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Allele-dependent thermodynamic and structural perturbations in ApoE variants associated with the correction of dyslipidemia and formation of spherical ApoE-containing HDL particles

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Abstract

Overexpression of ApoE4[Leu261Ala/Trp264Ala/Phe265Ala] mutant (ApoE4mutC) prevents hypertriglyceridemia and promotes formation of spherical ApoE-containing HDL in apoE^{-/-} or apoA-I^{-/-} mice. Although, a similar phenotype was observed with ApoE2[Leu261Ala/Trp264Ala/Phe265Ala] (ApoE2mutC), small differences in cholesterol distribution to IDL/LDL, HDL2 and HDL3 fractions and ApoE distribution to HDL2 and HDL3 fractions suggested that ApoE allelic background can influence mutant ApoE properties. To understand the structural basis behind the properties of ApoE2mutC and ApoE4mutC variants we analyzed their structural and thermodynamic integrity in comparison to their wild-type counterparts. Circular dichroism spectroscopy revealed a significantly reduced helical content for both mutants compared to wild-type. The presence of mutation only marginally affected the thermal stability of ApoE4 but greatly affected the thermal stability profile of ApoE2 leading to a previously uncharacterized intermediate stage. Both ApoE4mutC and ApoE2mutC were slightly stabilized against chemical denaturation compared to their wild-type counterparts. ApoE2mutC, in contrast to ApoE4mutC, exposed a larger hydrophobic surface to the solvent as determined by a fluorescent probe. Both mutants remodeled 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine vesicles with identical kinetics to the wild-type proteins. Given the known conformational differences between ApoE2 and ApoE4, our findings suggest that the 261–265 region may be involved in inter-domain interactions within the ApoE molecule. Overall, we show that substitution of Leu261, Trp264 and Phe265 with Ala in ApoE2 leads to more pronounced perturbations of thermodynamic stability and structure than in ApoE4. The minimal perturbations in ApoE4mutC may make it a more suitable candidate for therapeutic applications for the correction of remnant removal disorders and atheroprotection.

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Introduction

Human apolipoprotein E (ApoE) plays major role in lipid homeostasis in the circulation and brain ^{1, 2}. ApoE is a glycoprotein of 299 amino acid residues and has three common isoforms (ApoE2, ApoE3, ApoE4) in the general population, each differing in the amino acid positions 112 and 158 ³. ApoE3 (Cys112, Arg158) is the most common form ⁴. ApoE2 (Cys112, Cys158) is associated with a recessive form of type III hyperlipoproteinemia ⁵ and ApoE4 (Arg112, Arg158) is a significant risk factor for coronary heart disease and late-onset Alzheimer's disease ^{1, 6, 7}.

In plasma, ApoE is associated with chylomicron remnants, very low density lipoproteins (VLDL) and some high density lipoprotein (HDL) subpopulations ^{2, 8}. Lipoprotein-bound ApoE is the ligand for the LDL receptor as well as other receptors *in vitro* ⁹. *In vitro* and *in vivo* studies have shown that mutations in ApoE that diminish binding of ApoE-containing lipoproteins to the LDL receptor are associated with high plasma cholesterol and triglyceride levels and cause premature atherosclerosis in humans and experimental animals ^{10, 11}. Lipid free ApoE promotes ABCA1 mediated cholesterol efflux ¹² and participates in the biogenesis of ApoE containing HDL ¹³. In addition, reconstituted ApoE containing HDL promotes SR-BI mediated cholesterol efflux ¹⁴. These properties of ApoE may contribute to cell and tissue cholesterol homeostasis and protection from atherosclerosis ¹⁵.

Despite the beneficial functions of ApoE, previous studies showed that overexpression of any of the three ApoE isoforms in ApoE-deficient mice increases the rate of VLDL triglyceride secretion and is associated with hypertriglyceridemia ². In addition, plasma ApoE levels in humans and mice have been positively correlated with plasma triglyceride levels ^{8, 16}. The induction of hypertriglyceridemia in mice overexpressing full length ApoE can be prevented by expression of ApoE (at low or high concentrations) that lacks the carboxy-terminal region of residues 260–299 ¹⁷. Site-directed mutagenesis and gene transfer in mice localized the amino acids responsible for hypertriglyceridemia in the region of residues 261–269 of ApoE ¹⁸. This region was later narrowed to residues 261–265 ¹⁹. Specifically, it was shown by adenovirus-mediated gene transfer in apoE^{-/-} or apoA-I^{-/-} mice that substitution of residues Leu261, Trp264 and Phe265 by Ala in ApoE2 or ApoE4 did not induce hypertriglyceridemia ¹⁹. In addition, it was shown that both ApoE4[L261A/W264A/F265A] (ApoE4mutC) and ApoE2[L261A/W264A/F265A] (ApoE2mutC) mutants promote the formation of spherical ApoE-containing HDL in apoA-I^{-/-} mice ¹⁹. However, small differences in cholesterol distribution to IDL/LDL, HDL2 and HDL3 fractions and ApoE distribution to HDL2 and HDL3 fractions between ApoE4mutC and ApoE2mutC were observed, suggesting that the ApoE allelic background may influence the novel ApoE properties introduced by the mutation.

Previous studies have suggested that the unusual structural and biophysical properties of ApoE directly underlie the biological function of the protein and have provided insight into the mechanisms by which ApoE is involved in cardiovascular and neurological diseases ^{20–22}. To investigate the structural basis behind the properties of these ApoE mutants and to test whether the introduced mutation interferes with the structural and thermodynamic integrity of ApoE, we employed circular dichroism spectroscopy to compare the secondary structure and thermal denaturation profiles of the mutants in comparison to wild-type (WT) ApoE2 and ApoE4. We furthermore analyzed the chemical denaturation profile of the mutants, as well as the solvent accessible hydrophobic surfaces probed by 1-anilinonaphthalene-8-sulfonic acid (ANS) binding. Finally, we measured the kinetics of clearance of multilamellar 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) vesicles by each ApoE form. Overall, our data demonstrate that the mutation interferes with the thermodynamic and structural stability of the molecule in an allele-dependent manner. The

established structural and thermodynamic differences between ApoE2 and ApoE4 in combination with the allele-dependent effects of the L261A/W264A/F265A mutation, implicate the 261–265 region of ApoE in inter-domain interactions. Finally, the significantly smaller perturbations in the ApoE4mutC suggest it may constitute a preferred candidate for therapeutic applications for the treatment of dyslipidemia and atherosclerosis.

Materials and Methods

Protein expression

The construction of recombinant adenoviruses used for the expression of the ApoE mutants has been described ¹⁹. For protein expression, human astrocytoma HTB13 cells (ATCC number HTB-13, designation SW 1783) grown in roller bottles were infected with the appropriate recombinant adenovirus at a multiplicity of infection of 20 pfu/cell. The medium (Leibovitz L-15 containing 1% v/v penicillin and streptomycin and 2% v/v heat-inactivated horse serum) was discarded 24 hours post-infection and the cells in the roller bottles were rinsed with Dulbecco's phosphate-buffered saline (DPBS). The DPBS was discarded and Leibovitz L-15 medium containing 1% v/v penicillin and streptomycin was added. The medium was harvested 24 hours later and the harvest was repeated at 24-hour intervals for 4–6 days. The medium containing the recombinant protein was lyophilized to reduce total volume to ~200ml and then extensively dialyzed versus 25mM ammonium bicarbonate. The dialyzed medium was lyophilized and the dried recombinant protein was stored at –80°C.

Recombinant protein purification

The lyophilized medium containing ApoE was dissolved in 20mM Tris, 0.2M NaCl, pH 7.4 and ApoE was purified using a dextran-sulfate Sepharose column connected to an AKTA Fast-Protein Liquid Chromatography system (GE Healthcare). The column was equilibrated with 20mM Tris, 0.2 M NaCl, pH 7.4 and the protein was loaded onto the column through a 10mL loop with a flow of 0.2mL/min to allow binding of ApoE. Elution was performed with a 90mL, 0.2–1.0M NaCl gradient in 20mM Tris, pH7.4, at a flow rate of 1mL/min. Fractions of 1.5mL were collected and analyzed by SDS-PAGE to evaluate purity. The pure protein fractions were pooled, dialyzed extensively against 5mM ammonium bicarbonate and lyophilized.

Western blot analysis

Recombinant ApoE was analyzed by western blotting after 10% SDS-PAGE. The nitrocellulose membrane was probed with the mouse anti-human ApoE monoclonal antibody 6C5 (Ottawa Heart Institute Research Corporation, Ottawa, Canada) (1:1000). Proteins were visualized by chemiluminescence following incubation with goat anti-mouse IgG (Novagen, EMD Chemicals, San Diego, CA, USA) (1:5000) coupled to horseradish peroxidase.

Analysis of ApoE glycosylation

To deglycosylate the recombinant ApoE, 2.7µg WT ApoE2, WT ApoE4 or their mutant forms were incubated in 50 mM sodium citrate pH 6.0 and a total volume of 55µl at 37°C for 4h in the presence or absence of 10 units of *Clostridium Perfringens* Neuraminidase (New England Biolabs, P0720S). After the incubation, proteins were immediately analyzed on SDS-PAGE. For thermal denaturation analysis ApoE2mutC at a concentration of 0.1 mg/ml was treated with 370U/ml of *Clostridium Perfringens* Neuraminidase for 3.5hrs at 37°C in DPBS.

Preparation of ApoE samples before biophysical experiments

Before all analyses, lyophilized WT or mutant ApoE was reconstituted at a final concentration of 0.2mg/mL in 6M guanidine hydrochloride in DPBS containing 1mM DTT and incubated for 1h at room temperature. Samples were dialyzed extensively against DPBS at 4°C. After dialysis, the samples were centrifuged at 12000g for 20min to remove any precipitated protein. ApoE at the supernatant was quantitated by measuring absorbance at 280nm (ϵ_{280} E2wt and E4wt=1.3 mL mg⁻¹ cm⁻¹, ϵ_{280} E2mutC and E4mutC=1.1 mL mg⁻¹ cm⁻¹). The proteins were kept at low concentrations (~0.1mg/mL) on ice to avoid aggregation. All analyses were performed on freshly refolded protein.

Circular Dichroism Spectroscopy

We used a Jasco-715 spectropolarimeter to record the far-UV CD spectra of the ApoE samples from 200 to 260nm at 25°C. The instrument was connected to a Jasco PTC-348 WI Peltier temperature controller. The protein samples were at 0.1mg/mL concentration in DPBS (pH 7.4) containing 1mM DTT. We used a quartz cuvette with an optical path length of 1nm. Measurements were performed using the following parameters: bandwidth 1nm, response 8sec, step size 0.2nm and scan speed 50nm/min. Each spectrum was the average of 5 accumulations. Helical content was calculated using the molecular ellipticity at 208 or 222nm as described^{23, 24}.

For thermal denaturation measurements, we monitored the change in molar ellipticity at 222nm, while varying the temperature from 20°C to 80°C, at a rate of 1°C/min. Except when stated in the results, the thermal denaturation curve was fitted to a Boltzman sigmoidal model using the Graphpad Prism™ software. The cooperativity index, *n*, was calculated as previously described²⁵.

Chemical Denaturation Experiments

0.04 mg/mL of freshly refolded ApoE were added in a 4 mL quartz fluorimeter cuvette and the intrinsic tryptophan protein fluorescence was measured at 340nm after excitation at 295 nm. Small aliquots from an 8.0 M guanidine hydrochloride (Applchem) solution were gradually added and mixed by pipetting. After each addition of guanidine hydrochloride, the sample was incubated in the dark for 2 min before measuring the fluorescence signal. The experimental data were fitted to a three-state denaturation model as described²⁴.

ANS Fluorescence

1,8 ANS (1-anilinonaphthalene-8-sulfonic acid, Sigma-Aldrich) was dissolved in dimethylsulfoxide (DMSO) to a final concentration of 50 mM (ANS stock solution). Freshly refolded ApoE at 0.04mg/ml in DPBS pH 7.4, 0.1mM DTT was inserted into a 4 mL quartz fluorimeter cuvette (Hellma, Germany). Fluorescence measurements were performed in a Quantamaster 4 fluorescence spectrometer (Photon Technology International, New Jersey). The scan rate was 1 nm/s with excitation at 395 nm and emission range from 420 to 550 nm. After measuring the background protein fluorescence, 7.5 μ L of ANS stock solution were added in the cuvette and mixed so that the final ANS concentration was 250 μ M. A control ANS spectrum in the absence of protein was also recorded.

DMPC remodeling kinetics

Dimyristoyl-L- α -phosphatidylcholine (DMPC) remodeling assay was performed as described^{26, 27} with the following modifications: DMPC concentration was 97 μ g/ml and ApoE concentration was 43 μ g/ml. The reaction was performed in DPBS with 3.5% KBr (w/v), 0.1mM DTT, 0.1M guanidine hydrochloride, 0.1mM EDTA. Reaction kinetics were followed by the change in absorbance at 325nm using a Perkin-Elmer Lambda 35 UV/VIS

spectrophotometer. The cuvette was thermostated at $24 \pm 0.1^\circ\text{C}$ using the Perkin-Elmer PCB 1500 Peltier temperature controller. The contents were mixed by repeated pipetting for 3 sec every 2min. Experimental data were fitted to a two-phase exponential decay model using Graphpad Prism™.

Results

Protein expression and purification

Recombinant ApoE secreted into the HTB-13 cell medium after infection with the appropriate adenovirus was purified to homogeneity using a dextran-sulfate Sepharose column. Pooled fractions were analyzed on SDS-PAGE and by western blotting (Figure 1A). Although all proteins migrated at the expected molecular weight (~35kDa) subtle differences in their relative migration prompted us to investigate whether this was due to an inherent property of the variants. This was particularly evident for the ApoE2mutC that migrated to a slightly higher MW compared to ApoE2WT. Analysis by SDS-PAGE containing SDS below the CMC concentration did not eradicate the differences suggesting that they are not due to different SDS micellar bound forms (Supplemental Figure 1). Treatment of the proteins with *Clostridium Perfringens* Neuraminidase - an enzyme that removes terminal sialic residues from glycoproteins²⁸ - largely normalized the migration patterns for all variants, suggesting that changes in glycosylation were primarily responsible for the observed differences in electrophoretic mobility (Figure 1B). ApoE is found partially glycosylated in human plasma (~20%) at Thr194 and we have previously shown that recombinant ApoE produced by HTB-13 cells mimics the glycosylation pattern of plasma ApoE^{24, 28}.

Effects of the mutations on the secondary structure of ApoE

To investigate whether the mutations in the 261–265 region affected the secondary structure of ApoE we measured the circular dichroism spectra of the WT and mutant ApoE forms (Figure 2A,B). The mutations appeared to severely affect the helical content of ApoE, irrespective of the allelic background. On average, ApoE lost about 18% of its helical content as calculated by observed molecular ellipticity at 222nm (Table 1). The change was virtually identical for both E2 and E4 alleles. Such a drastic change cannot be explained solely on localized structural disruption in the region of each mutation, suggesting that the structural destabilization caused by the mutations is transmitted to other regions of the molecule.

Effects of the mutations on the thermal stability of ApoE

The significant loss of helical content due to the mutations prompted us to investigate their effects on the thermodynamic stability of the whole ApoE molecule. We measured the thermal denaturation profile of WT ApoE and mutant forms, by following the circular dichroism signal as a function of increasing temperature (Figure 2C,D). WT ApoE2 and WT ApoE4 underwent single transitions with a mid-point at 52.8°C and 43.6°C respectively (Table 1). Both molecules were thermally denatured with limited cooperativity, a hallmark of ApoE²¹. The ApoE2mutC however, underwent an apparent two-stage transition with an intermediate state within the $40\text{--}60^\circ\text{C}$ range (Figure 2C). The second step of this two-stage thermal transition had significantly elevated mid-point at 65.5°C and a higher cooperativity index indicating a more cooperative transition compared to the WT protein (Table 1). This unusual thermal denaturation profile for ApoE2mutC was not due to its hyper-glycosylated state since deglycosylation of the protein did not alter the denaturation profile (Supplemental Figure 2). In contrast, introduction of the same mutation in the ApoE4 background (ApoE4mutC) had only minor effects on the thermal denaturation properties of ApoE4 (Figure 2D and Table 1). This sharp contrast between the effects of introduction of the same

mutation in E2 or E4 allelic background may be related to the previously established structural differences between the two ApoE alleles ²⁹.

Effects of the mutations on the chemical stability of ApoE

We used the chemical chaotrope guanidine hydrochloride to follow the chemical denaturation profile of WT ApoE and mutant forms. WT ApoE is characterized by a two-stage transition with an intermediate state appearing between 1–2 M guanidine hydrochloride ^{21, 24}. Both WT ApoE forms underwent such a transition (Figure 3A,B). Introduction of the L261A/W264A/F265A mutations in both ApoE2 and ApoE4 led to a shift of the transitions towards higher guanidine hydrochloride concentrations (Figure 3A,B). Overall, it appears that the L261A/W264A/F265A mutations lead to a relatively small stabilization of ApoE versus chemical denaturation.

Probing the hydrophobic surface exposure by ANS binding

We used the changes in fluorescence of the probe ANS as an indicator of hydrophobic surface exposure of ApoE. ANS shows minimal fluorescence in water and undergoes a significant fluorescence enhancement and blue-shift when it interacts with ApoE due to ANS binding onto hydrophobic patches exposed on the protein ^{21, 24, 30}. Interaction of ANS with ApoE2mutC resulted in a larger increase in fluorescence compared to WT ApoE2 (Figure 3C and Table 1). In contrast, interaction of ANS with ApoE4mutC gave identical enhancement compared to WT ApoE4 (Figure 3D and Table 1). These results suggest structural alterations leading to additional hydrophobic surface exposure only when the L261A/W264A/F265A mutations are introduced in an E2 background.

DMPC remodeling kinetics

The kinetics of remodeling of DMPC by ApoE have been used previously as a functional indicator or the capability of the protein to interact with lipids ^{24, 27, 31}. To evaluate any effects of the mutations on the association of ApoE with phospholipids, we measured the kinetics of remodeling of DMPC vesicles in the presence of either WT or mutant ApoE2 and ApoE4 forms (Supplemental Figure 3A,B). Introduction of the L261A/W264A/F265A mutations did not appear to alter the kinetics of DMPC remodeling for either ApoE2 or ApoE4, suggesting that any structural alterations brought about by the mutation do not induce any rate limiting functional changes in the interaction of ApoE with phospholipids.

Discussion

ApoE is important for cholesterol and triglyceride homeostasis and the formation of apoE containing HDL ⁴. Using adenovirus-mediated gene transfer in appropriate mouse models, it was shown that when expressed at physiological levels apoE corrects the hypercholesterolemia in apoE^{-/-} mice ². However when overexpressed, ApoE not only fails to correct the hypercholesterolemia, but also causes hypertriglyceridemia ^{16, 17}. The residues responsible for the induction of hypertriglyceridemia have been localized within the 261 to 269 region ^{16, 17}. Two ApoE mutants (ApoE2mutC and ApoE4mutC), both carrying the L261A/W264A/F265A mutations within this region were found to prevent apoE-induced hypertriglyceridemia ¹⁹. These mutants can achieve the enhanced biological properties of a previously characterized mutant with 5 amino acid substitutions (L261A, W264A, F265A, L268A, V269A) indicating that the alterations at positions 268 and 269 are not an absolute requirement for achieving the desired phenotype in mice ^{18, 32}. ApoE has been shown to participate in the biogenesis of apoE-containing HDL particles by a pathway that requires the functions of ABCA1 and LCAT and is similar to the pathway of biogenesis of the classical HDL particle that contains apoA-I ¹³. Spherical HDL particles generated by this pathway may account at least for some of the atheroprotective functions of

apoE. However, overexpression of WT apoE4 promoted the formation of discoidal HDL particles, whereas overexpression of apoE4mutC or apoE2mutC, promoted the formation of spherical apoE-containing HDL particles^{18, 19}. As a result, it has been proposed that the apoE4mutC and apoE2mutC represent apoE variants with improved biological functions that may be therapeutically relevant^{18, 19}.

In this study, we investigated whether the improved biological functions bestowed to ApoE by the L261A/W264A/F265A mutation affect the structural and thermodynamic integrity of the molecule. The drastic loss of helical content upon introduction of the mutations in ApoE2 and ApoE4 in combination with the alterations in the chemical denaturation profile suggest that the mutations do indeed perturb the structure and thermodynamics of ApoE in a manner well beyond the localized area of the mutations. Interestingly, similar changes in helical content and chemical denaturation profile have been previously described for the deletion mutant ApoE4[Δ260–299], a mutant that lacks the 261–265 region, suggesting that this region may be at least partially responsible for these perturbations²¹. Additionally, the increased thermal stability and cooperativity found for ApoE2mutC is similar to the ApoE4[260–299] phenotype suggesting that the 261–265 region may be primarily responsible for such effects.

ApoE4 has been described to have both structural and functional differences to ApoE2, primarily stemming from different interactions between the N-terminal and C-terminal domain of the molecule, interactions considered very important for the physiological function of ApoE²². The finding that some effects of the L261A/W264A/F265A mutation depend on the allelic background (E2 versus E4) suggest that the 261–265 region may influence the thermodynamics of this domain interaction possibly by direct participation in atomic interactions between the two domains. Recently, position 264 has been mutated in conjunction with four other amino acids to generate a stabilized, monomeric form of ApoE used for the determination of the molecule's three-dimensional structure by NMR³³. Mapping of the 261–265 region in this structure reveals that the mutated amino acids are located towards the C-terminal end of an extended alpha-helix belonging to the C-terminal moiety of the molecule (Figure 4). This helix packs closely onto the N-terminal 4-helix bundle of ApoE and stabilizes the N-terminal/C-terminal interaction. Leu261 and Phe265 are exposed towards the solvent, possibly available to interact with lipids, and Arg264 (that replaces Trp264 in this structure) lies in the intra-helical junction³³. Interestingly, the space between the two helices is not sufficient to accommodate the naturally occurring tryptophan residue at that location, indicating that the NMR structure does not necessarily correspond to the WT ApoE structure in solution. As a result, the region 261–265 is unlikely to be as tightly packed against the N-terminal four-helix bundle in the WT protein in solution and the Trp264Ala mutation may facilitate this packing, leading to altered thermodynamics and function. Overall, the structural configuration of these amino acids in conjunction with the necessity to mutagenize position 264 to stabilize ApoE for structural analysis corroborate to the importance of those residues in mediating the overall domain organization and conformational plasticity of ApoE.

Bioengineered ApoE variants with improved biological functions have been proposed to hold promise for therapeutic applications in the correction of remnant removal disorders as well as for use of apoE containing HDL for stabilization of unstable plaques and atheroprotection^{18, 19}. To optimize the properties of the new apoE variants, it is important to characterize any structural and functional deviations from the WT protein and attempt to limit them while retaining the desired functions. Our analysis here of the ApoE[L261A/W264A/F265A] mutants suggests that the mutations induce significant structural and thermodynamic perturbations in the molecule of ApoE. However, these perturbations are far less pronounced in the E4 allelic background compared to E2. Furthermore, these

perturbations are also far less pronounced compared to ApoE[L261A,W264A,F265A,L268A,V269A] indicating that minimizing the number of mutations can help minimize structural and thermodynamic perturbations³². Since the thermal stability and hydrophobicity of ApoE are key components of its biological function, we propose that any efforts to evaluate the therapeutic potential of this variant are focused on the E4 allelic background.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We characterized the structure of an apoE mutant with improved biological properties.
- The mutation affects the secondary structure and stability of apoE.
- The structural changes greatly depend on the allelic background (apoE2 versus apoE4).
- Our findings implicate the 261–265 region of apoE in inter-domain interactions.
- The smaller perturbations in E4 background make it a better therapeutic candidate.

Figure 1.

Panel A: SDS-PAGE and western blot analysis of purified recombinant WT and mutant ApoE forms. *Panel B:* SDS-PAGE analysis of recombinant WT and mutant ApoE forms with or without treatment with neuraminidase (NA=neuraminidase).

Figure 2.

Circular dichroism analysis. *Panels A, B:* Circular dichroism spectra of the mutant ApoE2 (panel A) and ApoE4 (panel B) compared to the WT proteins. Spectra are the average of three experiments. *Panels C, D:* Characteristic thermal denaturation profiles of the mutant ApoE2 (panel C) and ApoE4 (panel D) compared to the WT proteins. Solid lines represent non-linear regression fits to a Boltzmann sigmoidal model, as explained in the Materials and Methods section, with the exception of the data for the ApoE2mutC that were fitted to a three-state denaturation model.

Figure 3.

Fluorescence analysis. *Panels A, B:* Chemical denaturation profiles of the mutant ApoE2 (panel A) and ApoE4 (panel B) compared to the WT proteins. Solid lines represent non-linear regression fits to a three-state unfolding model as described in the Materials and Methods section. *Panels C, D:* ANS fluorescence spectra in the presence or absence of ApoE2 (panel C) and ApoE4 (Panel D) WT or mutants. Spectra are the average of three measurements.

Figure 4.

Schematic representation of the location of the mutated residues in the recently determined NMR structure of ApoE3 (PDB code: 2L7B). Residues 261, 264 and 265 are located near the end of an extended helical segment of the C-terminal region of ApoE (on the right) that interacts with the N-terminal four-helix bundle (on the left). In this structure, the tryptophan residue at position 264 has been mutagenized to an arginine.

Table 1

Calculated biophysical parameters for WT and mutant ApoE forms. Averages are calculated from three to four separate experiments. The cooperativity index, n , is calculated as described ²¹. T_m and n parameters for ApoE2mutC are shown for both first and second transitions.

ApoE	% α -helix _{208nm}	% α -helix _{222nm}	T_m (°C)	n	ANS binding (fold increase)
ApoE2wt	47.9±1.25	52.4±0.8	52.8±1.2	6.1±0.7	3.2±0.23
ApoE2mutC	35.5±1.8 ^{*1}	43.0±0.4 ^{*2}	32±5, 65.5±0.4	7.9±0.5, 16.9±1.4	3.86±0.46 ^{*5}
ApoE4wt	45.8±1.3	50.7±0.3	43.6±0.7	5.3±0.3	3.16±0.11
ApoE4mutC	32.7±1.7 ^{*3}	41.5±1.1 ^{*4}	44.6±2.9	5.0±0.1	3.14±0.37

^{*1} $p=0.0015$,

^{*2} $p=0.0027$,

^{*3} $p=0.0086$,

^{*4} $p=0.0021$,

^{*5} $p=0.035$