Orientation and mode of lipid-binding interaction of human apolipoprotein E C-terminal domain

Vincent RAUSSENS*,†, Jessica DRURY†, Trudy M. FORTE†, Nicole CHOI†, Erik GOORMAGHTIGH*, Jean-Marie RUYSSCHAERT* and Vasanthy NARAYANASWAMI†

*Structure and Function of Biological Membranes, Université Libre de Bruxelles, CP-206/2, bd. Du Triomphe, B-1050 Brussels, Belgium, and †Lipid Biology in Health and Disease Research Group, Children’s Hospital Oakland Research Institute, 5700 Martin Luther King Jr. Way, Oakland, CA 94609, U.S.A.

INTRODUCTION

ApoE (apolipoprotein E) is a 299-residue exchangeable apolipoprotein that is a component of triacylglycerol-rich lipoproteins and a subclass of HDL (high-density lipoprotein). Transgenic studies [1,2], and clinical and biochemical analyses of apoE-deficient subjects [3], reveal the direct relevance of apoE in lipoprotein metabolism, cholesterol homeostasis and as a modulator of atherogenesis. The role of apoE in causing a reduction of plasma and cellular cholesterol levels is documented by its ability to: (i) act as a ligand for the LDL (low-density lipoprotein) receptor family [4], that leads to cellular uptake and clearance of remnant lipoprotein particles; and (ii) promote cholesterol efflux from peripheral tissues to nascent HDL particles in atherosclerotic lesions [5,6]. In the brain, apoE is involved in cholesterol transport from astrocytes to neurons by means of lipoproteins [7] and in neuronal regeneration following nerve injury [8,9]. In all of these instances, lipid association of apoE is a key factor in determining its functionality.

Lipid association of apoE is mediated by two independently folded domains: the NT (N-terminal) domain (residues 1–191) that bears low-affinity lipoprotein-binding ability and the CT (C-terminal) domain (residues 201–299) that accommodates high-affinity lipid-binding sites [10–12]. The prominent feature of the NT domain is its ability to interact with the LDL receptor via binding sites localized to helix 4 of the four-helix bundle architecture [13], an ability conferred by lipid association. In turn, lipid-binding interaction of apoE NT domain induces dramatic conformational alterations involving opening of the four-helix bundle at putative hinge regions, as determined from surface monolayer [14] and FRET (fluorescence resonance energy transfer) studies [15,16]. The structural re-organization triggered by lipid binding is an a priori requirement for receptor-binding competency of apoE NT domain, which probably bears significant physiological relevance [17,18]. Thus apoE has been proposed to exist in two different lipid-bound conformations: one where the NT domain is in a lipid-free helix-bundle state that is receptor-incompetent, and a second where it is in a lipid-bound, receptor-competent state following helix-bundle opening. Interestingly, both conformers involve ‘anchoring’ of apoE to the lipid particle via the CT domain [17,18]. However, the structural organization of the CT domain in the lipid-bound state and the mode of the lipid-binding interaction of this domain are poorly understood at present.

C-terminal truncation analyses demonstrate that deletion of residues 267–299 impaired 25% of the lipid-binding activity, while deleting residues 244–299 and shorter deletion variants abolished entirely binding to VLDL (very-low-density lipoprotein) and resulted in poor binding (~15–20%) to HDL [19]. Similar impaired lipoprotein association was identified in a naturally occurring apoE truncated variant, apoE-(1–209), with a premature stop codon at position 210 [20]. Other studies confirm that...
deleting CT residues 192–299 resulted in decreased lipid-binding affinity compared with intact apoE, with a decreased ability to promote cellular phospholipid and cholesterol efflux [21]. In addition to lipoprotein binding, apoE CT domain also mediates protein tetramerization in aqueous solutions via residues 267–299 [19,22,23], probably mediated by intermolecular coiled-coil interaction [24], a motif frequently encountered in protein oligomerization and assembly [25]. However, in the lipid-associated state, apoE has been proposed to exist as a monomer [26], although 5–7 apoE molecules appear to be required to saturate lipoprotein particles with a diameter of ∼25 nm [27].

In the present study, we report the mode of lipid binding of apoE CT domain and the organization of its helices in a lipid-associated state. We evaluate the structural organization of apoE CT domain following reconstitution into nascent lipoprotein particles by CD, ATR-FTIR (attenuated total reflectance Fourier transform IR) and fluorescence spectroscopy.

EXPERIMENTAL

Expression and purification of recombinant apoE CT domain

A pET22b vector encoding apoE (201–299) and bearing a His$_6$ tag at the NT end was used for expression [24]. Recombinant wild-type apoE CT domain was overexpressed in *Escherichia coli* and purified by affinity chromatography on a Ni-affinity matrix (HiTrap™ chelating column, Amersham Biosciences).

Cross-linking analysis

Cross-linking experiments were carried out using DSS (disuccinimidyl suberate) (Pierce Biotechnology, Rockford, IL, U.S.A.), which is a homobifunctional, primary amine-reactive cross-linker with spacer arm length of ∼12 Å (1 Å = 0.1 nm), as described previously [24]. Reaction mixtures contained 0.05 mg of lipid-free apoE CT domain (or DMPC (dimyristoylphosphatidylcholine)- or DMPG (dimyristoylphosphatidylglycerol)-bound, as described below) and various concentrations of cross-linker in 25 mM potassium phosphate, pH 7.4. Incubations were carried out for 30 min at room temperature (24°C). The reaction was quenched by the addition of 1 M Tris/HCl, pH 7.4, and/or non-reducing SDS/PAGE sample treatment buffer for 15 min, followed by electrophoresis on a 4–20% gradient gel to confirm cross-linking. DMSO was used as the solvent for DSS at concentrations not exceeding 2% of total reaction mixtures (v/v).

Lipid binding assay

The ability of apoE CT domain to transform phospholipid vesicles to discoidal bilayer particles was studied by monitoring the changes in right-angle light scattering in a PerkinElmer LS50B spectrofluorimeter [28]. Unilamellar vesicles of DMPG were prepared by extruding through a 200 nm membrane (Avanti Polar Lipids, Alabaster, AL, U.S.A.). Increasing concentrations of apoE CT domain (4–200 μg of protein) were added to 0.2 mg of DMPG vesicles in a thermostatically controlled cuvette containing 20 mM Tris/HCl, pH 7.2, and 150 mM NaCl at 32°C. The changes in light-scattering intensity were monitored at 560 nm (excitation and emission wavelengths) with a slit width of 3 nm.

Preparation and characterization of DMPC– or DMPG–apoE CT domain complex

DMPC– or DMPG–apoE CT domain discoidal bilayer complexes were prepared as described previously [29]. Briefly, sonicated or extruded phospholipid vesicles and apoE CT domain (2.5:1, w/w) were incubated in 50 mM Tris/HCl, pH 7.5, for 18 h at 24°C, followed by KBr density gradient ultracentrifugation to separate unbound protein and protein-free lipid vesicles from lipid-bound protein. Fractions containing both protein and lipid were pooled and concentrated. Protein assay was carried out using the bicinchoninic acid method (Pierce Biotechnology), and phospholipids were estimated using the phospholipid assay kit (Wako Chemicals GmbH, Neuss, Germany). Non-denaturing PAGE of the isolated lipoprotein complexes was carried out to evaluate the molecular mass and size of the particles on a 4–20% gradient gel for 20 h at 150 V and stained with Amido Black.

CD spectroscopy

Secondary-structural characterization was carried out on Applied Photophysics CD spectrometer equipped with a thermostatically controlled cuvette holder. The secondary-structure content of the samples was estimated using CDPRO software [30]. CD spectra were recorded between 250 and 190 nm at 0.5 nm intervals, and averaged over 20000 points.

IR spectroscopy

ATR-FTIR spectra were recorded on a Bruker IFS 55 IR spectrophotometer equipped with a reflectance accessory and a polarizer mount assembly with an aluminium wire grid on a KRS-5 element. The internal reflection element was a germanium ATR plate (50 mm × 20 mm × 2 mm) with an aperture angle of 45° yielding 25 internal reflections. The samples were dialysed against 5 mM Tris/HCl, pH 7.5, before FTIR analysis. Oriented multilayers were formed by slow evaporation of ∼30 μl of DMPC–apoE CT domain (∼0.5 mg/ml) on one side of the ATR plate under a gentle stream of nitrogen, yielding a semi-dry film bearing residual water molecules. The ATR plate was then sealed in a universal sample holder. Spectra were recorded at a 2 cm$^{-1}$ nominal resolution. A total of 256 accumulations were performed to improve the signal/noise ratio. The spectrometer was constantly purged with dry air. All measurements were made at 25°C. Before any analysis, the side-chain contributions to the spectra were subtracted [31].

Secondary-structure analysis by FTIR

Secondary-structure measurements were carried out on samples following deuteration for 1 h as described previously [32]. Briefly, Fourier self-deconvolution was applied to increase the resolution of the spectra in the amide I region. Least squares iterative curve fitting was performed to fit different components of the amide I band revealed by the self-deconvolution to the non-deconvolved spectrum between 1700 and 1600 cm$^{-1}$. The proportion of various secondary-structural elements was computed as reported in [32].

Orientation of the secondary structures on discoidal bilayer complexes

In an α-helix, the main transition dipole moment lies roughly parallel to the helical axis. It is therefore possible to determine the mean orientation of the α-helix structure from the orientation of the peptide bond C=O group [33]. To obtain this information, spectra of DMPC–apoE CT domain complexes were recorded with parallel and perpendicular polarized incident light with respect to a normal to the ATR plate. Polarization was expressed as the dichroic ratio $R_{\text{d}} = A_{\|}/A_{\perp}$. The mean angle between the helix axis and a normal to the ATR plate surface was then calculated from $R_{\text{d}}$. In these calculations, a 38° angle between the long axis of the α-helix and the C=O dipole moment was considered [34].
The γ(CH₃) transition at 1202 cm⁻¹, whose dipole lies parallel to the all-trans hydrocarbon chains, was used to characterize the lipid acyl chain orientation [35]. An estimation of the sample film thickness was obtained using the isodichroic ratio [36], based on which the angle for both lipids and protein α-helices was estimated.

**Fluorescence quenching**

We took advantage of the intrinsic fluorescence properties of apoE CT domain, attributed to the presence of three tryptophan residues, Trp²¹⁰, Trp²⁶⁴, and Trp²⁷⁶, to assess the depth of location of the fluorophores with respect to the phospholipid bilayer. Quenching of fluorescence emission of DMPC–apoE CT domain lipoprotein complexes was carried out as described previously [37,38] with 5-DSA (5-DOXYL stearic acid) or 12-DSA (12-DOXYL stearic acid), where the DOXYL group (quenching moiety) is located at different depths along the fatty acyl chain. Aliquots of 5-DSA or 12-DSA (1.3 mM stock in ethanol) were added directly to DMPC–apoE CT domain complexes (keeping the final concentration of ethanol ≤ 1%, v/v), and fluorescence intensities were measured at 340 nm. Effective quenching constants were calculated employing the Stern–Volmer equation, $F_0/F = 1 + K_{SV} \cdot [Q]$, where $F_0$ and $F$ are fluorescence intensities in the absence and the presence of various quencher concentrations [Q] respectively, and $K_{SV}$ is the Stern–Volmer quenching constant [39].

**Electron microscopy**

DMPC–apoE CT domain complexes were dialysed against ammonium acetate buffer and negatively stained with 2% sodium phosphotungstate, before examination by electron microscopy (Zeiss 10, 80 kV) as described previously [40].

**RESULTS**

**Lipid-binding activity of apoE CT domain**

A characteristic feature of apolipoproteins is their ability to interact with vesicular phospholipid bilayer structures (diameter ~200 nm) and convert them into small lipoprotein particles. This transformation or clearance of phospholipid vesicles has been monitored routinely as changes in intensity of right-angle light scattering [41–44]. We employed DMPG, a negatively charged phospholipid, to study the lipid-binding ability [28] of increasing concentration of apoE CT domain corresponding to lipid/protein mass ratios varying from 50:1 to 1:1. ApoE CT domain displays a concentration-dependent effect in its ability to cause vesicle clearance, as indicated by the decrease in right-angle light scattering intensity following addition of the protein. Figure 1 shows data for 10, 20, 50 and 100 µg of apoE CT domain added to 200 µg of DMPG, corresponding to lipid/protein mass ratios of 20:1, 10:1, 4:1 and 2:1 respectively. This indicates that isolated recombinant apoE CT domain retains lipid-binding ability, allowing us to examine the mechanism of lipid-binding interaction further.

**Estimation of reconstituted lipoprotein particle diameter**

Non-denaturing gradient PAGE analysis of DMPC–apoE CT domain demonstrated lipoprotein particles of 17 ± 2 nm Stokes diameter as the major species, corresponding to an apparent molecular mass of ~600 kDa (Figure 2). Particles 12 nm in diameter were noted to a minor extent (<10%). Electron microscopic analysis of DMPC–apoE CT domain complexes by negative staining (Figure 3) reveals discoidal structures with an average diameter of 16.6 ± 2.7 nm (n = 122), consistent with estimates obtained from native PAGE analysis. Similar discoidal complexes were noted earlier with other apolipoproteins, such as insect apolipoporphin III [42,45]. Compositional analysis DMPC/apoE CT domain particles yielded lipid/protein molar ratio of ~200:1. In the case of DMPG–apoE CT, particles with smaller diameter (~8 nm) were formed.

**Secondary-structure characterization of lipid-bound apoE CT domain**

The secondary-structure content of DMPC–apoE CT domain discoidal complexes was analysed by CD (Figure 4) and IR spectroscopy. Far-UV CD spectra of DMPC–apoE CT domain reveal...
DMPC–apoE CT domain complexes prepared and isolated as described in the Experimental section. The reconstituted lipoprotein particles were stained with 2% phosphotungstate for visualization. Scale bar, 100 nm.

Figure 4  Far-UV CD spectrum of DMPC–apoE CT domain complex

Far-UV CD spectrum of DMPC–apoE CT domain complex was recorded in 25 mM sodium phosphate, pH 7.0, from 250 to 190 nm at 24°C at 0.5 nm intervals, and averaged over 20 000 points.

Figure 5  IR spectrum of DMPC–apoE CT domain discoidal complexes in the 1700–1500 cm$^{-1}$ region (solid curve)

Secondary structure of DMPC–apoE CT domain complex was obtained by analysis of IR spectrum between 1700 and 1600 cm$^{-1}$ by curve fitting. The different curves used to fit the amide I peak are displayed as broken lines.

Table 1  Quenching of intrinsic fluorescence of DMPC–apoE CT domain complexes by 5-DSA and 12-DSA

<table>
<thead>
<tr>
<th>Quencher</th>
<th>$K_{SV}$ (M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-DSA</td>
<td>0.14 ± 0.03</td>
</tr>
<tr>
<td>12-DSA</td>
<td>0.06 ± 0.02</td>
</tr>
</tbody>
</table>

* $K_{SV}$ is obtained from a plot of $F_0/F$ against [Q].

Figure 6  IR spectra of DMPC–apoE CT domain complexes

The top spectrum was obtained with parallel polarized light and the middle spectrum with perpendicular polarized light. The bottom spectrum is the dichroic spectrum obtained by subtracting the middle spectrum (perpendicular polarization) from the top spectrum (parallel polarization). The optical density amplitude of the bottom spectrum has been increased three times with respect to the other spectra. Striped peaks are the most important for the orientation determination (see text).

Fluorescence quenching analysis

Lipid-based quenchers were employed to estimate the average depth of location of the three tryptophan residues in apoE CT domain with respect to the phospholipid bilayer in the lipoprotein particle. Increasing concentrations of DSA with the DOXYL group located at C-5 (5-DSA) or C-12 (12-DSA) was added directly to DMPC–apoE CT domain complexes in sodium phosphate, pH 7.0, to quench the intrinsic fluorescence emission of tryptophan residues in lipid-bound apoE CT domain. The higher $K_{SV}$ observed for 5-DSA (0.14 ± 0.03 M$^{-1}$) compared with that of 12-DSA (0.06 ± 0.02 M$^{-1}$) (Table 1) is indicative of a superficial location of the tryptophan residues proximal to the lipid/water interface.

Determination of helix orientation in DMPC–apoE CT domain

FTIR spectra of DMPC–apoE CT domain complex were recorded with parallel and perpendicular polarized light (Figure 6). The dichroic spectrum was obtained by subtracting the spectrum recorded with the perpendicular polarized light from that recorded with parallel polarized light using the lipid $\nu$(C=O) band as a
The conditions, while the upper band (37 kDa) contained a positive deviation. We used the peak at 1202 cm$^{-1}$ to quantify the lipid chain orientation. The measured dichroic ratio for this band is 3.6 with an isotropic dichroic ratio of 1.57, i.e. the dichroic ratio observed for an isotropically oriented sample. The lipid chain orientation was determined using the amide I band attributed to the peptide fragments encompassing discrete segments of apoE [49,50]. Whereas peptide fragments encompassing discrete segments of apoE (residues 263–286 and 267–286) display lipid-binding characteristics, we find it essential to examine the lipid-bound configuration of the entire functional unit encompassed in isolated apoE CT domain.

DISCUSSION

The lipid-binding interaction is an essential prerequisite for apoE to elicit its physiological role as a ligand for the LDL receptor family of proteins and to promote cholesterol efflux in atherosclerotic lesions. Interestingly, model lipid-bound complexes comprising phospholipids and apoE recapitulate the functional features of apoE on native spherical lipoprotein particles or nascent HDL. Furthermore, lipid binding of apoE is believed to be initiated by the CT domain, which bears a higher lipid-binding affinity than the NT domain, a concept supported by the lower free energy of stabilization for the former and the tendency of apoE to self-associate in a lipid-free state via the CT domain [10,11]. The two domains of apoE are independently folded structural and functional units, and isolated apoE CT domain retains the lipid-binding capability of the intact protein [49,50].

Cross-linking of apoE CT on discoidal particles

Finally, cross-linking of apoE CT domain was performed subsequent to formation of DMPC discoidal particles. Increasing concentrations of DSS, a cross-linker that bears the ability to access lysine residues from an aqueous and lipid environment, was employed, followed by SDS/PAGE analysis (Figure 7). At all concentrations of DSS employed, a band corresponding to dimeric apoE CT domain (apparent molecular mass of 32 ± 1 kDa) was the major cross-linked species present. Another minor band appeared at ~37 kDa. Densitometric analysis of the gel showed that the dimeric band (32 kDa) represented 30% of the proteins under all the conditions, while the upper band (37 kDa) contained <2% of the proteins. A similar pattern was noted in the case of DMPG–apoE CT domain (results not shown).

Figure 7 SDS/PAGE of DMPC–apoE CT domain discoidal complexes cross-linked with DSS

DMPC–apoE CT domain complexes (0.05 mg of protein) were incubated with increasing concentration of DSS for 30 min at room temperature. The reaction was stopped by addition of 1 M Tris/HCl, pH 7.4, and SDS sample treatment buffer, followed by electrophoresis in a 4–20% acrylamide gradient gel. Lane 1, molecular mass markers with the indicated masses in kDa; lane 2, DMPC–apoE CT domain; lanes 3–6, DMPC–apoE CT domain treated with 1-, 10-, 20-, and 50-fold molar excess DSS respectively.
neighbouring helix to form a coiled coil. In the case of lipid-free apoE CT domain, the ratio was calculated to be 1:0.1, indicative of coiled-coil helix formation [24]. Interestingly, lipid association did not induce any further increase in α-helicity or alteration in the 222/208 nm ratio. We suggest that the overall curvature bestowed on apoE CT domain helices as they circumscribe the discoidal bilayer contributes to the altered absorbance at 208 nm in lipid-associated apoE CT domain, with a resultant maintenance of the ratio at ~1:0.1.

In conjunction with fluorescence studies, FTIR analysis provides valuable information regarding the spatial disposition of the helices on the discoidal particles with regard to the lipid bilayer. Using parallax analysis of fluorescence quenching experiments, tryptophan residues 210, 264 and 276 in apoE CT domain are indicated to be located at the same depth in the bilayer, suggesting a perpendicular orientation of the helices. The fluorescence quenching approach provides a limited view of the spatial disposition of selected sites. On the other hand, ATR-FTIR analysis provides a discriminatory approach to determine not only the orientation of a secondary structure (using linear dichroism), but also the nature of this secondary structure (using the wavelength at which the dichroism appears, see above). Therefore, in the present study, we provide the first direct evidence that the entire helical segment of apoE CT domain is oriented perpendicular with respect to the phospholipid fatty acyl chains. This indicates that two helices (~15 Å diameter each) may be aligned adjacent to each other-shielding the hydrophobic part of the lipid bilayer (34 Å), circumscribing the perimeter of the lipid discoidal particles (the ‘belt model’). This conformation is in agreement with the cross-linking results of the present study that indicate apoE CT probably adopts a dimeric conformation on these particles. A similar orientation has been described for the receptor-binding domain of apoE [47,55]. Since the two domains of apoE are oriented similarly, it is likely that intact apoE also adopts such an extended belt conformation at the periphery of reconstituted discoidal lipoproteins. The perpendicular alignment of helices appears to be the trend for exchangeable apolipoproteins, with apoA-I (apolipoprotein A-I) [56–58] and insect apolipophorin III [46] displaying similar tendencies, while the parallel alignment (49) (the ‘picket-fence model’) appears to be of lesser relevance.

Taking the apparent molecular mass of the lipoprotein particle (~600 kDa), discoidal particle diameter of approx. 17 nm, in conjunction with the compositional analysis (lipid/protein molar ratio, 200:1) into consideration, an estimated 4–6 apoE CT domain molecules per discoidal particle in a fully extended belt-like conformation favours dimeric interactions. Such a mode of interaction gives us an indication of the structural aspects of recruitment of apoE to cholesterol-poor or nascent HDL particles at sites of atherosclerotic lesions, thereby increasing the capacity to acquire cholesterol [61,62]. Interestingly, it has been shown that the lipoprotein-binding surface of apoE CT domain offers a potential site for interaction with β-amyloid peptide, which is involved in the pathogenesis of Alzheimer’s disease [63]. With the emerging role of lipids in Alzheimer’s disease, understanding the mode of lipid-binding interaction of apoE is an important first step towards further studies addressing its potential role in amyloid formation.

This work was supported by a Pfizer International HDL Research Award, an Alzheimer’s Association and a Parkinson’s Disease Foundation grant (to V.N.). We thank Dr Cyril M. Kay and Robert Luty for CD measurements. V.R. and E.G. are, respectively, research associate and research director of the National Fund for Scientific Research (Belgium).

REFERENCES