyr-R61C/E255C/apoE4 complexes (10 µg of protein) were incubated with increasing concentrations of DMPC at 24 °C for 2 h. The reaction was stopped by the addition of SDS-PAGE sample treatment buffer followed by electrophoresis on 4–20% acrylamide gradient gel. The lane assignments are as follows: lane 1, DMPC/pyr-R61C/E255C/apoE4; lanes 2–4, DMPC/pyr-R61C/E255C/apoE4 treated with 0.46, 4.6, and 23 mM DMS, respectively. Arrows on the right indicate presence of two or four cross-linked apoE. The positions of molecular mass markers are indicated on the left, with the corresponding masses in kilodaltons. The presence of 0.4% lyso-PC, a loss in excimer fluorescence was noted, consistent with movement of the two domains away from each other on a micellar surface. Taken together, these results validate the use of pyrene excimer fluorescence to monitor alterations in tertiary contacts in apoE4.

Effect of Lipid Interaction of pyr-R61C/E255C/apoE4 on Pyrene Excimer Formation—The relative spatial positioning of R61C and E255C in pyr-R61C/E255C/apoE4 was examined on three different lipid surfaces: reconstituted discoidal particles, plasma HDL, and VLDL particles. Because several apoE molecules are expected to be present on lipid particles, the presence of excess WT apoE4 reduced the likelihood of intermolecular excimer fluorescence formation. In the first case, DMPC-pyr-R61C/E255C/apoE4 complexes (10 µg of protein) were incubated with increasing concentrations of DMPC at 24 °C for 2 h. The reaction was stopped by the addition of SDS-PAGE sample treatment buffer followed by electrophoresis on 4–20% acrylamide gradient gel. The lane assignments are as follows: lane 1, DMPC/pyr-R61C/E255C/apoE4; lanes 2–4, DMPC/pyr-R61C/E255C/apoE4 treated with 0.46, 4.6, and 23 mM DMS, respectively. Arrows on the right indicate presence of two or four cross-linked apoE. The positions of molecular mass markers are indicated on the left, with the corresponding masses in kilodaltons.
corresponding to a molecular mass of 68 kDa and higher appeared. This provides evidence of intermolecular cross-linking, suggesting the presence of approximately four molecules of apoE per lipoprotein particle. In addition, bands corresponding to monomeric apoE4 were present at all concentrations of DMS used, possibly representing intramolecular cross-linked apoE and apoE bearing covalently attached DMS.

Fluorescence emission analysis of DMPC-pyr-R61C/E255C/apoE4 particles indicated that excimer fluorescence was retained (Fig. 5). We conclude that excimer fluorescence arose from intramolecular proximity between R61C and E255C in the context of reconstituted lipoprotein particles. In recent investigations, several lines of evidence indicated formation of discoidal phospholipid bilayer complexes of DMPC/apoE3, with the α-helices circumscribing the periphery of the particles (30, 49, 50). Furthermore, examination of VLDL- and HDL-bound pyr-R61C/E255C/apoE4 revealed excimer fluorescence (Fig. 6), consistent with spatial proximity between R61C and E255C in the context of spherical particles.

Fluorescence Quenching—To assess the relative location of pyrene fluorophores with respect to the lipid in representative DMPC/pyr-R61C/E255C/apoE4 complexes, two different types of fluorescence quenchers were employed: water-soluble and lipid-based quenchers. KI has been routinely employed as an aqueous collisional quencher that yields information about the microenvironment of the fluorophores. KSV was calculated to be 4.4 ± 0.5 m⁻¹ and 22.0 ± 3.0 m⁻¹ for KI quenching of lipid-free pyr-R61C/E255C/apoE4 and DMPC/pyr-R61C/E255C/apoE4, respectively. The 5-fold increase in KSV upon lipid binding indicates a dramatic alteration in the microenvironment of the fluorophores. The ability of KI to quench pyrene fluorescence emission in DMPC/pyr-R61C/E255C/apoE4 suggests localization of the pyrene moieties toward the lipid/water interface of the phospholipid bilayer. This approach was complemented with the second class of quenchers, spin-labeled fatty acids, which are lipid-based collisional quenchers. 5-DSA and 12-DSA act as molecular rulers, enabling assessment of the depth of location of the fluorophores with respect to the phospholipid bilayer (51). The apparent KSV values for 5-DSA and 12-DSA were calculated to be 20.2 ± 5.8 × 10⁻³ m⁻¹ and 2.1 ± 0.2 × 10⁻³ m⁻¹, respectively. The 10-fold higher KSV for 5-DSA compared with 12-DSA indicates that the pyrene fluorophores are in a superficial location with respect to the membrane bilayer.

Taken together, we examined whether spatial proximity between positions 61 and 255 is retained upon interaction with a lipid surface by monitoring the signature spectral feature of pyrene. An apoE4 variant bearing Cys at these positions was constructed, which enabled us to selectively label with pyrene moiety employing the maleimide functional group. Secondary structural characterization indicated that the labeled apoE4 variant retained the overall fold of WT apoE4.

The Cys-to-Arg switch at position 112 in apoE4 compared with apoE3 has been proposed to direct its lipoprotein binding preference to VLDL-sized particles (23, 24), with the positive charge at this position being an important factor (27) in forming a salt bridge with Glu-109 (23). The salt bridge between positions 109 and 112 in turn displaces Arg-61 from a neighboring helix, thereby making it unavailable for salt bridge interaction with Glu-255 in the CT domain. This seems to be a unique case of salt bridge interaction because most salt bridges found in proteins (52), including those in apoE NT domain helix bundle (17), are formed between sequentially close residues. Human apoE is unique in that it bears Arg at position 61, whereas most other species have Thr at this position. Substitution of Arg-61 with Thr altered the lipoprotein preference of apoE4 from VLDL to HDL. Thus, the combination of positive charge at position 112 with Arg at position 61 seems to be responsible for mediating NT domain-CT domain interaction and the lipoprotein binding preference of apoE4. Therefore, in the present study, it was important for us to establish that substitution of Arg-61 and Glu-255 by Cys followed by covalent attachment with pyrene residues did not alter the lipoprotein-

![Fluorescence emission spectra of pyr-R61C/E255C/apoE4 bound to plasma lipoprotein fractions](image_url)

**FIG. 6.** Fluorescence emission spectra of pyr-R61C/E255C/apoE4 bound to plasma lipoprotein fractions. Fluorescence emission spectra of VLDL-pyr-R61C/E255C/apoE4 (curve a) or HDL-pyr-R61C/E255C/apoE4 (curve b) were recorded on fast performance liquid chromatography fractions 7 and 21, respectively.

![Schematic representation of lipid-binding interaction of apoE4](image_url)

**FIG. 7.** Schematic representation of lipid-binding interaction of apoE4. Lipid binding interaction of apoE4 (A) is proposed to trigger a conformational change involving movement of NT and CT domains and adoption of a looped-back extended helical conformation (B). Arg-61 and Glu-255 are represented as white circles. Lipid-free apoE4 is depicted as a monomer for the sake of simplicity, with the CT domain modeled as a series of helical structures (gray cylinders) linked to the NT domain (ribbon structure) (Dong et al., 1998) via a protease-sensitive loop (A). The lipid-bound conformation is probably valid for both discoidal HDL particles and spherical VLDL or HDL particles. The entire apoE molecule is depicted as a series of helical structures (gray cylinders) in lipid-bound state (B). In the case of discoidal particles, the helices are proposed to circumscribe a bilayer of phospholipid molecules with the helical axis oriented perpendicular to the plane of the bilayer. In the case of spherical particles, it is envisaged that the helices are embedded at the lipid/water interface near the phospholipid head groups as indicated previously for other exchangeable apolipoproteins (Sahoo et al., 1998).
Lipid-bound Conformation of Apolipoprotein E4

binding behavior of apoE4. Our studies indicated that we have effectively placed spectroscopic probes and retained NT domain-CT domain interaction in pyr-R61C/E255C/apoE4. We believe that we have maintained domain-domain interaction by pyrene-pyrene stacking interaction, as evidenced by the control experiment, which shows that the lipoprotein binding preference remains unaltered. The stabilization provided by pyrene excimer (dimer) formation is comparable with that contributed by surface or buried salt bridge (53, 54). In other studies, an increase in stability was noted when well-packed hydrophobic residues replaced a salt bridge triad (55).

In summary, we observe that in the lipid-free state, positions Arg-61 and Gln-255, representing the NT and CT domains, respectively, are proximal to each other in apoE4, as proposed earlier (23, 24). Upon interaction with phospholipid vesicles to form discoidal complexes, we propose that Arg-61 and Gln-255 lie spatially proximal to each other across the width of the phospholipid bilayer, such that each site is near the lipid/water interface (Fig. 7). In previous studies, it was suggested that apoE3 undergoes a dramatic conformational alteration upon lipid interaction, involving movement of the two domains away from each other (30, 56), accompanied by further opening of the helix bundle (49, 57) to yield a fully extended molecule circumferentially (Fig. 7). In previous studies, it was suggested that apoE4 participates in LDL receptor binding activity (13).

(58). It is interesting that the lipid-bound forms of both isoforms display LDL receptor binding activity (13).

For the apoE4 isoform, we propose that a similar movement of the helix bundle (49, 57) to yield a fully extended molecule circumferentially (Fig. 7). In previous studies, it was suggested that apoE4 participates in LDL receptor binding activity (13).

We observe that in the lipid-free state, positions Arg-61 and Gln-255, representing the NT and CT domains, respectively, are proximal to each other in apoE4, as proposed earlier (23, 24). Upon interaction with phospholipid vesicles to form discoidal complexes, we propose that Arg-61 and Gln-255 lie spatially proximal to each other across the width of the phospholipid bilayer, such that each site is near the lipid/water interface (Fig. 7). In previous studies, it was suggested that apoE3 undergoes a dramatic conformational alteration upon lipid interaction, involving movement of the two domains away from each other (30, 56), accompanied by further opening of the helix bundle (49, 57) to yield a fully extended molecule circumferentially (Fig. 7). In previous studies, it was suggested that apoE4 participates in LDL receptor binding activity (13).

We observe that in the lipid-free state, positions Arg-61 and Gln-255, representing the NT and CT domains, respectively, are proximal to each other in apoE4, as proposed earlier (23, 24). Upon interaction with phospholipid vesicles to form discoidal complexes, we propose that Arg-61 and Gln-255 lie spatially proximal to each other across the width of the phospholipid bilayer, such that each site is near the lipid/water interface (Fig. 7). In previous studies, it was suggested that apoE3 undergoes a dramatic conformational alteration upon lipid interaction, involving movement of the two domains away from each other (30, 56), accompanied by further opening of the helix bundle (49, 57) to yield a fully extended molecule circumferentially (Fig. 7). In previous studies, it was suggested that apoE4 participates in LDL receptor binding activity (13).

We observe that in the lipid-free state, positions Arg-61 and Gln-255, representing the NT and CT domains, respectively, are proximal to each other in apoE4, as proposed earlier (23, 24). Upon interaction with phospholipid vesicles to form discoidal complexes, we propose that Arg-61 and Gln-255 lie spatially proximal to each other across the width of the phospholipid bilayer, such that each site is near the lipid/water interface (Fig. 7). In previous studies, it was suggested that apoE3 undergoes a dramatic conformational alteration upon lipid interaction, involving movement of the two domains away from each other (30, 56), accompanied by further opening of the helix bundle (49, 57) to yield a fully extended molecule circumferentially (Fig. 7). In previous studies, it was suggested that apoE4 participates in LDL receptor binding activity (13).

We observe that in the lipid-free state, positions Arg-61 and Gln-255, representing the NT and CT domains, respectively, are proximal to each other in apoE4, as proposed earlier (23, 24). Upon interaction with phospholipid vesicles to form discoidal complexes, we propose that Arg-61 and Gln-255 lie spatially proximal to each other across the width of the phospholipid bilayer, such that each site is near the lipid/water interface (Fig. 7). In previous studies, it was suggested that apoE3 undergoes a dramatic conformational alteration upon lipid interaction, involving movement of the two domains away from each other (30, 56), accompanied by further opening of the helix bundle (49, 57) to yield a fully extended molecule circumferentially (Fig. 7). In previous studies, it was suggested that apoE4 participates in LDL receptor binding activity (13).

We observe that in the lipid-free state, positions Arg-61 and Gln-255, representing the NT and CT domains, respectively, are proximal to each other in apoE4, as proposed earlier (23, 24). Upon interaction with phospholipid vesicles to form discoidal complexes, we propose that Arg-61 and Gln-255 lie spatially proximal to each other across the width of the phospholipid bilayer, such that each site is near the lipid/water interface (Fig. 7). In previous studies, it was suggested that apoE3 undergoes a dramatic conformational alteration upon lipid interaction, involving movement of the two domains away from each other (30, 56), accompanied by further opening of the helix bundle (49, 57) to yield a fully extended molecule circumferentially (Fig. 7). In previous studies, it was suggested that apoE4 participates in LDL receptor binding activity (13).

We observe that in the lipid-free state, positions Arg-61 and Gln-255, representing the NT and CT domains, respectively, are proximal to each other in apoE4, as proposed earlier (23, 24). Upon interaction with phospholipid vesicles to form discoidal complexes, we propose that Arg-61 and Gln-255 lie spatially proximal to each other across the width of the phospholipid bilayer, such that each site is near the lipid/water interface (Fig. 7). In previous studies, it was suggested that apoE3 undergoes a dramatic conformational alteration upon lipid interaction, involving movement of the two domains away from each other (30, 56), accompanied by further opening of the helix bundle (49, 57) to yield a fully extended molecule circumferentially (Fig. 7). In previous studies, it was suggested that apoE4 participates in LDL receptor binding activity (13).

We observe that in the lipid-free state, positions Arg-61 and Gln-255, representing the NT and CT domains, respectively, are proximal to each other in apoE4, as proposed earlier (23, 24). Upon interaction with phospholipid vesicles to form discoidal complexes, we propose that Arg-61 and Gln-255 lie spatially proximal to each other across the width of the phospholipid bilayer, such that each site is near the lipid/water interface (Fig. 7). In previous studies, it was suggested that apoE3 undergoes a dramatic conformational alteration upon lipid interaction, involving movement of the two domains away from each other (30, 56), accompanied by further opening of the helix bundle (49, 57) to yield a fully extended molecule circumferentially (Fig. 7). In previous studies, it was suggested that apoE4 participates in LDL receptor binding activity (13).

We observe that in the lipid-free state, positions Arg-61 and Gln-255, representing the NT and CT domains, respectively, are proximal to each other in apoE4, as proposed earlier (23, 24). Upon interaction with phospholipid vesicles to form discoidal complexes, we propose that Arg-61 and Gln-255 lie spatially proximal to each other across the width of the phospholipid bilayer, such that each site is near the lipid/water interface (Fig. 7). In previous studies, it was suggested that apoE3 undergoes a dramatic conformational alteration upon lipid interaction, involving movement of the two domains away from each other (30, 56), accompanied by further opening of the helix bundle (49, 57) to yield a fully extended molecule circumferentially (Fig. 7). In previous studies, it was suggested that apoE4 participates in LDL receptor binding activity (13).

We observe that in the lipid-free state, positions Arg-61 and Gln-255, representing the NT and CT domains, respectively, are proximal to each other in apoE4, as proposed earlier (23, 24). Upon interaction with phospholipid vesicles to form discoidal complexes, we propose that Arg-61 and Gln-255 lie spatially proximal to each other across the width of the phospholipid bilayer, such that each site is near the lipid/water interface (Fig. 7). In previous studies, it was suggested that apoE3 undergoes a dramatic conformational alteration upon lipid interaction, involving movement of the two domains away from each other (30, 56), accompanied by further opening of the helix bundle (49, 57) to yield a fully extended molecule circumferentially (Fig. 7). In previous studies, it was suggested that apoE4 participates in LDL receptor binding activity (13).

We observe that in the lipid-free state, positions Arg-61 and Gln-255, representing the NT and CT domains, respectively, are proximal to each other in apoE4, as proposed earlier (23, 24). Upon interaction with phospholipid vesicles to form discoidal complexes, we propose that Arg-61 and Gln-255 lie spatially proximal to each other across the width of the phospholipid bilayer, such that each site is near the lipid/water interface (Fig. 7). In previous studies, it was suggested that apoE3 undergoes a dramatic conformational alteration upon lipid interaction, involving movement of the two domains away from each other (30, 56), accompanied by further opening of the helix bundle (49, 57) to yield a fully extended molecule circumferentially (Fig. 7). In previous studies, it was suggested that apoE4 participates in LDL receptor binding activity (13).

We observe that in the lipid-free state, positions Arg-61 and Gln-255, representing the NT and CT domains, respectively, are proximal to each other in apoE4, as proposed earlier (23, 24). Upon interaction with phospholipid vesicles to form discoidal complexes, we propose that Arg-61 and Gln-255 lie spatially proximal to each other across the width of the phospholipid bilayer, such that each site is near the lipid/water interface (Fig. 7). In previous studies, it was suggested that apoE3 undergoes a dramatic conformational alteratio...