Modulation of apolipoprotein E structure by domain interaction: Differences in lipid-bound and lipid-free forms

Danny M. Hatters‡§, Madhu S. Budamagunta¶, John C. Voss¶, Karl H. Weisgraber‡§||

‡From the Gladstone Institutes of Cardiovascular and Neurological Diseases, San Francisco, California 94158. §Cardiovascular Research Institute and ||Department of Pathology, University of California, San Francisco, California 94143, ¶Department of Biological Chemistry, School of Medicine, University of California, Davis, California 95616.

Running Title: FRET and EPR Analysis of ApoE4

Address correspondence to: Karl H. Weisgraber, Gladstone Institute of Neurological Disease, 1650 Owens Street, San Francisco, California, 94158, Tel. 415 734-2000; Fax. 415 355-0824; E-mail: kweisgraber@gladstone.ucsf.edu

Interaction of the amino- and carboxyl-terminal domains in apolipoprotein (apo) E, referred to as domain interaction, is predicted to be more pronounced in apoE4 than in apoE3 and to underlie the association of apoE4 with Alzheimer’s and cardiovascular diseases. However, direct physical proof for the domain interaction concept is lacking. To address this issue, fluorescence resonance energy transfer and electron paramagnetic resonance (EPR) spectroscopy were used to probe the spatial proximity of the two domains of apoE. Both methods demonstrated that the two domains are closer in both lipid-free and phospholipid-bound apoE4 than in apoE3 as a result of domain interaction. In addition as shown by EPR, the domains of apoE4 move apart to resemble more closely the distance in apoE3 when the isoforms are bound to triglyceride-rich emulsion particles. These results demonstrate that domain interaction is a structural property of apoE4 and that apoE adopts different conformations when complexed to different lipids.

Apolipoprotein (apo) E mediates lipoprotein transport and cellular uptake of lipoproteins in plasma and the central nervous system. The protein contains two independently folded structural domains and exists as a stable tetramer in the lipid-free state (1, 2). As determined by x-ray crystallography, the amino-terminal domain is a four-helix bundle (3) and the carboxyl-terminal domain is predicted to contain a series of helices (2, 4). Of the three common allelic isoforms, apoE4, increases the risk for cardiovascular and Alzheimer’s diseases (5–9). By 85 years of age individuals homozygous for apoE4 have a 50–90% chance of developing Alzheimer’s disease, whereas apoE4 heterozygotes have a ~45% risk, and the general population just 20% (5, 7).

The three common apoE isoforms differ at only two amino acid positions: apoE2 (C112, C158), apoE3 (C112, R158), and apoE4 (R112, R158) (10). These sequence differences dictate different structural and biophysical properties that result in functional differences that are predicted to affect disease. One such property is the concept of domain interaction, originally proposed based on the differential binding of apoE4 to large triglyceride-rich very-low-density lipoproteins (VLDL) and apoE3 to smaller high-density lipoproteins (HDL) associated with the influence of positive charge at position 112 on binding (11). Based on x-ray crystallographic analysis of the amino-terminal domains of apoE3 and apoE4 and mutagenesis analysis of the carboxyl-terminal domain, domain interaction was later defined to include the formation of a putative salt bridge between R61 and E255 that was suggested to bring the two domains into close proximity (12, 13). It was predicted that the presence of arginine at position 112 in apoE4 promotes the interaction of R61 with E255, whereas the cysteine in apoE3 and apoE2 result in an alternate packing of the R61 side-chain that was predicted to interact less with E255 (12). The importance of R61 and E255 for domain interaction was demonstrated by site-directed mutagenesis in which replacement of either R61 with threonine or E255 with alanine alters the binding preference of apoE4 from VLDL to HDL (13). An in vivo mouse model of domain interaction has been generated by gene targeting.
replacing the threonine codon in the wild-type Apoe gene with an arginine codon at the position corresponding to 61 in human apoE (14).

Recently, fluorescence resonance energy transfer (FRET) between yellow- and cyan-fluorescent proteins fused to the amino- and carboxyl-terminal domains of apoE respectively, was used to assess the distance between the two domains in living neuronal cells (15). These data show greater FRET efficiency in an apoE4 construct than that of an apoE3 construct, consistent with closer contact between the two domains in apoE4 (15). However, to date, direct structural proof for the domain interaction concept and how it modulates protein conformation and lipid binding is unknown.

In this study, the distances between the amino-terminal and carboxyl-terminal domains of apoE4 and apoE3 derivatives were probed using FRET and electron paramagnetic resonance (EPR) spectroscopy. Based on the EPR data, a model of the spatial proximity of the amino-terminal and the carboxyl-terminal domains is proposed.

Materials and Methods

(1-oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-methyl) methanethiosulfonate (MTE) from Toronto Research Chemicals (cat# O875000); QuickChange Multi-Site XL mutagenesis kit from Stratagene; 5-(((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (AEDANS) from Molecular Probes (cat# I14); Egg yolk phosphatidylcholine (EYPC), triolein, tris(carboxylethyl)phosphine (TCEP), and N-ethylmaleimide (NEM) from Sigma; dimyristoylphosphatidylcholine (DMPC) from Avanti Polar Lipids; YM-10 centriprep filtration devices from Amicon; bovine α-thrombin from Haematologic Technologies; Slide-A-Lyzer dialysis cassettes (MWCO 10,000) from Pierce.

ApoE mutant construct setup, expression and purification. Mutations were introduced into the cDNA encoding apoE4 with the QuickChange mutagenesis kit (Stratagene) and a modified pET32a construct as a template (16). All sequences were verified by DNA sequencing. ApoE was expressed in E. coli. The cells were harvested and lysed, and cellular debris pelleted as described (16). ApoE was purified as described (17). After the final gel filtration chromatography step in 4 M guanidine hydrochloride, the apoE-containing eluent from the column was collected and concentrated using a YM-10 centriprep and stored at -20 °C. Protein concentrations were determined by the extinction coefficient calculated from amino acid composition with a formula for denatured proteins (18). Calculated molar extinction coefficients at 280 nm was $4.495 \times 10^4$ M$^{-1}$cm$^{-1}$ for all constructs except $2.788 \times 10^4$ M$^{-1}$cm$^{-1}$ for clones FC1 and FC2 and $3.936 \times 10^4$ M$^{-1}$cm$^{-1}$ for mutants with the 264C mutation.

Refolding of cysteine containing apoE and labeling with MTE, AEDANS or NEM. Denatured stock protein (2.5 ml of 3 mg/ml in 4 M guanidine hydrochloride, 0.1% β-mercaptoethanol, 1 mM EDTA, 10 mM Tris/Cl, pH 7.4) was dialyzed into ice-cold refolding buffer (100 mM ammonium bicarbonate, 1.5 M guanidine hydrochloride, 1 mM EDTA) with 5 mM DTT for 2 h at 4 °C using a Slide-A-Lyzer. The buffer was changed to ice-cold refolding buffer with 1 mM DTT and dialyzed a further hour at 4 °C. Samples were buffer exchanged into ice-cold refolding buffer using a PD-10 column (Pharmacia). For spin labeling, 19 µl of 37.8 mM MTE (prepared in acetonitrile) was added to the sample with rapid mixing immediately after elution of apoE from the PD-10 column. For AEDANS or NEM labeling, 95 µl of 46 mM AEDANS or NEM (freshly prepared in dimethylsulfoxide) was added instead of MTE.

Samples were incubated overnight at 4 °C (in the dark for AEDANS labeling) and the free label and high-molecular mass protein were removed by gel filtration chromatography on a 2.5 × 120 cm column packed with Superdex 200 resin (Pharmacia), pre-equilibrated in 150 mM NaCl, 0.25 mM EDTA, 10 mM Tris, pH 7.4 (TBS). Two peaks typically eluted early in the fractionation range, the first representing high molecular mass protein, and the second peak, which contains tetrameric/dimeric apoE, was collected. The apoE was concentrated using a centriprep YM-10 at 4 °C. For unlabeled samples, protein was refolded by directly loading stock protein in 4 M guanidine hydrochloride, 0.1% β-mercaptoethanol, 1 mM EDTA, 10 mM Tris, pH 7.4 onto the Superdex 200 column and after elution and collection of the second peak, 0.1 mM TCEP was added to maintain the cysteines in a reduced state. After concentration, protein was kept at a concentration of 1 mg/ml up to several days at 4 °C, and the aggregation state of the protein was monitored using analytical gel filtration chromatography on a
Superdex 200 10/300 GL column (Pharmacia) at various time points during the course of the experiments.

**FRET.** The apoE constructs for FRET, FC1 and FC2 were prepared both unlabeled and labeled with AEDANS. Labeling was determined to be ~1:1 stoichiometric as determined from the absorbance spectra of the labeled proteins and the extinction coefficients of AEDANS (6,100 M$^{-1}$ cm$^{-1}$ at 337 nm (19)) and the protein. This was confirmed by electrospray mass spectrometry of the labeled constructs, which indicated an increase in the deconvoluted masses of both FC1 and FC2 by 309 mass units and the lack of detectable masses corresponding to unlabeled protein (unlabeled calculated masses of 34,272.1 and 34,217.63 for FC1 and FC2 respectively). TCEP (0.1 mM) prevented disulfide formation in the unlabeled samples for at least one week under these conditions as assessed by non-reducing SDS-PAGE. Fluorescence spectra were collected using protein concentrations of 50 µg/ml in TBS with a Perkin Elmer LS-5B fluorescence spectrometer and emission and excitation bandwidths set at 3 nm.

For the samples complexed to DMPC, samples were prepared as follow. DMPC (5 mg/ml) in TBS was sonicated for 10 min with a small probe and added to apoE tetramer (1 mg/mL) at a mass ratio of 3.75:1, and the mixture was incubated overnight at 24 °C. The complex was separated from residual free DMPC and unbound apoE by density gradient centrifugation: the apoE•DMPC solution (1.65 ml) was added on top of d = 1.21 g/ml (1.65 ml) and d = 1.12 g/ml (1.65 ml) potassium bromide solutions in 10 mM Tris, pH 7.4, in a 13 × 51-mm polyallomer tube. Samples were spun overnight at 55,000 rpm in a SW41 rotor (Beckman/Coulter) and were mostly between 20 and 40 nm in radius as assessed by sucrose gradient fractionation and dynamic light scattering as described (22). Concentration of phospholipid was established using an enzymatic assay kit and emulsions were added to apoE at a final phospholipid concentration of 14.5 mM (Waco). Based on previous binding data ($K_d = 0.5—1.2$ µM and a binding capacity of 0.8—3.0 amino acids per phospholipid), this phospholipid concentration is predicted to result in ~90% of apoE bound to the emulsions (22, 23).

To determine the labeling efficiency, the number of spins per mg protein was determined from the integrated intensity of the sample in 5.2 M guanidine hydrochloride solution, prepared by adding guanidine hydrochloride (97 mg) to 125 µl

\[ E = 1 - \frac{Q_{DA}}{Q_D} \]  

where $Q_{DA}$ is the quantum yield of the donor in the presence of AEDANS, and $Q_D$ is the quantum yield of the donor lacking AEDANS. Quantum yields were determined using tryptophan in TBS as a reference assuming $Q=0.14$ (21).

The spectral overlap integral (\( J \)) was determined by

\[ J = \int F_D(\lambda)\epsilon(\lambda)\lambda^4 d\lambda \]  

where $\epsilon(\lambda)$ is the molar extinction coefficient of the acceptor at wavelength $\lambda$ and $F_D(\lambda)$ is the fluorescence of the donor normalized against the total fluorescence on the wavelength scale.

**EPR spectroscopy.** Refolded and labeled samples were concentrated to 1.5—3 mg/ml with a centriprep YM-10. Samples were prepared in TBS at final concentrations of 1 mg/ml for apoE alone and bound to DMPC, and 0.9 mg/ml for apoE with emulsions. After preparation, all samples were incubated overnight at 4 °C before EPR spectroscopy.

For the DMPC samples, a DMPC solution (50 mg/ml solubilized in TBS by incubation at 42 °C for 30 min) was added to apoE at a final concentration of 5 mg/ml and equilibrated at 24 °C for 30 min.

For the emulsion-bound apoE, unfractionated emulsions of EYPC and triolein were prepared in TBS as described (22). Emulsions were concentrated by centrifugation at 40,000 rpm in 11 × 34 mm polycarbonate tubes in a TLA100.2 rotor (Beckman/Coulter) and were mostly between 20 and 40 nm in radius as assessed by sucrose gradient fractionation and dynamic light scattering as described (22). Concentration of phospholipid was established using an enzymatic assay kit and emulsions were added to apoE at a final phospholipid concentration of 14.5 mM (Waco). Based on previous binding data ($K_d = 0.5—1.2$ µM and a binding capacity of 0.8—3.0 amino acids per phospholipid), this phospholipid concentration is predicted to result in ~90% of apoE bound to the emulsions (22, 23).

To determine the labeling efficiency, the number of spins per mg protein was determined from the integrated intensity of the sample in 5.2 M guanidine hydrochloride solution, prepared by adding guanidine hydrochloride (97 mg) to 125 µl
stock protein. All sites were spin labeled at 96% or greater efficiency. For comparing the distances between position 76 and 241, the labeling efficiency of protein containing the R61T mutation was 99.4% of the protein containing the native arginine.

EPR spectra were collected on a JEOL X-band spectrometer equipped with a loop gap resonator. Approximately 4 µl of sample were placed in a sealed quartz capillary tube. Spectra were acquired at 20–22 °C with a single 60s scan over 100 G at a microwave power of 2 milliwatts, and a modulation amplitude optimized to the natural line width of the attached nitroxide.

Interspin distance calculations. Distances within the range of 10–22 Å were determined from the spectral broadening of the double-labeled sample, compared with the composite spectrum from the two corresponding single labeled samples. Distances were calculated by Fourier deconvolution of the spectrum for the dipolar broadening as represented by a Pake pattern (24, 25). The interspin distance was calculated using a computer program (24) kindly provided by C. Altenbach, which determines the splitting that best reconstructs the broadened spectrum for discrete populations of interacting spins. Before analysis, spectra were examined for labeling efficiency (efficiency for all samples was within 2% of a standard reference), and normalized to the same number of spins.

Molecular modeling. Modeling and visualization was carried-out using Insight II (Accelrys, Inc.) and PyMol (DeLano Scientific LLC; http://www.pymol.org) software. Molecular models were constructed using the crystal structure of the amino-terminal domain of apoE4 (3) and residues 238–265 of the carboxyl-terminal domain fixed as a rigid helix. Distances obtained from double-spin labeled samples were used to model the placement of the carboxyl-terminal fragment relative to amino-terminal four-helix bundle. The calculated distances represent the distance between the ring nitrogens of the dipolar-coupled pair. The spin labeled side chains where placed in a conformation relative to a backbone of fixed secondary structure that is found to predominate for single component spectra (26). While positions 76, 241, and 263 appear dominated by a single component, position 77 shows two components. Thus the spin labeled side chain at position 77 is likely to adopt more than one conformation, though the model employs only the most likely conformation.

RESULTS

Domain interaction as monitored by FRET

The structural proximity of the two domains of apoE was investigated using FRET based on the construct in (Fig. 1A). These derivatives of apoE utilize the native four amino-terminal tryptophans as FRET donors. The carboxyl-terminal tryptophans at positions 210 and 276 were replaced with phenylalanine, and the tryptophan at position 264 was replaced with a cysteine, which was used to covalently attach AEDANS as a FRET acceptor.

Two variants of the FRET construct, FC1 and FC2, were prepared to assess the influence of domain interaction on the conformation. FC1 is a mimic for apoE4. FC2 is a derivative of FC1 containing a threonine substitution at position 61, which abolishes domain interaction (12). Hence, FC2 is a mimic for apoE3. After AEDANS labeling, both proteins remained tetrameric over the time frame of the experiment as determined by a single peak eluting from a gel filtration column (Fig. 2). Fluorescence spectra of FC1 and FC2 were first collected in a lipid-free tetrameric state with selective excitation of the tryptophans at a wavelength of 292 nm (Fig. 3A). AEDANS fluorescence occurred concurrently to the tryptophan fluorescence, indicating FRET. Differences in the emission intensities suggests that FRET is greater in FC1 than FC2 (Fig. 3A). Unlabeled FC1 and FC2 gave identical spectra, indicating that the tryptophans are collectively in identical microenvironments for both constructs and that the differences observed in the labeled samples are attributed solely to different FRET efficiencies (Table I). Independent protein preparations and labeling gave the same result, with an average difference in the emission intensity ratio 470 nm/350 nm of 21.7% (± 3.7% s.d., P = 0.002, paired Student’s t-test) between FC1 and FC2. These data are consistent with the amino-terminal and carboxyl-terminal domains of apoE4 being closer together than those of apoE3 in the lipid-free state, as predicted by domain interaction.

Spectra were also measured for FC1 and FC2 complexed with the phospholipids, dimyristoylphosphatidylcholine (DMPC) and
dipalmitoylphosphatidylcholine (DPPC) (Fig. 3B and C). Upon binding, apoE remodels the phospholipid into small particles, resembling HDL particles found in cerebrospinal fluid (27). As observed with the lipid-free protein, FC1 and FC2 retained differential FRET efficiencies in both lipid complexes, demonstrating that conformational differences in apoE4 and apoE3 are sustained in a phospholipid-bound state (Fig. 3B and C; Table I).

As a control, fluorescence spectra were measured for FC1 and FC2 in 1% SDS to disrupt tertiary structure (Fig. 3D). The FC1 and FC2 spectra were identical, and the AEDANS fluorescence was substantially reduced, indicating a loss of FRET in both FC1 and FC2. Similarly, addition of 7 M guanidine hydrochloride resulted in diminished AEDANS fluorescence and a shift in the tryptophan emission intensities to longer wavelengths, consistent with conformational denaturation and its associated reduction in FRET (data not shown).

Domain interaction as probed by EPR spectroscopy

Since all four tryptophans in the amino-terminal domain were used as donors in the FRET studies, only relative distances can be compared between the domains in apoE4 and apoE3. To determine the actual spatial relationship of the amino- and carboxyl-terminal domains, EPR spectroscopy was employed. Five positions, two in the amino-terminal domain and three in the carboxyl-terminal domain, were chosen for placement of cysteine residues (Fig. 1B). The five single cysteine mutants were produced. In addition, six double mutants were produced with one cysteine in each of the domains (Fig. 1B). All spin-labeled single and double mutants were evaluated for aggregation and tetramer formation by gel filtration. Only those mutants that existed primarily as tetramers and remained soluble for the time frame of the measurements were used in the analysis.

EPR line shapes of single-labeled proteins

The EPR spectra of apoE4 containing a single site-directed spin label are shown in (Fig. 4) (red and black traces). The amino-terminal spin labels at positions 76 and 77 showed moderately high mobility. This is consistent with their expected orientation on the exterior surface of the four-helix bundle along a fixed α-helical backbone, based on the crystal structure of the amino-terminal domain (3). Position 77 showed a slight broad component in the low field region of the spectrum, which likely arises from transient contact with 74Y.

The EPR spectrum of the carboxyl-terminal spin label at position 263 is consistent with a surface-exposed side chain attached to a backbone of fixed secondary structure (28). However, the spin label at position 264, which falls along the same face of a projected α-helix, showed substantial self-interaction. This was attributed to a dipolar interaction between the same sites (in the tetramer) and immobilization of the spin-labeled side chains due to the quaternary contact.

Distances between labels in the amino- and carboxyl-terminal domains of apoE4

To model both the alignment and orientation of the two domains, analysis was performed on the tetrameric, double-cysteine mutants of apoE. Spin labels were attached to a cysteine substituted at either position 76 or 77 and a cysteine substituted at positions 241, 263, or 264 (Fig. 1B). Evidence of dipolar broadening (or lack thereof) can be used to model the proximity between the amino- and carboxyl-terminal domains in apoE4 in these double-cysteine labeled samples. (Fig. 4) shows spectra normalized to the same number of spins for each double-labeled spectrum (blue trace) overlaid with their two corresponding single-labeled samples (black or red traces), and the sum of the single-labeled samples (green trace). Thus, the spectrum of the double-labeled protein should approximate the spectral sum of the two corresponding single-labeled proteins where no intramolecular spin-spin interaction occurs.

Interspin distances in the range of 8–25 Å influence the strength of the dipolar interactions and can be calculated from Fourier deconvolution of Pake patterns in the broadened spectrum (24, 25). Three of the double-cysteine samples, with spin labels at 76-241, 76-263, or 77-241, showed significant broadening and were analyzed by deconvolution which gave interspin distances of 14.1, 21.2, and 18.7 Å, respectively. Uncertainty in these measurements is on the order of 3-5 Å, considering error inherent in the theoretical treatment (19) of the broadened spectra and the fidelity of the regenerated spectrum to the
observed spectrum (18). Given the low level of broadening apparent in other pairs, deconvolution analysis was not suitable for the signal-to-noise levels of these data. In these cases, we can say that the spin labels of the 76-263, 77-239, 77-263, and 77-264 pairs are all separated by at least 22 Å. These distances were then used to construct a model (Fig. 5). This model assumes that the carboxyl-terminal region that encompasses the mutations is a single straight helix, as predicted (4) and that the four-helix bundle structure of the amino-terminal domain remains in the intact protein, which is consistent with denaturation studies (29). Given these two assumptions, an antiparallel orientation of the carboxyl-terminal helix relative to helix 2 is most consistent with the distances obtained (Fig. 5).

Deconvolution also provides information on the fractional population of interacting spins, which can be used to infer conformational heterogeneity. For the three pairs examined here, the optimal deconvolution was obtained when 60–65% of the spins interact. This is consistent with either conformational heterogeneity of lipid-free apoE or interconversion between different conformations where the two regions are farther apart.

The double-labeled 76-241 apoE displayed the strongest spin interactions and was used to assess the effect of domain interaction on the spatial proximity of the amino- and carboxyl-terminal domains. EPR spectra were collected of the 76-241 double cysteine mutant with the additional arginine to threonine mutation at position 61 to abolish domain interaction (13). Only minor spectral broadening was observed when the data were overlaid with the spectral sum of the single-labeled samples, suggesting the sites are separated by more than 22 Å in the context of the R61T substitution. The average spectrum of three separately purified and labeled samples are shown in Fig. 4A, boxed, where the loss of interspin interactions in the R61T mutant is readily apparent. The relative broadening in the R61 protein was reproduced in each experiment, with an average central (M\textsubscript{c}=0) line intensity of 57% (± 0.98% s.d., P = 0.0002, paired Student’s t-test) of the R61T mutant.

To determine if the spin-spin interactions are inter- or intramolecular, the nitroxide-labeled 76-241 was mixed with 76-241 apoE lacking spin-labels (labeled instead with N-ethyl-maleimide (NEM)) at various percentages ranging from 25% to 100% of the fixed total protein concentration of 1 mg/ml. In the case of intermolecular interactions in the tetramer, a loss of spectral broadening is predicted upon dilution of the spin labels after normalizing the number of spins. However, no significant changes occurred in any of the mixtures of nitroxide-labeled 76-241 apoE and NEM-labeled 76-241 apoE after normalizing the data for the same number of spins (Fig. 4C). Furthermore, addition of SDS to denature the 100% and 25% nitroxide-labeled 76-241 apoE resulted in identical changes to the spectra, which indicates that spin dilution does not influence spectral broadening (data not shown).

Influence of binding to phospholipid on domain interaction

To assess the influence of lipid binding on the conformational dynamics and domain interaction, the 76 and 241 spin-labeled proteins were examined in complex with DMPC. DMPC induced a slight increase in the mobility of the position 76 or 241 single-labeled proteins, indicating conformational change at these sites (Fig 5A). Like that observed for the lipid-free tetramer, the spectrum of the double-labeled 76-241 mutant showed a strong dipolar interaction as assessed by overlaying the sum of the single mutants (Fig. 6A). Disrupting domain interaction with the R61T mutation reduced spectral broadening, which indicates an increased distance between these positions (Fig. 6A). This effect of the R61T mutation on spectral broadening is similar to the lipid-free tetramer, suggesting that domain interaction maintains a closer proximity between positions 76 and 241 in both lipid-free and DMPC bound conformations (Fig. 6A).

We also determined if the spin-spin interactions were inter- or intramolecular in the DMPC-bound state. Mixtures of nitroxide- and NEM-labeled 76-241 apoE in complex with DMPC did not change the spectral broadening after normalization to the number of spins, suggesting that only intramolecular interactions drive the differences in spectra of the R61T and R61 variants (Fig. 6C).

The effect of triglyceride-rich emulsion particles on apoE conformation was also examined (Fig. 6B). Phospholipid-triolein emulsions mimic large triglyceride-rich lipoproteins (i.e., VLDL and chylomicrons (23, 30)). In contrast to the effect of DMPC, the 76-241 double-labeled apoE had little
spectral broadening when mixed with emulsions as assessed by overlaying the sum of the 76 and 241 single-labeled proteins (Fig. 6B). This result indicates that the distance between positions 76 and 241 increases compared to that of DMPC-bound and lipid-free apoE. Also concordant with this result is that abolishing domain interaction with the T61 mutation did not result in further change in the EPR spectrum.

**DISCUSSION**

To date, the concept of domain interaction was inferred from x-ray crystallography and scanning mutagenesis and the effect of various mutations on changing the apoE4 lipoprotein binding distribution to one resembling that of apoE3. Domain interaction in apoE4 was postulated to contribute to the association of apoE4 with cardiovascular disease, as well as various neurodegenerative diseases, including Alzheimer’s disease (13, 31). However, structural data supporting the interaction of the amino-terminal and carboxyl-terminal domains in apoE4 was lacking. FRET and EPR are established structural tools for measuring distances in proteins and obtaining information about local environments at specific sites within a protein (32, 33). Using both FRET and EPR as structural probes of the amino- and carboxyl-terminal domain distances in apoE4 and apoE3 mimics, we demonstrate that the domains on the apoE4 background are closer spatially than on an apoE3 (R61T) background, establishing the concept of domain interaction as a structural feature of apoE4.

Our data also provides insight into conformational change upon lipid binding. The shifts in the tryptophan fluorescence upon binding to DMPC suggest a repositioning of the tryptophans to a more hydrophobic environment, which is consistent with burial of the tryptophans within the phospholipid acyl chains (Fig. 3). Such a conformational change would be consistent with fluorescence and infra-red spectroscopy studies supporting a “belt” model that describes a pair of amphipathic helices wrapping the edge of a phospholipid bilayer (34–38).

Our FRET data cannot be used to determine meaningful distances (using the Förster theory) due to the presence of four tryptophan donors. However, the FRET efficiency ($E$) increased slightly upon binding to phospholipid (Table I) suggesting that the relative distances between the tryptophans and AEDANS decreases when apoE binds to DMPC. Previous FRET experiments with an apoE3 derivative containing a sole tryptophan residue at position 264 and an AEDANS attached to cysteine 112 shows FRET efficiencies decreased from $E = 0.28$ to 0, which corresponds to an increase in distance from 28 Å to beyond FRET detection ($> 80$ Å) upon binding to DMPC (39). At first appearance this contradicts our results, however, (Fig. 7) shows one plausible α-helical hairpin conformation where helix 3, which contains residue 112 is bent 180 degrees with respect to helix 2, which contains all the mutations for EPR. This model (Fig. 7) is similar to that proposed from a recent study of pyrene excimer formation in the pyrene-labeled apoE4 R61C, E255C double cysteine mutant (40).

A key finding is that domain interaction in apoE4 results in a closer distance between the amino- and carboxyl-terminal domains in the lipid-free tetramer, as well as when it is bound to DMPC or DPPC. This contrasts to that of apoE in the presence of triglyceride-rich emulsions, which shows a lessened spin-spin interaction between 76 and 241 and suggests that either domain interaction is much reduced by binding to the emulsion surface, or that apoE adopts a substantially different conformation. Either way, the differential effects of emulsions and DMPC on the EPR spectra suggests that at least apoE4 adopts different conformations when complexed to phospholipid alone versus triglyceride-rich emulsions.

Our data complement previous studies that support conformational heterogeneity of lipid-bound apoE. Based on the modulation of apoE receptor binding activity by the presence of the C-apolipoproteins or lipoprotein lipid composition, a model of two alternative VLDL-bound apoE conformations was proposed, a receptor-inactive and a receptor active conformation (41–43). Another study reported that although apoE3 and apoE4 have similar binding affinities to large emulsions, apoE4 has a higher binding capacity than apoE3, which suggests that apoE4 has a more compact binding footprint than apoE3 (22). Another study reported that apoE4 bound with a reduced enthalpy on apoE4-crowded emulsion surfaces compared to apoE4-poor emulsion surfaces, which suggests the presence of (at least) two alternate conformations on emulsions (44).
These studies, and our data, point to conformational heterogeneity being modulated by domain interaction and lipoprotein composition as a mechanism for apoE3 and apoE4 binding specificity to different lipoprotein classes. In particular, domain interaction may influence the binding kinetics of lipid-free apoE to lipids, or the binding foot-print size. Further studies are required to understand the conformational changes that accompany emulsion binding. For studies with emulsions a major limitation for FRET studies is solution turbidity and hence EPR is more useful to provide insight in apoE conformations on triglyceride-rich lipoproteins.

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**FOOTNOTES**

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FIGURE LEGENDS

Fig. 1. Design and rationale of fluorescence and EPR mutants on a model of apoE. The amino terminal domain is represented in red helices and the carboxyl-terminal domain is modeled in green helices. The two domains are separated by a flexible linker region (A) FRET setup. The four endogenous tryptophans in the amino-terminal domain (shown in purple boxes) were FRET donors. An AEDANS molecule was attached to cysteine at residue 264 (blue box) and served as a FRET acceptor. The other tryptophans in the linker region and carboxyl-terminal domain were mutated to phenylalanine. (B) EPR setup. Cysteines were introduced at the indicated sites, either singly or paired with one mutation from each domain.

Fig. 2. Gel filtration analysis of AEDANS-labeled FC1 and FC2 apoE. FC1 and FC2 tetramer eluted as a single peak on a Superdex 200 column during the timeframe of the experiment (two days; storage at a protein concentration of 1 mg/ml and temperature 4 °C). Arrow indicates the column void volume and the calibrated elution volume of aggregated apoE.

Fig. 3. Fluorescence spectra of unlabeled and AEDANS-labeled FC1 and FC2 apoE. (A) Lipid-free tetrameric apoE. (B) ApoE•DMPC complexes. (C) ApoE•DPPC complexes. (D) ApoE and 1% SDS.

Fig. 4. EPR spectra of lipid-free single- and double-spin labeled apoE. Spectra from site-directed spin labels are distinguished by color. Sum of singles represents a summation of the spectra from the two single-labeled samples. Within a set, each spectrum represents the same number of spins. Each set is scaled to the spectrum of highest amplitude in each set. (A) EPR spectra from pairs involving the amino-terminal domain position 76. A direct comparison of the normalized 76-241 spectra in the presence and absence of the R61T mutation is shown in the boxed plot. (B) EPR spectra from pairs involving position 77 in the amino-terminal domain. (C). Effect of spin dilution on the broadening of nitroxide-labeled 76C-241C. Compared is the spectrum from a sample containing 100% nitroxide labeled protein (black line) to the spectrum of a sample containing 25% nitroxide labeled protein + 75% NEM-labeled protein (red line). The NEM-containing spectrum has been FFT filter smoothed and its amplitude has been multiplied by 4 to normalize for the same spin number.

Fig. 5. A ribbon model showing the arrangement of the apoE4 carboxyl-terminal domain (green) relative to its amino-terminal domain (red). Shown is the span of the carboxyl-terminal region examined in this study, which is projected as a straight α-helix, and positioned according to distances derived by dipolar coupling between spin-labeled sites introduced at the sites shown. Also shown are the R61 and E255 side chains that modulate domain interaction (gray).

Fig. 6. Effect of lipid binding on the EPR spectra of spin-labeled apoE4. Within a set, each spectrum represents the same number of spins and the spectra are scaled relative to the spectrum of highest amplitude within that set. (A) Comparison of lipid-free, DMPC-complexed, and emulsion-complexed apoE4 with spins labels located at the indicated positions. (B) Effect of the R61T mutation on the EPR spectrum of 76-241 in the presence of DMPC or emulsion. The sum of singles spectra were generated by summing the spectra of single-labeled samples for the respective lipid. (C) Effect of spin dilution on the broadening of nitroxide-labeled 76C-241C in complex with DMPC. Compared is the spectrum from a sample containing 100% nitroxide labeled protein (black line) to the spectrum of a sample containing 25% nitroxide labeled protein + 75% NEM-labeled protein (red line). The NEM-containing spectrum has been FFT filter smoothed and its amplitude has been multiplied by 4 to normalize for the same spin number.

Fig. 7. Schematic cartoon of the conformational heterogeneity of apoE. (A) Lipid free conformation. (B) DMPC-bound conformation. Distances between residues 76 and 241 and between the four amino-terminal tryptophans and residue 264 are similar for A and B. Residue 112 is further away from residue 264 in B than A. Residues 61 and 255 are close together in A and B.
Table I.

Parameters of the FRET experiments.

| Protein and Conformation | Quantum yield | FRET Efficiency | Spectral Overlap |
|--------------------------|---------------|-----------------|------------------|
|                          | No AEDANS $Q_D$ | With AEDANS $Q_{DA}$ | $E$ | $J$ (cm$^3$/mol) |
| FC1 (tetramer)           | 0.13          | 0.09            | 0.28             | $1.91 \times 10^{-14}$ |
| FC2 (tetramer)           | 0.13          | 0.10            | 0.23             | $1.91 \times 10^{-14}$ |
| FC1 (DMPC bound)         | 0.16          | 0.11            | 0.30             | $1.92 \times 10^{-14}$ |
| FC2 (DMPC bound)         | 0.16          | 0.12            | 0.24             | $1.92 \times 10^{-14}$ |
A. tryptophan mutated to cysteine

B. tryptophan mutated to phenylalanine

Non-mutated tryptophans

Figure 1
Figure 2
Figure 3
Figure 4
Figure 6

A.

- 76C
- + DMPC
- + emulsion

- 241C
- + DMPC
- + emulsion

- 76C-241C
- + DMPC
- + emulsion

- 76C-241C + R61T
- + DMPC
- + emulsion

B.

DMPC

- 76C-241C
- 76C+241C sum of singles
- 76C-241C + R61T

Emulsion

- 76C-241C
- 76C+241C sum of singles
- 76C-241C + R61T

C.

- 100% nitroxide
- 25% nitroxide × 4
A. Lipid-free

B. DMPC-bound

Figure 7
Modulation of apolipoprotein E structure by domain interaction: Differences in lipid-bound and lipid-free forms
Danny M. Hatters, Madhu S. Budamagunta, John C. Voss and Karl H. Weisgraber

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