Published in final edited form as:

J Mol Biol. 2009 February 13; 386(1): 261–271. doi:10.1016/j.jmb.2008.12.040.

Insight on the Molecular Envelope of Lipid-Bound Apolipoprotein E from Electron Paramagnetic Resonance Spectroscopy

Danny M. Hatters^{1,2,3,†}, John C. Voss^{4,†}, Madhu S. Budamagunta⁴, Yvonne N. Newhouse¹, and Karl H. Weisgraber^{1,2,3,*}

¹Gladstone Institutes of Cardiovascular and Neurological Diseases, 1650 Owens Street, San Francisco, CA 94158, USA

²Cardiovascular Research Institute, University of California, San Francisco, San Francisco, CA 94143, USA

³Department of Pathology, University of California, San Francisco, San Francisco, CA 94143, USA

⁴Department of Biological Chemistry, School of Medicine, University of California, Davis, Davis, CA 95616, USA

Abstract

Although a high-resolution X-ray structure for the N-terminal domain of apolipoprotein E (apoE) in the lipid-free state has been solved, our knowledge of the structure of full-length apoE in a lipid-bound state is limited to an X-ray model fitting a molecular envelope at 10-Å resolution. To add molecular detail to the molecular envelope, we used cysteine mutagenesis to incorporate spin labels for analysis with electron paramagnetic resonance (EPR) spectroscopy. Twelve cysteine residues were introduced singly and in pairs at unique locations throughout apoE4 and labeled with an EPR spin probe. The labeled apoE4 was combined with dipalmitoylphosphatidylcholine, the particles were purified, and spectra were determined for 24 combinations (single and double) of the cysteine mutants. Data on the conformation, mobility, distance, and surface exposure of regions revealed by the cysteine probes were modeled into the molecular envelope of apoE bound to dipalmitoylphosphatidylcholine that had been determined by X-ray analysis. This EPR model of apoE in a native lipid-bound state validates the structural model derived from X-ray analysis and provides additional insight into apoE structure-function relationships.

Keywords

protein structure; models; spin labels; lipoproteins

Introduction

Apolipoprotein E (apoE) is one of several exchangeable apolipoproteins involved in lipid transport within the cardiovascular and central nervous systems. In plasma, apoE associates

© 2008 Elsevier Ltd. All rights reserved

*Corresponding author. Gladstone Institutes of Cardiovascular and Neurological Diseases, 1650 Owens Street, San Francisco, CA 94158, USA. E-mail address: kweisgraber@gladstone.ucsf.edu..

[†]D.M.H. and J.C.V. contributed equally to this work.

Present address: D. M. Hatters, Department of Biochemistry and Molecular Biology, The University of Melbourne, Melbourne, Victoria, Australia.

Supplementary Data Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2008.12.040

with both high- and lower-density lipoproteins and targets lipoprotein uptake through its binding to members of the low-density lipoprotein receptor (LDLR) family on the surface of cells.^{1,2} In the central nervous system, apoE has been postulated to play a role in lipid remodeling and neuronal repair.³⁻⁵ Of the three common structural isoforms of apoE, apoE4 is associated with an increased risk for Alzheimer's disease and other forms of neurodegeneration.⁶⁻⁸

The conformation of apoE when associated with lipoproteins and the relationship with lipid-unassociated forms are not well understood. However, lipid binding is necessary for high-affinity binding to the LDLR, which suggests that apoE adopts a unique conformation in this state.⁹ This conclusion is supported by studies indicating that apoE has a substantially different conformation when associated with lipids.¹⁰⁻¹⁶ We also showed that lipid-free apoE, which normally forms a tetramer, readily associates into neurotoxic proto-filament-like fibrils that could play a pathological role in disorders, such as Alzheimer's disease.¹⁷

In the lipid-free state, apoE adopts and displays two independently folded domains that are separated by a more flexible linker region.^{18,19} The 191 N-terminal residues or so form a four-helical bundle.²⁰ The structure of the C-terminal domain has not been determined to high resolution, but it adopts an independently folded, α -helix-rich domain that is separated from the N-terminal bundle by a more flexible, protease-sensitive linker region.²¹

Less is known about the structure of lipid-associated apoE. Recently, we determined a low-resolution model of the structure of apoE complexed with dipalmitoylphosphatidylcholine (DPPC) based on 10-Å diffraction data of crystals of the co-complex.¹⁰ Unlike the lipid-free state, the protein is predicted to adopt an α -helical hairpin conformation in the shape of a helical horseshoe in which the apex of the hairpin juxtaposes all known elements of the LDLR-binding region. This model is supported by small-angle X-ray scattering studies in which two helical horseshoes bind to a spheroidal particle.¹⁶

In an earlier study, we used electron paramagnetic resonance (EPR) spectroscopy of site-directed spin labels to probe the conformation of the lipid-free tetrameric protein.¹¹ Here, we applied EPR spectroscopy to establish helix orientations and side-chain proximities in the context of the helical hairpin structure of apoE•DPPC.

Results

ApoE•DPPC complex preparation

We prepared complexes of apoE and DPPC by using established cholate/dialysis methods.^{22,23} Previously, we showed that apoE complexed with DPPC binds the LDLR effectively and that, once formed, apoE•DPPC complexes were relatively stable and readily formed crystals.²³ The apoE4•DPPC complexes have a density similar to that of high-density lipoprotein (HDL) (Fig. 1a).

One concern in site-directed spin-labeling EPR experiments is that engineering cysteine substitutions and subsequent modification by the spin label may significantly alter protein folding, stability, or behavior. However, experimental and theoretical calculations reveal that the nitroxide ring is well tolerated in proteins because of its ambivalent hydrophobicity, its compact size when conjugated to a Cys residue (total side-chain volume comparable with Tyr), and its ability to assume a limited number of rotamers.²⁴⁻²⁶ These are evidenced by studies on T4 lysozyme where nitroxide labels incorporated at either exposed or buried sites result in no significant perturbation of the backbone.

Our nitroxide-labeled mutants of apoE were complexed to DPPC and screened for a number of features to ensure that the protein retained key structural and lipid-binding features. In summary, complexes composed of mutant apoE used in this study were of similar size and morphology with nonmutated apoE4 when assessed by gel-filtration chromatography (not shown) and electron microscopy (Supplementary Fig. S1), providing confidence that the mutations used in this study retain key biological and structural properties. Conversely, mutants that did not display these properties were excluded from further analysis.

EPR spectroscopic measurements

We sought to assign amino acid residues to specific locations within the three-dimensional molecular envelope determined from diffraction studies on apoE4•DPPC.¹⁰ EPR spectroscopy of site-directed spin labels was used to obtain a set of constraints within the envelope. To access the side-chain dynamics within different regions of DPPC-bound apoE4, we introduced spin-labeled side chains into apoE4 at 12 unique positions: 18, 76, 77, 94, 120, 239, 241, 247, 263, 264, 278, and 289. All EPR spectra were normalized to the same number of spins (Fig. 2). The line shape of each spectrum reflects the dynamics unique to the specified location, ranging from the narrow lines of position 18 (indicating a high range of motion for both the side chain and the backbone) to the very broad spectrum of position 247 (revealing a strongly immobilized side chain indicative of a tightly packed environment).

Each sample was also prepared by mixing (1:1) (1-oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-methyl)-methanethiosulfonate (MTS-SL)-labeled protein with the EPR-silent *N*-ethyl maleimide (MalNET)-labeled protein before DPPC particle assembly (see Materials and Methods) to determine if any spectral broadening of the singly labeled proteins arises from intermolecular magnetic interactions between labels. In each of the 12 samples, broadening was not influenced by spin dilution (examples are shown below); thus, broad spectra represent minimal averaging of the hyperfine anisotropy through rapid reorientation of the spin label. Since highly mobile and highly immobile sites represent surface and buried sites, respectively, they can be applied as constraints when considering the arrangement of apoE4•DPPC in a helical hairpin structure. The constraints, based on spin-label dynamics (Table 1), can be used to classify each of the spin-labeled positions into four categories of molecular order.

A second set of constraints was collected to determine the alignment of the strands within the hairpin. Here, we probed for dipolar broadening in the continuous-wave EPR spectrum, which is evident for spins that are within 20 Å of one another.²⁷ Double-Cys substitutions were introduced into apoE4 and the resultant EPR spectra were analyzed for evidence of dipolar broadening to identify proximal regions within apoE4•DPPC. Figure 3 shows the results of 12 combinations of apoE4•DPPC-containing spin labels at two sites. The presence of a dipolar interaction can be observed by comparing the spectrum of the doubly labeled sample with the summation of the spectra acquired from the two sites individually. Figure 3a compares (for an identical number of spins) the spectrum for the doubly labeled protein (black trace) with the resultant spectrum generated by adding the spectra acquired from the two sites in the singly labeled protein (red trace). Of the 12 combinations measured, only 5 (18-289, 76-239, 76-241, 77-239, and 94-247) displayed significant dipolar broadening. The interaction in the 76-241 pair is most prominent (reflecting a distance separating the spin labels of ~15 Å). This mirrors previous work that established close proximity of the 76-241 pair in both the lipid-free state and the DMPC-associated state.¹¹ Working within the structural envelope determined by X-ray diffraction, the proximity of this pair alone can establish the alignment of the two strands in the horseshoe structure, assuming a side-by-side association of elongated, mostly helical structures. Although weaker (on the order of 16-18 Å), interactions at other sites both in the central region (76-239, 77-239, and 94-247) and near the termini (18-289) are consistent with an alignment that places the region of 162-169 in the hairpin loop connecting the two strands.

X-ray and biochemical studies revealed that each DPPC particle contains two apoE4 molecules.¹⁰ To determine whether any of the significant dipolar broadening could be due to intermolecular interaction, we diluted the number of spin-labeled apoE molecules per DPPC particle by assembling particles with a mixture of doubly nitroxide-labeled protein and doubly MalNEt-labeled protein (1:1). Intermolecular interaction between spin labels would be substantially affected by this spin dilution: only 25% of the particles contain two spin-labeled species. However, spin dilution had no effect on the shape or amplitude of the 76-239, 76-241, 77-239, and 94-247 pairs (when normalized to the same number of spins) (Fig. 3b). In summary, 5 of the 12 nitroxide pairs were within 2 nm, and their interactions were largely, if not exclusively, intra-molecular.

Model building

The dimensions of the protein envelope obtained by X-ray diffraction suggest an arrangement of apoE4•DPPC in which one strand of the helical hairpin is in contact with the lipid surface, while the outer strand runs along the surface of the inner strand. Like apoA-I, apoE contains repeating 11/3 amphipathic sequence motifs, which facilitate both protein-protein and protein-lipid interactions along a curved surface.²⁸ However, unlike the canonical models for apolipoprotein association with lipid particles that have the hydrophobic faces of the amphipathic helices in contact with lipid, this arrangement would allow for only one strand of the hairpin to be partitioned into lipid. In addition, the side-chain mobilities, as determined by EPR, showed that residues oriented toward the hydrophobic face were the most immobilized (e.g., S94, V239, and A247), suggesting that protein-protein contact on apoE4•DPPC is established along hydrophobic faces of the helices.

While we cannot exclude the possibility that these immobilized side chains result from protein-lipid interactions rather than protein-protein interactions, results from other studies on surface-binding amphipathic peptides and proteins indicate that nitroxide interactions with neutral phospholipids do not appreciably immobilize the nitroxide moiety.^{29,30} This suggests that the head-group region in phosphatidylcholine lamellar structures is at least as disordered as surface-exposed spin-labeled side chains and thus provides confidence that our highly immobilized nitroxide labels result from protein-protein interactions.

Thus, to construct our model, we used the dihedral angle parameters for the backbone to generate a curved 11/3 helix as described by Segrest *et al.*²⁸ These backbone angles generate a curved amphipathic helix with the hydrophobic residues oriented inward. In this 11/3 helix motif, hydrophobic residues occupied positions 1, 5, and 8 (Fig. 4). However, to create a structure in which the protein-protein contact is facilitated by hydrophobic residues, we exchanged the angle assignments for the C-terminal half of apoE4 such that the hydrophobic residues are presented on the outer surface of the curved helix.

We then used the interactive modeling program ProteinShop to adjust backbone dihedral angles for all stretches of sequence that agree with the 11/3 motif (i.e., with positions 1, 5, and 8 being hydrophobic). Unassigned regions were initially defined to have an extended-coil backbone structure. This produces a series of curved amphipathic helical segments connected by regions of random coil. Treating the random-coil regions as flexible loops, we then used ProteinShop to calculate dihedral angles within the random-coil regions that accommodate the positioning of the helical segments in three-dimensional space. This positioning of the helical segments obeys the following constraints:

- i. A helical backbone fold is only given to regions of sequence that are consistent with the 11/3 sequence motif.
- ii. Hydrophobic side chains of residues 76 and 241 are separated by 1.3-1.5 nm.

- iii. Alignments of helices from the N- and C-terminal halves of the protein are made along the hydrophobic faces.
- iv. The inner and outer strands of the helix are stacked such that the arms of the horseshoe have dimensions consistent with the density envelope determined by X-ray diffraction [i.e., 20 Å (width) by 10 Å (thickness)].

The resultant model is shown in Fig. 5. The interface of the two strands via hydrophobic residues is highlighted in Fig. 5a, where nonpolar residues are shown in red and polar residues are shown in blue. The alignment of positions 76 and 241 is achieved by introducing a hairpin loop in the central coil segment predicted for residues 162-169. The rendering of helical (red cylinders) and coil (green ribbon) structures is shown in Fig. 5b. Also, the sites of proteolysis were confirmed in the apoE4•DPPC particles.¹⁶ There is a strong correlation between positions that are susceptible to proteolytic cleavage and the ends of predicted helical domains.

The molecular model can also be evaluated on the basis of the observed side-chain dynamics at the 12 targeted positions. Figure 6 shows the spin-labeled side chains displayed in a space-filling model of apoE4•DPPC. The broadest spectra (94, 239, and 247; Fig. 2) are largely buried within the structure, whereas the other sites occupy surface-exposed positions, a placement consistent with the EPR results. In addition, position 18 is the only residue predicted to be well within a coil domain, an assignment that agrees with experimental data (Fig. 2).

Discussion

The EPR approach used in these studies has provided important new conformational details of apoE4 bound to DPPC that were not observable in the low-resolution models from X-ray crystallography and small-angle X-ray scattering.^{10,16} The conformational, mobility, distance, and surface exposure data of regions revealed by the 12 cysteine probes throughout apoE bound to DPPC were modeled into the molecular envelope of apoE bound to DPPC determined by X-ray analysis. This EPR model of apoE in a native lipid-bound state provides the most detailed structural information available to date.

The docking of apoE4 on the head-group surface of DPPC allows for the hydrophobic surfaces of the protein to interact within the hairpin, producing strongly immobilized spectra at positions 94, 238, and 247 and stabilizing the protein-protein interaction of the N- and C-terminal regions. The 11/3 helices in apoE provide for a continuous curved surface that is consistent with the circular horseshoe molecular envelope. The EPR model supports the prediction from X-ray models that apoE interacts with phospholipids differently from apoA-I and that the arrangement of apoE on DPPC particles differs significantly from the association of apoA-I on discoidal particles.^{10,16} In contrast, in the lipid-bound state of apoA-I, the protein surrounds the periphery of a lamellar phospholipid disc, with the hydrophobic faces of the helices shielding the aliphatic lipid chains from solvent. EPR of site-directed spin labels in lipid-bound apoA-I shows little evidence for stable protein-protein interactions between the lipid-associated helical segments.³¹⁻³³ In addition, although membrane proteins represent the majority of systems studied by site-directed spin-labeling EPR, the only examples in the literature that show strong immobilization are due to head-group interactions involving Ca⁺ chelation. It is important to note that the protein-protein interactions we see are along the hydrophobic face (i.e., van der Waals) and are therefore very close-range interactions that will indeed result in limited mobility for interfacial side chains. In addition, the spin-labeled side chain can participate in these interactions if it is oriented toward the interface. Electrostatic interactions are longer in range (so there is more space in the interface), and the spin-labeled side chain (being neutral) will not participate.

These differences in lipid binding provide a potential explanation for why apoA-I- and apoE-containing HDLs behave differently in reverse cholesterol transport, an important pathway for removing cholesterol from the body.³⁴ A first step in this process is the transfer of cholesterol from peripheral cells to the surface of HDL particles. The cholesterol is then esterified by lecithin-cholesterol acyltransferase and transferred to the hydrophobic core of the particle, expanding the size of the particle. With its unique form of phospholipid interaction, apoE would be predicted to be less sensitive than apoA-I to the size of the hydrophobic core. This finding is consistent with previous observations that apoA-I HDL particles have limited ability to support core expansion, whereas apoE-containing particles more easily support core expansion.^{35,36}

In these studies, we focused only on the apoE4 isoform (the other two common isoforms are apoE2 and apoE3). Unlike apoE3 and apoE2, apoE4 displays domain interaction. In this prominent structural feature, the apoE4 N- and C-terminal structural domains interact through a salt bridge between arginine 61 and glutamic acid 255, resulting from the influence of Arg112 in apoE4 on the conformation of Arg61 (Fig. 7).^{37,38} Domain interaction does not occur to the same degree in apoE3 and apoE2 because both contain Cys112, which does not induce an Arg61 conformation that promotes interaction with Glu255. In addition, apoE4 with mutations Arg61Thr or Glu255Ala does not display domain interaction, and these mutants function in a manner similar to apoE3.^{37,38} In a mouse model specific for domain interaction, domain interaction resulted in lower apoE levels in the brain and was associated with synaptic, functional, and cognitive deficits.^{39,40}

Previously, we determined that the distances between the cysteine probes at positions 76 and 241 were closer in lipid-free apoE4 (11 Å) than in apoE4 Arg61Thr (>23 Å), which provided physical evidence supporting the concept of domain interaction.¹¹ Interestingly, these distance differences were maintained when apoE4 and apoE4 Arg61Thr were bound to DPPC and were confirmed in the current studies. These results indicate that domain interaction in lipid-free apoE influences the final conformation of apoE4 and apoE3 bound to DPPC particles. Similarly, domain interaction in apoE4 accounts for its preference for very-low-density lipoprotein particles.^{37,38,41}

X-ray crystallography studies showed that Arg112 in helix 3 of the four-helix bundle of the N-terminal domain is in close proximity to Arg61 in helix 2 in the lipid-free state (Fig. 7). The proximity of Arg112 influences the conformation of the Arg61 side chain, positioning it to interact with Glu255. Several studies demonstrated that the N-terminal four-helix bundle undergoes an extensive reorganization in binding to lipids.^{12,42,43} What is interesting from the current studies is that while Arg61 and Glu255 maintained close proximity bound to DPPC, Arg112, the residue responsible for the interaction of Arg61 with Glu225 in the lipid-free state, was now distant from the Arg61/Glu225 pair (Fig. 7). This suggests that the influence of domain interaction on the conformation of apoE4 on DPPC particles or its preference for very-low-density lipoproteins must occur in the early stages of lipid association before the influence of Arg112 is lost as the four-helix bundle undergoes structural reorganization.

The primary distance constraint in the modeling was the strong 76-241 interaction, although other pairs showing lesser interaction (on the order of 17- 20 Å) each has a separation distance in the model consistent with the observed, but weak, dipolar interaction. Furthermore, all pairs that show no interaction by EPR are separated by >2 nm. The model now accurately positions the region of amino acids 162-169 in the hairpin loop that connects the two helical strands in the circular horseshoe model. As suggested previously, a potential hairpin loop in this region would bring regions enriched in basic residues (134-150 and 172) and known to be important in the interaction of apoE with the LDLR in juxtaposition.¹⁰ The repositioning of these residues

into close proximity in the lipid-bound state provides an explanation for the requirement of lipid association for high-affinity binding of apoE to the LDLR.⁹

In summary, the EPR model of apoE•DPPC lipoprotein particles generated in these studies provides new structural details for this protein, which plays a central role in lipid transport and whose isoforms have a differential impact on disease. Details revealed in the model provide new and important insight on the structure and function of apoE.

Materials and Methods

Materials

Materials included MTS-SL (Toronto Research Chemicals, catalog number O875000), a QuikChange multisite XL mutagenesis kit (Stratagene), MalNEt (Sigma) and sodium cholate (Sigma, catalog number C-1254), DPPC (Avanti Polar Lipids), YM-10 centriprep and centricon filtration devices (Amicon), Slide-A-Lyzer dialysis cassettes (molecular weight cutoff= 10,000; Pierce), PD-10 columns (GE), BioRad Protein Assay reagent (catalog number 500-0006), and a Superdex 200 10/300 GL column (Pharmacia).

ApoE mutagenesis, expression, and purification

Recombinant apoE was expressed in *Escherichia coli* and purified as described previously.^{11,44} Mutations were made in the expression plasmid with the QuikChange Mutagenesis kit (Stratagene), and the cDNA sequences of the vector were verified with DNA sequencing.

Site-specific spin labeling

ApoE [5 mL of 1 mg/mL in 100 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% β -mercaptoethanol, and 5 M guanidine hydrochloride, pH 7.4] was dialyzed into 100 mM ammonium bicarbonate, 1.5 M guanidine hydrochloride, 1 mM EDTA, and 1 mM DTT, pH 7.8, for 4 h at 4 °C with a Slide-A-Lyzer. After dialysis, the apoE was buffer exchanged into 100 mM ammonium bicarbonate, 1.5 M guanidine hydrochloride, 1 mM EDTA, and 100 μ M tris(carboxylethyl)phosphine, pH 7.8, with two PD-10 columns in parallel, according to the manufacturer's directions. The resulting 7-mL volume was divided into two fractions. The larger portion (5.25 mL) was spin labeled with MTS-SL by quickly mixing 42 μ L of a 37.8 mM stock (prepared in acetonitrile) with the 5.25-mL solution. The remaining portion (1.75 mL) was labeled with MalNEt by rapidly mixing with 23.75 μ L of MalNEt (46 mM stock prepared in DMSO). Samples were incubated overnight at 4 °C in the dark.

Purification of apoE•DPPC complexes

The labeled apoE samples were dialyzed into TBS (10 mM Tris, 150 mM sodium chloride, 1 mM EDTA, and 0.05% sodium azide, pH 7.4) for 4 h at 4 °C. In the meantime, a solution of DPPC (7.7 mg/mL) and that of sodium cholate (18.46 mg/mL) in TBS were solubilized by incubation at 45 °C for 4 h. After dialysis of the apoE, a portion (1.75 mL) of the spin-labeled apoE was transferred to the tube containing MalNEt-labeled apoE (to give a 50% MTE:50% MalNEt sample). DPPC/cholate solution (325 μ L) was added to each of the two apoE samples (100% MTE labeled and 50% MTE labeled), and the resultant solutions were incubated at room temperature for 10 min and then again at 39 °C for a further 4 h. The samples were exhaustively dialyzed into cold TBS over 5 days at 4 °C with the Slide-A-Lyzer cassettes. After dialysis, the sample density was raised to 1.21 g/mL by adding potassium bromide, and the solutions were laid beneath a three-step gradient of $d = 1.19$ g/mL (10 mL), $d = 1.17$ g/mL (10 mL), and $d = 1.10$ g/mL (10 mL) in a QuikSeal tube (Beckman/Coulter). The tubes were sealed and spun in a 60Ti rotor at 55,000 rpm for 16 h at 15 °C with the brake off. The tubes were

fractionated into 15-17 fractions by piercing the bottom of the tube with a needle and drawing off the liquid with a peristaltic pump.

Fractions were assayed for protein with the BioRad Protein Assay reagent and, in some samples, for phospholipid with a quantitative enzymatic colorimetric method (Wako Chemicals, catalog number 990-54009E). Tubes containing lipid-apoE complexes were pooled ($d \approx 1.10$ - 1.15 g/mL) and concentrated to 800 μ L with a YM-10 centriprep filtration device. The sample was further purified for homogenous particle size by gelfiltration chromatography on a Superdex 200 10/300 GL column with TBS as the elution buffer and a flow rate of 0.5 mL/min. Fractions corresponding to the major peak were collected and pooled (10-12 mL of elution volume for all mutants). Samples were concentrated to approximately 20 μ L with a YM-10 centricon. Protein concentrations were determined using the BioRad assay with bovine serum albumin as the mass standard.

EPR spectroscopy

EPR measurements were carried out in a JEOL X-band spectrometer fitted with a loop-gap resonator as described previously.³³ Briefly, an aliquot (5 μ L) of apoE•DPPC complex (1 mg/mL of apoE) in TBS was loaded into a sealed quartz capillary and placed in the resonator. Spectra were acquired at room temperature (20-22 °C) from a single 60-s scan over a field of 100 G at a microwave power of 2 mW and a modulation amplitude optimized to the natural line width of the individual spectrum (0.5- 1.5 G). The spectra as displayed are all normalized to the same number of spins using the sample unfolded in 5 M guanidine hydrochloride as a reference, where line broadening is minimized to facilitate the integrated intensity calculations. Based on the spin count for the protein concentrations measured, all sites label with an efficiency of >90%.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported in part by the National Institutes of Health through grants PO1 AG022074 and RO1 AG028793. We thank John Carroll and Chris Goodfellow for graphics, Gary Howard for editorial assistance, Jinny Wong for negative stains, and Linda Turney for manuscript preparation.

Abbreviations used

apoE, apolipoprotein E; LDLR, low-density lipoprotein receptor; DPPC, dipalmitoylphosphatidylcholine; HDL, high-density lipoprotein; MTS-SL, (1-oxy-2,2,5,5-tetramethyl-3-pyrroline-3-methyl)-methanethiosulfonate spin label; MalNEt, *N*-ethyl maleimide.

References

1. Weisgraber KH. Apolipoprotein E: structure- function relationships. *Adv. Protein Chem* 1994;45:249–302. [PubMed: 8154371]
2. Mahley RW. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science* 1988;240:622–630. [PubMed: 3283935]
3. Nathan BP, Bellosta S, Sanan DA, Weisgraber KH, Mahley RW, Pitas RE. Differential effects of apolipoproteins E3 and E4 on neuronal growth *in vitro*. *Science* 1994;264:850–852. [PubMed: 8171342]
4. Weisgraber KH, Pitas RE, Mahley RW. Lipoproteins, neurobiology, and Alzheimer's disease: structure and function of apolipoprotein E. *Curr. Opin. Struct. Biol* 1994;4:507–515.

5. Mahley RW, Huang Y. Apolipoprotein E: from atherosclerosis to Alzheimer's disease and beyond. *Curr. Opin. Lipidol* 1999;10:207–217. [PubMed: 10431657]
6. Strittmatter WJ, Saunders AM, Schmechel D, Pericak-Vance M, Enghild J, Salvesen GS, Roses AD. Apolipoprotein E: high-avidity binding to β -amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. *Proc. Natl Acad. Sci. USA* 1993;90:1977–1981. [PubMed: 8446617]
7. Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, et al. Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science* 1993;261:921–923. [PubMed: 8346443]
8. Saunders AM, Strittmatter WJ, Schmechel D, St George-Hyslop PH, Pericak-Vance MA, Joo SH, et al. Association of apolipoprotein E allele ϵ 4 with late-onset familial and sporadic Alzheimer's disease. *Neurology* 1993;43:1467–1472. [PubMed: 8350998]
9. Innerarity TL, Pitas RE, Mahley RW. Binding of arginine-rich (E) apoprotein after recombination with phospholipid vesicles to the low density lipoprotein receptors of fibroblasts. *J. Biol. Chem* 1979;254:4186–4190. [PubMed: 220235]
10. Peters-Libeu CA, Newhouse Y, Hatters DM, Weisgraber KH. Model of biologically active apolipoprotein E bound to dipalmitoylphosphatidylcholine. *J. Biol. Chem* 2006;281:1073–1079. [PubMed: 16278220]
11. Hatters DM, Budamagunta MS, Voss JC, Weisgraber KH. Modulation of apolipoprotein E structure by domain interaction. Differences in lipid-bound and lipid-free forms. *J. Biol. Chem* 2005;280:34288–34295. [PubMed: 16076841]
12. Narayanaswami V, Szeto SSW, Ryan RO. Lipid association-induced N- and C-terminal domain reorganization in human apolipoprotein E3. *J. Biol. Chem* 2001;276:37853–37860. [PubMed: 11483594]
13. Gianturco SH, Gotto AM Jr, Hwang S-LC, Karlin JB, Lin AHY, Prasad SC, Bradley WA. Apolipoprotein E mediates uptake of S_f 100–400 hypertriglyceridemic very low density lipoproteins by the low density lipoprotein receptor pathway in normal human fibroblasts. *J. Biol. Chem* 1983;258:4526–4533. [PubMed: 6300089]
14. Weisgraber KH, Mahley RW, Kowal RC, Herz J, Goldstein JL, Brown MS. Apolipoprotein C-I modulates the interaction of apolipoprotein E with β -migrating very low density lipoproteins (β -VLDL) and inhibits binding of β -VLDL to low density lipoprotein receptor-related protein. *J. Biol. Chem* 1990;265:22453–22459. [PubMed: 2266137]
15. Narayanaswami V, Ryan RO. Molecular basis of exchangeable apolipoprotein function. *Biochim. Biophys. Acta* 2000;1483:15–36. [PubMed: 10601693]
16. Peters-Libeu CA, Newhouse Y, Hall SC, Witkowska HE, Weisgraber KH. Apolipoprotein E-dipalmitoylphosphatidylcholine particles are ellipsoidal in solution. *J. Lipid Res* 2007;48:1035–1044. [PubMed: 17308333]
17. Hatters DM, Zhong N, Rutenber E, Weisgraber KH. Amino-terminal domain stability mediates apolipoprotein E aggregation into neurotoxic fibrils. *J. Mol. Biol* 2006;361:932–944. [PubMed: 16890957]
18. Aggerbeck LP, Wetterau JR, Weisgraber KH, Wu C-SC, Lindgren FT. Human apolipoprotein E3 in aqueous solution: II. Properties of the amino- and carboxyl-terminal domains. *J. Biol. Chem* 1988;263:6249–6258. [PubMed: 3360782]
19. Wetterau JR, Aggerbeck LP, Rall SC Jr, Weisgraber KH. Human apolipoprotein E3 in aqueous solution: I. Evidence for two structural domains. *J. Biol. Chem* 1988;263:6240–6248. [PubMed: 3360781]
20. Wilson C, Wardell MR, Weisgraber KH, Mahley RW, Agard DA. Three-dimensional structure of the LDL receptor-binding domain of human apolipoprotein E. *Science* 1991;252:1817–1822. [PubMed: 2063194]
21. Hatters DM, Peters-Libeu CA, Weisgraber KH. Apolipoprotein E structure: insights into function. *Trends Biochem. Sci* 2006;31:445–454. [PubMed: 16820298]
22. Matz CE, Jonas A. Micellar complexes of human apolipoprotein A-I with phosphatidylcholines and cholesterol prepared from cholate-lipid dispersions. *J. Biol. Chem* 1982;257:4535–4540. [PubMed: 6802835]

23. Newhouse Y, Peters-Libeu C, Weisgraber KH. Crystallization and preliminary x-ray diffraction analysis of apolipoprotein E-containing lipoprotein particles. *Acta Crystallogr., Sect. F* 2005;61:981–984.
24. Langen R, Oh KJ, Cascio D, Hubbell WL. Crystal structures of spin labeled T4 lysozyme mutants: implications for the interpretation of EPR spectra in terms of structure. *Biochemistry* 2000;39:8396–8405. [PubMed: 10913245]
25. Guo Z, Cascio D, Hideg K, Kálái T, Hubbell WL. Structural determinant of nitroxide motion in spin-labeled proteins: tertiary contact and solvent-inaccessible sites in helix G of T4 lysozyme. *Protein Sci* 2007;16:1069–1086. [PubMed: 17473014]
26. Fajer MI, Li H, Yang W, Fajer PG. Mapping electron paramagnetic resonance spin label conformations by the simulated scaling method. *J. Am. Chem. Soc* 2007;129:13840–13846. [PubMed: 17948993]
27. Mchaurab HS, Oh KJ, Fang CJ, Hubbell WL. Conformation of T4 lysozyme in solution. Hinge-bending motion and the substrate-induced conformational transition studied by site-directed spin labeling. *Biochemistry* 1997;36:307–316. [PubMed: 9003182]
28. Segrest JP, Jones MK, Klon AE, Sheldahl CJ, Hellinger M, Loof H-D, Harvey SC. A detailed molecular belt model for apolipoprotein A-I in discoidal high density lipoprotein. *J. Biol. Chem* 1999;274:31755–31758. [PubMed: 10542194]
29. Qin Z, Cafiso DS. Membrane structure of protein kinase C and calmodulin binding domain of myristoylated alanine rich C kinase substrate determined by site-directed spin labeling. *Biochemistry* 1996;35:2917–2925. [PubMed: 8608129]
30. Nielsen RD, Che K, Gelb MH, Robinson BH. A ruler for determining the position of proteins in membranes. *J. Am. Chem. Soc* 2005;127:6430–6442. [PubMed: 15853351]
31. Oda MN, Forte TM, Ryan RO, Voss JC. The C-terminal domain of apolipoprotein A-I contains a lipid-sensitive conformational trigger. *Nat. Struct. Biol* 2003;10:455–460. [PubMed: 12754494]
32. Martin DD, Budamagunta MS, Ryan RO, Voss JC, Oda MN. Apolipoprotein A-I assumes a “looped belt” conformation on reconstituted high density lipoprotein. *J. Biol. Chem* 2006;281:20418–20426. [PubMed: 16698792]
33. Lagerstedt JO, Budamagunta MS, Oda MN, Voss JC. Electron paramagnetic resonance spectroscopy of site-directed spin labels reveals the structural heterogeneity in the N-terminal domain of apoA-I in solution. *J. Biol. Chem* 2007;282:9143–9149. [PubMed: 17204472]
34. Glomset JA. The plasma lecithin:cholesterol acyltransferase reaction. *J. Lipid Res* 1968;9:155–167. [PubMed: 4868699]
35. Gordon V, Innerarity TL, Mahley RW. Formation of cholesterol- and apoprotein E-enriched high density lipoproteins *in vitro*. *J. Biol. Chem* 1983;258:6202–6212. [PubMed: 6343371]
36. Koo C, Innerarity TL, Mahley RW. Obligatory role of cholesterol and apolipoprotein E in the formation of large cholesterol-enriched and receptor-active high density lipoproteins. *J. Biol. Chem* 1985;260:11934–11943. [PubMed: 2995353]
37. Dong L-M, Wilson C, Wardell MR, Simmons T, Mahley RW, Weisgraber KH, Agard DA. Human apolipoprotein E. Role of arginine 61 in mediating the lipoprotein preferences of the E3 and E4 isoforms. *J. Biol. Chem* 1994;269:22358–22365. [PubMed: 8071364]
38. Dong L-M, Weisgraber KH. Human apolipoprotein E4 domain interaction. Arginine 61 and glutamic acid 255 interact to direct the preference for very low density lipoproteins. *J. Biol. Chem* 1996;271:19053–19057. [PubMed: 8702576]
39. Zhong N, Scearce-Levie K, Ramaswamy G, Weisgraber KH. Apolipoprotein E4 domain interaction: synaptic and cognitive deficits in mice. *Alzheimer's & Dementia* 2008;4:179–192.
40. Ramaswamy G, Xu Q, Huang Y, Weisgraber KH. Effect of domain interaction on apolipoprotein E levels in mouse brain. *J. Neurosci* 2005;25:10658–10663. [PubMed: 16291938]
41. Raffai RL, Dong L-M, Farese RV Jr, Weisgraber KH. Introduction of human apolipoprotein E4 “domain interaction” into mouse apolipoprotein E. *Proc. Natl Acad. Sci. USA* 2001;98:11587–11591. [PubMed: 11553788]
42. Lu B, Morrow JA, Weisgraber KH. Conformational reorganization of the four-helix bundle of human apolipoprotein E in binding to phospholipid. *J. Biol. Chem* 2000;275:20775–20781. [PubMed: 10801877]

43. Fisher CA, Narayanaswami V, Ryan RO. The lipid-associated conformation of the low density lipoprotein receptor binding domain of human apolipoprotein E. *J. Biol. Chem* 2000;275:33601–33606. [PubMed: 10906325]
44. Morrow JA, Arnold KS, Weisgraber KH. Functional characterization of apolipoprotein E isoforms overexpressed in *Escherichia coli*. *Protein Expression Purif* 1999;16:224–230.

Fig. 1. Preparation of homogeneous apoE4•DPPC particles. (a) Step 1: Separation of apoE4•DPPC complexes that are of different densities by potassium bromide density gradient fractionation. Fractions corresponding to $d \approx 1.10$ - 1.15 g/mL were collected for each mutant analyzed in this study. (b) Step 2: Further separation for particle size by Superdex 75 gel-filtration chromatography. Fractions eluting at 10-12 mL were collected for each mutant analyzed in this study.

Fig. 2.
EPR scans (100 G) of spin-labeled side chains in apoE4•DPPC. The sequence positions of the targeted locations are indicated. All spectra were normalized to the same number of spins.

Fig. 3.

EPR spectra of apoE4•DPPC containing two site-directed spin labels. (a) Each spectrum of doubly labeled protein (black trace) is compared with the composite spectrum (red trace) generated by adding the spectra of the two sites from samples labeled at only one position to identify broadening caused by dipolar coupling. (b) Doubly spin-labeled proteins (black trace) were compared with the same spin-labeled protein diluted 1:1 with MalNEt-labeled protein (red trace) to determine whether any of the observed broadening depends on intermolecular interactions. All spectra were normalized to the same number of spins.

kveqavetepepelrqqteqwsgqrwelalgrfwdylrwvqtlseqvqeellssqvtqelralmdetmkelkaykselee
 10 11 1 2 3 4 5 6 7 8 9 10 11 5 6 7 8 9 10 11 12 3 4 5 6 7 8 9 10 11 1 2 3 4 5 6 7 8 9 10 11 12 3

qltpvaeetrarlskelqaaqarlgadmedvcgrlvqyrgevqamlgqsteelrvrlashlrklrkrllrdaddlqkrla
 8 9 10 11 1 2 3 4 5 6 7 8 9 10 11 12 3 4 5 6 7 8 9 10 11 1 2 3 4 5 6 7 8 9 10 11 12 3 4 5 6 5 6 7 8 9 10 11 12 5 6 7 8 9 10 11 12

vyqagaregaerglsairerlgplveqgrvraatvgslagqplqeraqawgerlrarmee mgstrdrlddevkeqvaevr
 1 2 3 4 5 6 7 8 9 10 8 9 10 11 12 3 3 4 5 6 7 8 9 1 2 3 4 5 6 7 8 9 10 11 1 2 3 4 5 6 7 1 2 3 4 5 6 7 8 9 10 11 12 3

akleeqaqqirlqaeafqarlkswfepivedmqrqwaglvekvaavgtsaapvpsdnh
 1 2 3 4 5 6 7 8 9 10 11 1 2 3 4 5 6 7 8 9 10 11 5 6 7 8 9 10 11 1 2 3 4 5 6 7 8 9 10 11 12 3

Fig. 4.

Identification of sequence within apoE4 that displays the 11/3 amphipathic helix motif.

Regions that contain a sequence consistent with the 11/3 helix motif (where positions 1, 5, and 8 within the 11-mer repeat are hydrophobic) are shown in black text. Sequences with little agreement with the 11/3 motif (magenta) are assigned as a random coil in the model. For predicted helical segments, the assigned position within the 11-mer repeat is given just beneath the respective amino acid. The bending of helical segments is achieved by setting the dihedral angles as follows: In the N-terminal half (blue, hydrophobic residues oriented inward), positions 2, 4, 9, and 11 have $\phi = -58.7^\circ$ and $\psi = -48.8^\circ$; positions 3, 6, 7, and 10 have $\phi = -55.8^\circ$ and $\psi = -49.9^\circ$; and positions 1, 5, and 8 have $\phi = -61.6^\circ$ and $\psi = -51.7^\circ$. In the C-terminal half (green, hydrophobic residues oriented outward), positions 2, 4, 9, and 11 have $\phi = -58.7^\circ$ and $\psi = -48.8^\circ$; positions 3, 6, 7, and 10 have $\phi = -55.8^\circ$ and $\psi = -49.9^\circ$; and positions 1, 5, and 8 have $\phi = -61.6^\circ$ and $\psi = -51.7^\circ$.

Fig. 5. Molecular model of apoE4•DPPC based on constraints derived from EPR data and the molecular envelope measured by X-ray diffraction. Placement of helical segments in three-dimensional space was carried out with the ProteinShop software, which adjusts dihedral angles within the coil segments to accommodate the positioning of the helical segments. (a) The hydrophobic residues are shown in red, whereas the polar residues are shown in blue. (b) Rendering of the secondary structure and the location of confirmed proteolytic cleavage sites in apoE4•DPPC.

Fig. 6.
Location of spin-labeled side chains in a space-filled model of apoE4•DPPC. Four views of the model are presented.

Fig. 7.
Model of the conformational differences between lipid-free apoE4 and lipid-bound apoE4.

Table 1

Structural constraints of apoE complexed with DPPC based on spin-label dynamics

Motional freedom/environment	Residue(s)
Very high/Random coil	18
High/Helix, open surface	76, 77, 120, 241, 263, 278, 289
Moderate/Helix, partially restricted	120, 264
Low/Helix, buried	94, 239, 247