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Nuclear Magnetic Resonance Spectroscopy Studies of the Lipid-bound, Receptor Active Conformation of the Apolipoprotein E Amino-terminus

by

Paul Stephen Hauser

A dissertation submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

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in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Dr. Robert Ryan, Advisor and Co-Chair
Dr. Jen-Chywan Wang, Co-Chair
Dr. Joseph Napoli
Dr. David Wemmer

Spring 2010
Abstract

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Apolipoprotein (apo) E is an exchangeable apolipoprotein that is critical for the trafficking of lipid and cholesterol nutrients in the brain and peripheral circulation. ApoE is a 299 amino acid (37 kDa) protein comprised of two independently folded functional domains, the carboxy-terminal lipid-binding domain and the receptor-binding amino-terminal (NT) domain that only displays receptor competent activity upon association with lipid. In the absence of lipid, the isolated NT domain (residues 1-183) of apoE adopts an amphipathic four α-helix bundle architecture that is characteristic of several other related apolipoproteins.

Various models have been advanced that describe the predicted conformational change of the protein upon lipid binding. Experiments have shown that the α-helical secondary structure is preserved if not enhanced upon lipid binding and yet it is known that the protein undergoes a dramatic conformational change in the transition to the lipid-bound state. Low-resolution experiments have provided insight into the mechanism and possible path of this transition, but a high-resolution determination of the lipid-bound conformation of apoE has not been accomplished. Using a combination of unique protein engineering methods and nuclear magnetic resonance (NMR) spectroscopy, this thesis advances the understanding of the lipid-induced conformational change of the apoE N-terminus.

Recombinant apoE NT (residues 1-183) is a representative model for apolipoprotein helix bundle conformational flexibility in the presence of lipid and on the surface of lipoprotein particles. The 22 kDa domain is predominantly α-helical, monomeric, and comparably stable relative to the native protein. This domain readily forms discoidal particles in the presence of phospholipids, which imparts low-density lipoprotein (LDL) receptor activity to the protein.

A protein engineering approach was used to further define the structural determinants of apoE NT that are necessary for lipid binding. A short helix connecting helix 1 and 2 in the four-helix bundle was replaced by a sequence predicted to adopt a β-turn. The resulting stable recombinant protein was not compromised in its ability to function as
a ligand for the LDL receptor, yet the protein displayed greatly enhanced binding affinity for lipid as assessed by phospholipid solubilization studies, a lipophilic fluorescent dye binding assay, and protection against phospholipase induced aggregation of human LDL fractions.

In order to define the detailed lipid-induced conformational change in apoE, a protein engineering approach termed segmental isotope labeling was deemed necessary to simplify the system for analysis by NMR. Using expressed protein ligation (EPL) methodology, a hybrid apolipoprotein was constructed from two independently generated fragments, apoE residues 1-111 and a 91 amino acid apolipophorin protein fragment. This protein ligation experiment tested the novel use of a pelB leader sequence for the generation of an N-terminal cysteine-containing protein fragment required for the joining of protein fragments by EPL.

Expressed protein ligation techniques were alternatively adapted to create an intact, semisynthetic apoE NT domain using apoE(1-111) and apoE(112-183) protein fragments. This semisynthetic protein displayed nearly identical structural and function properties as wild-type apoE NT by circular dichroism spectroscopy, guanidine denaturation studies, and functional lipid and LDL receptor binding studies. Stable isotope-labeled (\(^{15}\)N) apoE(112-183) was produced and ligated to unlabeled apoE 1-111 protein to create a segmental isotope-labeled protein. NMR experiments of the segmental protein further confirmed a structural and functional correspondence between wild-type, fully \(^{15}\)N-labeled and segmental isotope-labeled apoE NT while affirming that the segmental system dramatically simplified the NMR system for examining the protein region containing the LDL receptor recognition sequence.
Dedication

This thesis is dedicated to my life companion and wife, Alexandra Lockett, for all her consistent compassion, patience, and careful observation of my work throughout the entire duration of my thesis work. Thank you for your loving support and what a formative experience it was to walk beside you as this process unfurled.
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CHAPTER 1: Literature Review

Apolipoprotein E (apoE) is a potent modulator of plasma lipoprotein and cholesterol levels whose cellular mode of action is mediated by interaction with members of the low-density lipoprotein (LDL) receptor family. Transgenic mice over-expressing apoE exhibit decreased plasma cholesterol levels and are resistant to diet-induced atherosclerosis, while apoE knock-out mice manifest highly elevated plasma cholesterol levels and increased susceptibility to diet-induced atherosclerosis compared to their wild-type counterparts. In addition, the prototypical LDL receptor has been identified as both binding tightly to apoE and regulating blood cholesterol homeostasis via receptor-mediated endocytosis. The putative LDL receptor-binding domain of apoE (residues 134-150) contains a cluster of solvent accessible positively charged residues based on the lipid-free crystal structure of the N-terminal (NT) domain. However, it is recognized that lipid association is a requirement for productive apoE-LDL receptor interaction. This strongly suggests that apoE-LDL receptor interaction is conformation dependent, requiring apoE to adopt a structure that makes the receptor-binding domain available for interaction with the ligand binding sites in the receptor. Using information about apoE structural organization and lipid-binding properties, I propose to use a novel segmental isotopic labeling method to probe the lipid-free and lipid-associated structures of apoE using nuclear magnetic resonance (NMR) spectroscopy.

1.1 Physical Characteristics of ApoE

The importance of apoE was first suggested by studies describing its relatively high abundance in lipoprotein particles isolated from hyperlipoproteinemic patients as compared to normal individuals (Havel and Kane 1973). This has spurred continued and consistent studies into the structure and function of apoE as it has emerged as a central regulator of cholesterol homeostasis and thus central to the understanding of atherosclerosis.

ApoE is synthesized with an 18 amino acid N-terminal signal peptide that undergoes intracellular processing before secretion of a mature 37 kDa glycoprotein containing 299 amino acids (Rall, Weisgraber et al. 1982; McLean, Elshourbagy et al. 1984; Zannis, McPherson et al. 1984). The protein is encoded by a 3.6 kbp, four exon gene at a single locus on chromosome 19 (Olaisen, Teisberg et al. 1982; Paik, Chang et al. 1985). It is expressed in a wide variety of tissues including brain, spleen, lung, adrenal gland, ovary, kidney with the highest expression levels occurring in liver (Mahley 1988). The wide tissue distribution of this protein has led to the supposition that apoE participates in a myriad of activities related to and disparate from lipid metabolism. Indeed, support for this concept has been born out by studies linking apoE function to cholesterol homeostasis, cardiovascular disease, immunity, normal brain function, and Alzheimer’s disease (AD) (Mahley, Weisgraber et al. 2009).

1.1.1 Domains
Aided by early structural and functional studies investigating its role in disease and cholesterol homeostasis, apoE represents the canonical example of a two domain exchangeable apolipoprotein. The first indication that apoE contained two structural domains came from work by Wetterau et al. (Wetterau, Aggerbeck et al. 1988). Studies monitoring the secondary structure of the protein in the presence of chaotropes showed a biphasic curve with transition midpoints at 0.7 and 2.5M GuHCl, suggesting the presence of two structurally independent domains with differing stabilities.

Limited proteolysis of the full-length protein with a variety of proteolytic enzymes resulted in 22 kDa and 10 kDa fragments that were shown to correspond to the N- and C-terminal domains, respectively (Wetterau, Aggerbeck et al. 1988). This finding was consistent with the concept that the domains are separated by a flexible, protease sensitive loop. The conclusion that the smaller C-terminal domain may facilitate lipid binding (in a manner similar to the apoA family and apoC-II) came from the observed lower stability and greater conformational flexibility of this region of the protein. Analytical ultracentrifugation studies concluded that, like other exchangeable apolipoproteins, full-length apoE forms multimeric complexes in aqueous solution (Yokoyama, Kawai et al. 1985). The full length protein showed a propensity for forming tetrameric complexes that was attributed primarily to the presence of the C-terminus since the isolated N-terminal domain remained monomeric up to 15 mg/mL (Aggerbeck, Wetterau et al. 1988), further supporting the role of the C-terminus in initiating lipid binding.

1.1.2 Glycosylation
Early studies of apoE focused on characterizing the biochemical composition and differences among isolated populations of apoE from various organisms and tissues. It was recognized early on that more than one form of apoE existed and this was confirmed by seminal isoelectric focusing experiments that separated apoE into more than one band (Utermann, Langenbeck et al. 1980; Utermann, Steinmetz et al. 1982). Experiments in which neuraminidase, a glycosidase that cleaves sialic acid residues, treatment reduced some 2-D gel bands and increased others suggested that apoE was modified by glycosylation (Zannis and Breslow 1981). Modifications were further characterized and identified as galactose, glucosamine, galactosamine, sialic acid, N-acetylglucosamine, and N-acetylgalactosamine (Jain and Quarfordt 1979). Amino acid analysis identified Thr 194 as the sole residue modified by glycosylation and glycosylation was found not to be a necessary requirement of apoE expression (Wernette-Hammond, Lauer et al. 1989). While some studies have attempted to link alterations in glycosylation of apoE to changes in disease phenotypes, the exact role of the specific sugar moieties remains unknown. Hypotheses have been advanced that suggest the sugar moieties prevent proteolytic cleavage of the sensitive loop region in which it is contained (Mahley 1988) while others suggest these modifications may affect receptor binding or in vivo lipoprotein particle preference (Ji, Fazio et al. 1994).

1.1.3 Isoforms
The first experiments that suggested that apoE might have sequence heterogeneity came from pioneering experiments to isolate and characterize proteins found on very low-density lipoprotein (VLDL) particles by ion-exchange chromatography (Shore and Shore 1973). Confirmation that some of the multiple chromatography peaks showed similar
amino acid sequence and electrophoretic mobility hinted at subtle differences that were further supported by 2-D gel electrophoresis experiments (Zannis and Breslow 1980). The speculation that one of the isoforms may be playing a role in the development of hyperlipoproteinemia was confirmed once the three major isoforms of apoE were identified (Weisgraber, Rall et al. 1981; Utermann 1987). Variation in the human APOE gene locus results in three common alleles, ε2, ε3, and ε4 with allelic frequencies of 8%, 77%, and 15%, respectively. The three isoforms, apoE2, apoE3 and apoE4, differ only by the amino acid composition at positions 112 and 158,apoE3 (the most common allelic variant) contains a cysteine at position 112 and an arginine at 158. On the other hand, apoE2 contains cysteine at positions 112 and 158 while apoE4 contains arginines at these two sites.

i) ApoE2

Studies linking an apoE isoform containing a slightly more acidic isoelectric point to the development of type III hyperlipoproteinemia and premature atherosclerosis turned out to be the apoE2 variant whose LDL receptor binding activity was measured to be only 1% compared to the more common apoE3 isoform (Davignon, Gregg et al. 1988). Though genotypically rare (less than 1% in North American populations), the incidence of hyperlipoproteinemia among homozygotes is still quite low (less than 5%), suggesting that additional environmental factors are required to trigger the phenotype (Mahley 1995).

Studies to reveal the mechanism behind the reduced LDL receptor binding efficiency of the apoE2 isoform focused on understanding the contribution of Arg 158 to interactions with the receptor. Using a thrombin-treated protein to isolate the 22 kDa N-terminal domain followed by treatment with cysteamine to modify cysteine residues to positively charged lysine analogs, it was shown that when complexed with lipid apoE2 LDL receptor binding increased 100-fold to normal, to apoE3-like levels (Innerarity, Weisgraber et al. 1984). However, only an 8-fold receptor binding increase was observed for cysteamine-modified, full-length apoE2, suggesting that while the charged Arg does play a role in receptor binding, the orientation of the residues in the lipid bound state may be more important than the presence of any single charged group (Weisgraber, Innerarity et al. 1982). This also indicates that intramolecular interaction within the protein may depend on Arg 158 for proper folding or lipid interaction, which may then contribute to receptor interactions. Additionally, removal of the C-terminus eliminates any intramolecular interactions between the N- and C-termini, which could otherwise impose structural constraints that may play a role in lipid and/or receptor binding.

One strong mechanistic explanation for the difference between apoE2 and E3 with respect to receptor binding came from an X-ray crystallographic comparison of the isoforms (Dong, Parkin et al. 1996). In structures of the two isoforms, it was revealed that a salt bridge between Arg 158 and Asp 154 in apoE3 was absent in apoE2 as a result of the isoform specific differences. As a result, an alternative salt bridge formed between Arg 150 and Asp 154 in apoE2 effectively eliminating the availability of Arg 150 for interaction with the LDL receptor. An alanine to arginine substitution at position 150 in apoE3 decreased receptor binding to 24% of normal (Lalazar, Weisgraber et al. 1988) and although much higher than the 1% binding seen in apoE2, this finding did suggest that salt bridge alterations can strongly affect receptor binding presumably by changing the orientation or
patterning of basic residues in the receptor recognition sequence of apoE. These authors further hypothesized that dietary factors (such as a high fat diet) influence lipoprotein particle size and lipid composition which in turn dictates apoE2 Arg 150 side chain conformation and, consequently, the presence or absence of the Arg 150-Asp 154 salt bridge (Dong, Parkin et al. 1996). The authors further speculate that the resulting effects of the Arg 150-Asp 154 salt bridge on receptor binding activity under various dietary regimes explain the need for secondary dietary factors to induce type III hyperlipoproteinemia in apoE2 homozygotes.

ii) ApoE4

While apoE3 is considered the “wild-type” isoform in humans because of its high allelic frequency and lack of strong association with a human disease phenotype, apoE4 appears to be the ancestral form since the Arg112 and Arg 158 positions are strongly conserved across almost all animal species containing apoE (Mahley 1995). The apoE4 isoform is associated with decreased plasma concentrations compared to apoE3 and elevated plasma cholesterol and LDL levels leading to increased incidence of cardiovascular disease (Davignon, Gregg et al. 1988; Wilson 1995). Additionally and most strikingly, inheritance of apoE4 is correlated with cerebral amyloid angiopathy, tauopathies, dementia with Lewy bodies, Parkinson’s disease, multiple sclerosis and a higher incidence and significantly earlier onset of AD among carriers (Corder, Saunders et al. 1993). These collective disease phenotypes have been causally linked to what is now commonly referred to as domain interaction within apoE4, wherein the presence of arginine at position 112 instead of cysteine results in altered amino acid orientations within the protein that allow the N- and C-termini to interact via a specific salt bridge (Dong, Wilson et al. 1994; Dong and Weisgraber 1996; Mahley, Weisgraber et al. 2009). The resulting domain interaction is unique to apoE4 and has been hypothesized to be a causative factor linking this isoform to cardiovascular and AD phenotypes. The reduced plasma apoE4 levels compared to apoE3 presumably results from two-fold higher clearance rates, which causes a reduction in LDL receptor levels and subsequently reduced LDL clearance (Gregg, Zech et al. 1986) and predisposes the carrier to hypercholesterolemia. It has also been noted that apoE4 shows a preference for larger lipoprotein particles, such as VLDL and chylomicron remnants, which has been noted as the causative factor for the association of apoE4 with excess plasma LDL and lipoprotein components (Gregg, Zech et al. 1986; Weisgraber 1990).

The molecular basis for domain interaction was elucidated by comparison of X-ray crystallographic structures comparing apoE3 and apoE4 N-terminal domains (Dong, Wilson et al. 1994). The apoE4 structure showed that the intramolecular interaction is mediated by a salt bridge between Arg 61 and Glu 255. This isoform specific effect is the result of a salt bridge between Glu 109 and Arg 112 in apoE4 that causes Arg 61 to reorient and protrude slightly from the helix bundle, thereby becoming available for salt bridge formation with Glu 255 in the C-terminal domain (Dong and Weisgraber 1996). The interaction between the N- and C-terminal domains results in a more compact structure and has been associated with increased molten globule like properties, though a full mechanistic understanding of how this intramolecular interaction dictates lipoprotein particle preference and decreased structural stability remains unclear.
Similarly, domain interaction has been proposed as the causative factor for cellular effects leading to AD pathology in neural cells though the exact mechanism for how or why the domain interaction leads to the propagation of neurological defects remains elusive (Weisgraber and Mahley 1996; Mahley and Huang 1999; Huang, Weisgraber et al. 2004). Evidence linking apoE4 structural characteristics to neuropathology includes a unique susceptibility of apoE4 to neuron-specific proteolysis, resulting in the generation of neurotoxic protein fragments (Sanan, Weisgraber et al. 1994; Huang, Liu et al. 2001; Harris, Brecht et al. 2003). The protease sensitivity of apoE4 and subsequent bioactive fragment accumulation in the cytosol of neurons has been shown to alter cytoskeletal organization and disrupt mitochondrial energy balance but whether this is necessary and sufficient for the progression of AD pathology remains unknown.

1.1.4 ApoE Structural Organization

Exchangeable apolipoproteins are structurally related by their high amphipathic α-helical content that is critical to their functioning as surface exposed lipoprotein components and receptor ligands. Their opposing hydrophobic and hydrophilic helical faces allow the proteins to exist in both lipid-free and lipid-associated states and permits their activity as natural protein detergents for solubilizing lipophilic molecules (Segrest, Jones et al. 1992). Secondary structure prediction (Chou and Fasman 1974; Chou and Fasman 1974) and analyses of apoE by circular dichroism (CD)(Agerbeck, Wetterau et al. 1988) and Fourier transform infrared spectroscopy (FITR) (De Pauw, Vanloo et al. 1995) have demonstrated that apoE consists predominantly of α-helices (62% in aqueous solution) with a limited amount of β-strand secondary structure predicted in the C-terminal domain (Figure 1-1).

Understanding the relationships between the structure and function of apoE was greatly enhanced by determination of the x-ray crystal structure of the isolated N-terminus of apoE (Wilson, Wardell et al. 1991) and more recently by a more complete NMR structure of the same N-terminal domain (Sivashanmugam and Wang 2009) (Figure 1-2). The two structures show a prominent four extended amphipathic α-helix bundle arranged in an anti-parallel manner that folds to form an elongated, globular protein in the aqueous state. The boundaries of the four major helices: helix 1 residues 24-42, helix 2 residues 54-81, helix 3 residues 87-122 and helix 4 residues 130-164 are augmented by helix 1’ residues 44-53 and, in the NMR structure, helices N and C (residues 12-22 and 173-181 respectively). A high-resolution structure of the isolated C-terminal domain remains unsolved, though it does contain a predominance of α-helical content that, like regions of the N-terminal domain (residues 172-192), has been shown to increase in helicity upon exposure to lipid or receptor interaction.

1.2 Lipoprotein Metabolism

The transport and delivery of lipids and cholesterol required for cellular maintenance and metabolism must be highly regulated to mitigate the detrimental effects of the deposition of excess fatty acids and cholesterol in the circulatory system, liver, and adipose tissues. Transport of insoluble cholesterol, lipid and fat-soluble vitamins through the circulation is accomplished by the formation of plasma lipoprotein particles. These
spherical particles contain an outer shell of amphipathic phospholipids and proteins surrounding a core containing esterified cholesterol, triglycerides and fat-soluble vitamins. Lipoprotein particles are synthesized by either intestinal cells as chylomicrons or the liver as VLDL and contain dietary-derived and de novo synthesized or repackaged lipid metabolites, respectively. In the periphery, lipoprotein particles are lipolysed in the circulation by the action of lipases to form successively smaller, denser particles known as remnant particles (intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and chylomicron remnants). Lipolytically released metabolites are taken up by nearby cells of the artery wall, whereas the remnant particles are eventually cleared from the circulation by the liver, primarily by the action of the LDL receptor-mediated endocytosis.

1.2.1 Classification of Lipoproteins

The protein components of lipoproteins provide both a structural and metabolic control function as they encapsulate the core lipophilic metabolites for delivery and transport to peripheral tissues. If such apolipoproteins can exist free of lipid and transfer between lipoprotein classes they are referred to as exchangeable apolipoproteins and include apoE and the apoA and apoC classes. ApoB48 and B100 are the only two non-exchangeable apolipoproteins, which prevents them from leaving a lipoprotein without losing particle integrity and ensures that they travel with the de novo synthesized lipoprotein particle until recycling and degradation. Apolipoproteins act in a regulated manner to influence and direct lipoprotein metabolism in their various roles as ligands for receptors, activators or inhibitors of metabolizing enzymes, and structural determinants of lipoprotein size and composition. In humans, apoE, apoB48 and B100 are only ligands for the LDL receptor and collectively form the structural scaffolds for VLDL, IDL, LDL and chylomicron particles. ApoCII is a known activator of lipoprotein lipase (LPL), the enzyme that is responsible for the hydrolysis of lipids found on chylomicrons and VLDL particles. ApoCIII antagonizes LPL to prevent lipolysis-driven uptake of chylomicrons and VLDL metabolites. ApoA1 is primarily found on high density lipoprotein (HDL) secreted from liver and is responsible for reverse cholesterol transport, the process by which cholesterol and lipid is picked up from loaded peripheral cells for delivery back to the liver for processing and redistribution. ApoE is found on all lipoprotein classes except LDL and in circulation functions predominantly as an LDL receptor ligand for the uptake and removal of remnant particles by the liver.

1.2.2 Dysfunctions of Lipoprotein Metabolism

Dysfunction in lipoprotein clearance or lipoprotein metabolism results in a number of human diseases known as dyslipidemias. Of particular importance to this thesis is the role that apoE plays in the process of lipid and cholesterol homeostasis via its activity as ligands for the LDL receptor. Disruptions in the proper functioning of the LDL receptor have been shown to result in the dyslipidemia known as familial hypercholesterolemia (FH) (Brown and Goldstein 1974).

i) Familial Hypercholesterolemia

FH is an autosomal dominant inheritance genetic disorder characterized by elevated LDL cholesterol levels in plasma, xanthomas and xanthelasma, and premature onset of atherosclerosis resulting from the accelerated atherosclerotic lesion formation (Goldstein
Characterizations of FH patients led to the discovery of mutational defects in the cell surface expressed LDL receptor that caused deficiencies in remnant lipoprotein clearance (Brown and Goldstein 1986; Goldstein and Brown 2009). Further research of FH patients showed that LDL receptor mutations result in disease phenotypes through direct effects on ligand binding as well as problems with LDL receptor protein synthesis, Golgi to cell surface LDL receptor transport, endocytosis and clathrin coated pit formation, or retrieval and recycling of the LDL receptor from the endosome (Brown and Goldstein 1974; Goldstein and Brown 1974; Hobbs, Russell et al. 1990). Continued investigation of the LDL receptor pathway has resulted in considerable insight into the molecular determinants of proper LDL receptor function.

### 1.2.3 LDL Receptor Pathway

Work to determine the molecular defects in cells isolated from familial hypercholesterolemic patients led Brown and Goldstein to the discovery and elucidation of the pathway for the clearance of cholesterol rich lipoprotein remnants (Brown and Goldstein 1986) (Figure 1-3). The first observation that was noted from assays of cells from FH patients was that the normal reduction of HMG-CoA reductase activity enzyme activity upon the addition of an exogenous cholesterol was deficient (Brown and Goldstein 1974). Cells from FH patients had 50- to 100-fold elevations in HMG-CoA levels and the addition of LDL to the media failed to reduce intracellular HMG-CoA activity. Once normal LDL binding and uptake was shown to be active at low (µM) concentration, relatively independent of temperature, and saturable, it was presumed that the process must be receptor mediated (Goldstein and Brown 1974). The definitive experiment showed that normal cells had high- affinity cell surface binding and rapid uptake of 

125I-LDL, whereas FH cells lacked such binding, demonstrating the existence of receptor-mediated uptake of lipoprotein-derived cholesterol that resulted in negative feedback inhibition of cholesterol synthesis (Goldstein and Brown 1974; Goldstein and Brown 2009). From the defects seen in FH patients, it was shown that high-affinity binding of lipoprotein substrates to the LDL receptor was the rate-limiting step in the LDL degradation pathway. This led to the discovery of the LDL receptor and its connection to cholesterol homeostasis by the canonical sterol regulatory element-binding protein (SREBP) pathway (Schneider, Beisiegel et al. 1982; Brown and Goldstein 1999). Further genetic, biochemical, and cellular metabolism work resulted in our current detailed understanding of the LDL receptor pathway involving the binding of lipoprotein substrates, clathrin coated pit and vesicular formation, endosomal formation, intracellular cholesterol release and homeostatic maintenance, independent receptor recycling, lysosomal protein degradation, and the de novo LDL receptor synthesis pathway (Brown and Goldstein 1986).

### 1.2.4 ApoE Receptor Binding

One of the fundamental roles of apoE is to function as a ligand for cell-surface receptors of the LDL receptor family. ApoE has been shown to be a high affinity ligand for the LDL receptor, LRP1, apoER2 and the VLDL receptor (Figure 1-4). Studies to understand the elevated affinity of HDL particles for the LDL receptor determined that apoE copy number on rHDL particles correlated with receptor binding strength (Pitas, Innerarity et al. 1980). When the ratio of active to inactive apoE was varied on DMPC particles containing an average of four apoE per particle, the LDL receptor binding activity
could be modulated accordingly such that when the number of active apoE per particle approached one, the binding affinity approximated that of LDL. Additional competition experiments between HDL_c and LDL showed that approximately four times as many LDL particles were bound than HDL_c suggesting that HDL_c is able to bind four receptors per particle. Thus, the higher affinity binding by HDL_c appears to be the result of multiple receptor binding and the formation of multiple receptor contacts per particle in accordance with apoE copy number per particle. This hypothesis is further supported by studies that show optimal receptor binding is achieved when spherical lipid microemulsion particles contain at least four apoE molecules per particle (Funahashi, Yokoyama et al. 1989). This model implies that the high density arrangement of apoE (and greater lipid-dependent presentation of the LDL receptor recognition sequences) on discoidal and spherical particle allows for increased LDL receptor binding efficiency compared to a larger particle containing a single apoB-100. As important as the lipid induced conformational change may be, providing a scaffold for lipid-bound apoE molecules to interact with multiple cell surface receptors may be just as critical.

Significant understanding of the functional importance of apoE binding to the LDL receptor was gleaned from the generation of apoE transgenic (Shimano, Yamada et al. 1992) and homozygous null mice (Piedrahita, Zhang et al. 1992; Zhang, Reddick et al. 1992). The overexpressing, transgenic mice were created by integrating the rat apoE gene into mouse genome. Analysis of these mice showed that the apoE was primarily expressed in liver and associated with plasma lipoproteins as predicted. On a standard chow diet, the mice displayed markedly reduced cholesterol and triglyceride levels (43% and 68% respectively) and the reduction was correlated with reduced amounts of circulating VLDL and LDL lipoproteins owing to increased clearance of TG-rich particles mediated by apoE. On a high cholesterol diet, the mice were significantly more resistant to hypercholesterolemia and development of atherosclerotic lesions than control mice. One caveat of the study was its overexpression of rat apoE, which was shown to have characteristics more similar to human apoE4 than apoE3, and suggested that lipoprotein clearance might be reduced compared to human apoE3 overexpressing mice. Support for this hypothesis came when human apoE3 overexpressing mice were generated and shown to be hypertriglyceridemic with elevated VLDL and decreased LDL and HDL compared to wild-type mice (Huang, Liu et al. 1998). The mice displayed increased hepatic VLDL production and the apoE3-enriched VLDL contained elevated levels of apoC-II (a known inhibitor of lipoprotein lipase) resulting in lower peripheral lipase activity and contributing to the hypertriglyceridemic phenotype. The observed stimulation of VLDL triglyceride production and VLDL lipolysis inhibition in transgenic mice emphasized the importance of maintaining moderate levels of plasma apoE for promoting normal lipoprotein metabolism.

Additional support for the role of apoE in the maintenance of cholesterol and lipoprotein homeostasis came from transgenic mice overexpressing a receptor binding deficient form of the protein, apoE (Arg112, Cys142) (Fazio, Horie et al. 1994). Interestingly, on a chow diet, apoE was found to primarily associate with HDL_1 lipoprotein subclasses, which appeared to retard the clearance of these molecules from plasma. However, on a high fat diet, this mutant apoE primarily associated with apoB-containing lipoproteins leading instead to increased plasma and VLDL cholesterol caused by deficient receptor clearance. These studies confirmed the promiscuity of apoE lipoprotein binding and implicated apoE as a mediator of both HDL and VLDL metabolism.
Working from the hypothesis that human apoE3 displays a lipoprotein preference that is different from mouse apoE, Sullivan et al. created a mouse model in which a gene replacement was made to substitute the mouse apoE gene for the human cDNA (Sullivan, Mezdour et al. 1997). In wild-type mice, apoE is predominantly found on HDL sized particles. In mice homozygous for the human apoE3 gene, its distribution shifted from HDL to larger particles and there was a concomitant decrease in β-migrating and apoA-1-containing HDL particles. On a high fat diet, the transgenic human apoE mice displayed five-fold higher total plasma cholesterol levels compared to the 1.5-fold increase seen in wild-type mice. Consistent with this increase, the apoE replacement mice had 13-fold larger atherosclerotic plaques after 3 months on a high fat diet compared to their wild-type counterparts (Sullivan, Mezdour et al. 1997). While the consequences of the gene replacement were pronounced, it cannot be ruled out that a difference in affinity of the human apoE3 for mouse LDL receptor protein, HSPG’s, or lipolytic enzymes may have contributed to the observed phenotypic effects. This study stressed that despite the relatively high sequence identity between human and mouse apoE (70%), the species-specific activity and processing can play an important role in the proper functioning of lipoprotein metabolism.

Understanding of the physiological role of apoE was significantly advanced by the creation of mice lacking the apoE locus. The absence of apoE in these mice recapitulated many of in vivo phenotypes of atherosclerosis and this knockout remains a highly valuable model for the continued study of the disease (Zhang, Reddick et al. 1992). The initial characterizations of apoE null mice found that the mice were physically and behaviorally normal despite having a five-fold elevation in plasma cholesterol levels compared to wild-type littermates. It was also found that apoE knockouts had a 55% reduction in HDL cholesterol levels, while triglyceride levels were 68% higher, which parallels trends seen in apoE deficient humans.

1.2.5 Receptor Recognition Sequence

Just as Brown and Goldstein were elucidating the LDL receptor endocytosis pathway for the uptake of apoB-containing LDL particles, Mahley and coworkers noticed limited but highly conserved sequence similarity between apoB and the “arginine-rich” apoE found on HDLc particles (Mahley, Weisgraber et al. 1975; Bersot, Mahley et al. 1976). Shortly thereafter, Mahley et al. demonstrated the importance of this stretch of charges residues by showing that the treatment of apoE with cyclohexanedione (an arginine-specific modifier) abolished all receptor activity (Mahley, Innerarity et al. 1977).

From these early studies, the highly conserved LDL receptor recognition sequence was localized to the N-terminus of apoE and residues 3359–3367 of apoB. Analysis using cyanogen bromide to digest apoE at methionine residues found that an isolated peptide containing residues 126-218 bound the LDL receptor with the same affinity as LDL once complexed with DMPC-containing rHDL particles (Innerarity, Friedlander et al. 1983). The receptor binding region was further defined when an antibody that recognized residues 139-169 was shown to abolish receptor binding (Weisgraber, Innerarity et al. 1983). Yet further refinement came when mutational analysis in which positively charged residues 142, 145, 146, and 158 were substituted for neutral amino acids markedly reduced apoE binding to the LDL receptor (Mahley 1988). This data and sequence analysis combined to
define the putative LDL receptor recognition sequence as residues 136-152 of the apoE N-terminus.

Mutagenesis studies by Lalazar and colleagues investigated the contribution of individual amino acids in the recognition sequence to high affinity receptor interaction (Lalazar, Weisgraber et al. 1988). Substitution of basic amino acids with alanine reduced the ability of apoE to compete with LDL for receptor binding. No single substitution fully abolished receptor binding and the addition of arginines at certain positions increased binding activity, leading to the suggestion that cooperativity among positively charged residues contributes to functional receptor interaction. While it is implied that the degree of direct ionic interaction and changes in the presentation of basic amino acids is responsible for receptor binding activity, the authors recognized that there may be alterations in salt bridge formation that may be responsible for modulation of receptor interactions.

The region of apoE primarily responsible for receptor recognition was also probed by binding truncation mutants to DMPC and measuring receptor activity (Lalazar and Mahley 1989). While apoE(1-170) and apoE(1-174) fragments maintained only 1% and 19% of respective LDL receptor binding activity, apoE(1-183) retained 85% of binding compared to full-length protein. This was the first detailed study implicating residues outside of the putative LDL receptor recognition sequence (residues 136-152) in promoting receptor activity. Subsequent mutagenesis analysis noted the striking contribution of arginine 172 to receptor binding activity (Morrow, Arnold et al. 2000). This study confirmed the general importance of residues 170-183 by showing that their removal reduced binding activity to 15% of full-length apoE3 levels, but strikingly, a 98% drop in binding activity was seen with a single Arg172Ala substitution. Notably, an Arg172Lys substitution showed only 6% of normal activity, suggesting that arginine is required at this position to preserve the apoE conformation necessary for receptor competent orientation.

1.2.6 LDL Receptor Structure and Function

Early studies to determine the in vivo ligands of the LDL receptor demonstrated that transgenic mice overexpressing the LDL receptor showed a greater than 90% reduction in plasma apoE and apoB-100 levels, while apoA-1 levels remained the same (Hofmann, Russell et al. 1988). Additional evidence came from LDL receptor null mice that showed dramatically elevated plasma LDL and cholesterol levels due to impaired receptor-mediated clearance of apoE and apoB containing lipoproteins (Ishibashi, Brown et al. 1993). By binding to the LDL receptor, apoE and apoB containing lipoproteins are cleared from the plasma in a mechanism designed to regulate blood cholesterol levels (Yokode, Hammer et al. 1990; Ishibashi, Goldstein et al. 1994). Upon binding to the LDL receptor at neutral pH, the receptor and ligand complexes are internalized into vesicles that become acidified in their conversion to endosomal compartments. At this lowered pH, the receptor releases the lipoprotein ligand in the endosomes, enabling lysosomal degradation of LDL, release of cholesterol and recycling of the receptor (Brown and Goldstein 1986; Goldstein and Brown 2009).

The LDL receptor is the prototypical member of a large class of integral membrane cell surface receptors that acts through a ligand-activated, clathrin coated pit-mediated endocytosis to internalize plasma lipoproteins. The 839 amino acid LDL receptor protein is
composed of five distinct regions i) an amino terminal ligand binding domain (LBD) containing seven cysteine-rich repeat modules of roughly 40 amino acids, ii) an epidermal growth factor (EGF) precursor domain containing three EGF-like, cysteine-rich repeats and a β-propeller domain that mediate ligand release in the endosome via a pH-dependent conformational change preceding receptor recycling (Davis, Goldstein et al. 1987), iii) a domain with an O-linked sugar domain, iv) a single-pass transmembrane domain, v) and a cytoplasmic tail that contains the NPxY sequence believed to cause the clathrin coated pit clustering of these receptors (Rudenko and Deisenhofer 2003).

The first modules of the LDL receptor to be structurally characterized were the cysteine-rich repeat modules of the LBD: R1 and R2 by NMR (Daly, Djordjevic et al. 1995; Daly, Scanlon et al. 1995), R5 by x-ray crystallography (Fass, Blacklow et al. 1997) and R6 by NMR (Clayton, Brereton et al. 2000; North and Blacklow 2000) (Figure 1-5). Each of the roughly 40 amino acid long modules form a globular structure that contains two lobes held together by extensive disulfide bonding. The C-terminal loops of each module contain a cluster of acidic residues that fold to create a coordination site for a single calcium ion that was first definitively shown in R5 by x-ray crystallography (Fass, Blacklow et al. 1997). Structural elucidation of tandem repeat pairs of R1-R2 (Kurniawan, Atkins et al. 2000) and R5-R6 (North and Blacklow 1999; Beglova, North et al. 2001) show an absence of interaction between neighboring repeat domains. Tryptophan and tyrosine-based fluorescence and isothermal titration calorimetry techniques have been used to measure $K_d$ values of calcium binding in the nanomolar to micromolar range that collectively confirm the presence of high-affinity calcium binding sites within each of the repeat domains (Dirlam-Schatz and Attie 1998; North and Blacklow 1999; North and Blacklow 2000; Simonovic, Dolmer et al. 2001). All of the repeat domains in the LBD contribute to apoE binding, however the 4-5 module pair is sufficient for interaction with apoE (Fisher, Abdul-Aziz et al. 2004) and thus has been postulated to provide the greatest contribution to a functional receptor interaction with apoE ligands.

The entire EGF precursor domain is comprised of two EGF-like repeat modules (A and B) each containing 40-50 residues that form two pairs of short antiparallel β strands linked to a β-propeller motif followed by a third EGF-like repeat (C). In solution, the EGF-like A-B pair forms a rigid rod (Kurniawan, Aliabadizadeh et al. 2001; Saha, Boyd et al. 2001) and the EGF-like C module has been shown to pack tightly against the β propeller (Jeon, Meng et al. 2001). In the A-B tandem structure, the extreme C-terminus of repeat B was shown to be unstructured, showing motion on the nanosecond timescale (Saha, Boyd et al. 2001), which leads to the suggestion that this portion of the module becomes stabilized upon interaction with the β propeller of the EGF precursor domain (Kurniawan, Aliabadizadeh et al. 2001). Calcium binding sites in LDL receptor repeats A and B display low micromolar $K_d$ values and the orientation of module A with respect to B seems to be strongly influenced by calcium binding in the B module, whereas module C does not have a calcium binding site (Jeon, Meng et al. 2001; Kurniawan, Aliabadizadeh et al. 2001; Malby, Pickering et al. 2001; Saha, Boyd et al. 2001). NMR relaxation data indicated that calcium binding in module B creates a rigid linker between the last cysteine of module A and the first cysteine of module B (Kurniawan, Aliabadizadeh et al. 2001; Saha, Boyd et al. 2001). The six bladed structure of the β propeller motif was first predicted by sequence analysis (Springer 1998) and then shown by crystallography to contain a single β sheet and
four antiparallel β strands per blade (Jeon, Meng et al. 2001). A Tyr-Trp-Thr-Asp (or YWTD-like) consensus sequence repeats six times roughly every 40 residues and is found on strand 2 of each propeller blade to provide stabilizing interactions both within each blade and between blades.

Understanding the structural determinants of ligand binding to the LDL receptor was significantly advanced by the x-ray crystallography work of Rudenko et al. showing a structure of the soluble, extracellular portion of the LDL receptor (lacking the transmembrane domain and cytoplasmic tail) at pH 5.3 (Rudenko, Henry et al. 2002) (Figure 1-6). In addition to confirming all the known structural features of the LDL receptor, this crystal structure provided strong support for a comprehensive model to explain the mechanism of LDL receptor ligand release in the endosomal compartment upon endocytosis. In the structure solved at lowered pH, the LBD modules R4 (residues 127-163) and R5 (residues 176-210) bind to the β propeller (residues 377-642) of the EGF precursor domain via extensive intramolecular contacts that cause the elongated molecule to fold upon itself, presumably to drive off bound lipoprotein ligands in the endosomal space as has been suggested to occur on the basis of biochemical experiments. An extensive network of hydrophobic and charged interactions and several salt bridges were observed between the R4 (Ala130, Ile140, Pro141, Trp144, and Asp151) and R5 (Trp193, Asp196, Gly198, and Asp200, His190, Ser191, and Lys202) modules and the β propeller domain (Trp515, Thr517, Gln540, Trp541, Lys560, His562, His586, Glu581, and Lys582). Of particular note are the abundance of highly conserved R4 and R5 residues that are responsible for β propeller contacts, including previously identified FH-associated His to Tyr substitutions at position 190 of R5 and residue 562 in the β propeller, suggesting a highly functional relevance to this observed binding interaction. The authors further suggested that the formation and strength of the interface salt bridging could change as a function of pH and side chain pKₐ of the aspartates, glutamates and histidines within the contact region (Rudenko, Henry et al. 2002; Rudenko and Deisenhofer 2003). Based on crystallographic measurements, the extended length of the modular R1-R7 repeat domains is approximately 270 Å, which would provide significant surface area for accommodation of apoE and apoB-containing lipoprotein substrates of various sizes. The exact mechanism for ligand binding remains controversial and unresolved primarily because the prevailing model promoting the importance of the acidic residues in the LBD repeat modules for ligand binding was called into question when many of these residues were found to buried and involved in intramolecular calcium coordination. From this structure and biochemical binding experiments, it is clear that the LDL receptor is suited to recognize lipoprotein ligands with high affinity, presumably by the structural organization and flexibility of the recognition domain that can bend at flexible linkers to bind and surround particles of varying size and protein composition.

1.3 Neurobiology and apoE

The importance of apoE for the maintenance of cellular cholesterol homeostasis does not appear to be limited to the periphery as its role in neurological phenomena such as neuronal plasticity, neurite outgrowth, and synaptogenesis continues to be elucidated. While plasma apoE originates from the liver and macrophages, apoE found in the central
nervous system (CNS) is produced locally in the brain. Exchange between liver and brain does not appear to take place owing to the blood-brain barrier (BBB) and experiments demonstrating a failure to detect liver derived apoE in the cerebrospinal fluid (CSF) of liver transplant recipients (Linton, Gish et al. 1991). While apoE is the predominant apolipoprotein found in the CNS, other apolipoproteins, such as apoJ, apoD, apoAI and apoAIV are also present. In the brain, apoE is primarily synthesized by astrocytes (Boyles, Pitas et al. 1985; Pitas, Boyles et al. 1987), although microglia and neurons have been shown to synthesize apoE under select physiological and pathological conditions (Han, Einstein et al. 1994; Xu, Gilbert et al. 1999; Aoki, Uchihara et al. 2003). Under normal conditions, glial cells produce two- to three-times more cholesterol than neurons, in line with the elevated apoE expression. It has been demonstrated that apoE is predominantly associated with lipoproteins in the brain, though the astrocyte secreted apoE particles differ significantly from peripheral apoE-containing lipoproteins in that they are discoidal HDL-like particles composed of phospholipid and unesterified cholesterol (Fagan, Holtzman et al. 1999; Ladu, Reardon et al. 2000). It is presumed that certain astrocyte-secreted apoE-containing lipoproteins acquire a core of esterified cholesterol on their way to the CSF as both discoidal and HDL-like spherical lipoproteins have been isolated from the CSF (Fagan and Holtzman 2000; Ladu, Reardon et al. 2000). The stability of apoE depends upon its proper lipidation as evidenced by a marked reduction in brain apoE levels upon deletion of the Abca1 gene in mice, the product of which lipidates astrocyte-secreted apoE (Hirsch-Reinshagen, Zhou et al. 2004; Wahrle, Jiang et al. 2004).

1.3.1 Synaptic Plasticity and Spine Integrity

There is mounting evidence that apoE can affect brain morphology and suggests that apoE plays a crucial role in the aging process. Studies to examine apoE-null mice within the context of neurological aging continue to be useful for understanding its role in natural aging despite the fact that conflicting results on its role and actions in the brain persist. In some studies of apoE knock-out mice, the animals displayed no signs of synaptic degeneration (Cambon, Davies et al. 2000), showed normal brain histology, an absence of neurodegenerative markers (Moghadasian, McManus et al. 2001), and normal cholinergic activity and neuronal function (Anderson and Higgins 1997; Anderson, Barnes et al. 1998; Bronfman, Tesseur et al. 2000; Puolivali, Miettinen et al. 2000). However, other studies have shown that apoE null mice develop mild to severe spatial learning and memory deficits as assessed by Morris Water Maze experiments (Oitzl, Mulder et al. 1997; Champagne, Dupuy et al. 2002). When memory impairment was observed in these mice, cholinergic deficits were also observed, highlighting the importance of apoE in cognition and memory (Gordon, Grauer et al. 1995; Chapman, Sabo et al. 2000). ApoE has also been implicated in age-related neuropathology from studies demonstrating that apoE knockout mice are more susceptible to neurodegeneration compared to their wild-type counterparts (Walker, Parker et al. 1997; Robertson, Dutton et al. 1998).

The predominant presence of apoE-containing lipoproteins in the brain suggests the protein plays a critical role in lipid and cholesterol trafficking and clearance. It is known that brain cholesterol is required for synapse development (Mauch, Nagler et al. 2001), dendrite formation (Goritz, Mauch et al. 2005), long term potentiation (Koudinov and Koudinova 2002), and axonal guidance (de Chaves, Rusinol et al. 1997; Posse De Chaves, Vance et al. 2000). Cholesterol delivered to neurons on apoE-containing particles
dramatically increases synapse formation (Mauch, Nagler et al. 2001) by increasing the formation of synaptic vesicles and upregulating the machinery necessary to release the vesicles (Thiele, Hannah et al. 2000; Ullian, Christopherson et al. 2004). Cholesterol depletion or a lack of cholesterol delivery causes synaptic and dendritic spine degeneration and results in failed neurotransmission and decreased synaptic plasticity (Koudinov and Koudinova 2001). Following significant neuronal cell damage, cell death or terminal differentiation in the central nervous system, large amounts of lipid and cholesterol are lost (presumably by a clearance mechanism) from the site of membrane and myelin degeneration (Rawlins, Villegas et al. 1972). In response to these events, apoE protein expression is upregulated in astrocytes and brain macrophages in parallel with the clearance of cholesterol and lipid debris from the site of injury (LeBlanc and Poduslo 1990; Poirier, Hess et al. 1991). This evidence suggests a role for apoE as a scavenger of lipophilic molecules for nerve regeneration, CNS re-innervation or downstream recycling and storage of lipophilic metabolites (Rawlins, Hedley-Whyte et al. 1970; Poirier, Baccichet et al. 1993). The LDL receptor is also induced at the processes of a regenerating peripheral nerve suggesting the importance of lipoprotein uptake and redistribution during nerve growth and regeneration (Boyles, Notterpek et al. 1990). Additionally, it has been shown that the cholesterol-containing apoE-lipoproteins secreted by astrocytes are essential for the formation of synapses in vitro through a mechanism that is dependent upon functional apoE-dependent receptors (Mauch, Nagler et al. 2001).

With the observed isoform-specific effects of apoE on the onset and progression of neurodegenerative disease, several studies have focused on the differential effects of apoE3 and apoE4 on synaptic plasticity and synaptogenesis. A few studies have shown that both apoE3 and apoE4 have the ability to reverse the presynaptic deficits and cognitive impairment seen in apoE null-mice (Masliah, Samuel et al. 1997), but most evidence suggests that apoE4 is less efficient than apoE3 in promoting neurological repair and maintaining proper brain function. Buttini and colleagues showed that expression of apoE3, but not apoE4, protects against neuronal damage and the age-related neurodegeneration seen in apoE-null mice (Buttini, Orth et al. 1999). In contrast to apoE3-expressing mice, apoE4 mice display synaptic deficits and lower excitatory synaptic transmission even in the absence of neuropathology (Wang, Wilson et al. 2005). In addition, apoE4 expressing mice have impaired long term potentiation, decreased numbers of synapses per neuron (Cambon, Davies et al. 2000), and reduced dendritic spine formation (Ji, Gong et al. 2003) compared to apoE3-expressing counterparts. ApoE4-mice also do not recover as efficiently from traumatic brain injury (Sabo, Lomnitski et al. 2000) and are more susceptible to cerebral ischemia (Horsburgh, McCulloch et al. 2000). Despite these defects, most studies demonstrate that both apoE3 and apoE4-expressing mice perform better on cognitive tests compared to apoE-null mice (Veinbergs, Mallory et al. 1999). However, when comparing dendritic spine morphology, the phenotype of apoE4-expressing mice did not differ significantly from apoE-null mice suggesting a loss of function effect of apoE4 on dendritic spine maintenance in the mouse and human brain (Ji, Gong et al. 2003). Interestingly, this effect seems to be age-dependent as the apoE4-dependent reduction in dendritic spine formation was only observed in mice aged for at least 1-2 years, which suggests that the apoE isoform effects might relate to the increased risk of dementia in aged humans expressing apoE4. It was further shown that in aged patients with and without AD, apoE4 gene dose is inversely correlated with dendritic spine
density (Ji, Gong et al. 2003). It seems evident that apoE plays an important role in the proper maintenance of the nervous system despite the fact that the mechanism and degree of necessity remains unclear; especially in light of the fact that apoE does not appear to be indispensable since apoE-null mice do not show gross defects in normal nerve repair and development and only develop neurodegenerative phenotypes upon significant aging.

1.3.2 Alzheimer’s Disease

There is strong circumstantial evidence that apoE plays a central, if not direct, role in the pathogenesis of AD. The clinical symptoms of AD are a progressive loss of cognition, dementia, and the presence of extracellular amyloid plaques and intracellular neurofibrillary tangles consisting of hyperphosphorylated tau protein in the brain tissues of patients (Auld, Kornecook et al. 2002; Selkoe 2004). The apoE4 isoform has been shown to be a significant risk factor for the development of the disease, including early and late onset familial (Corder, Saunders et al. 1993; Strittmatter, Weisgraber et al. 1993) and sporadic forms (Rebeck, Reiter et al. 1993; Saunders, Strittmatter et al. 1993; Frisoni, Govoni et al. 1995; Ishii, Tamaoka et al. 1997). Among individuals possessing the late onset form of AD, the ε4 allele is present at 2- to 3-fold higher rates than among the general population and some studies show that up to 65% of all clinically diagnosed cases carry at least one ε4 allele (Saunders, Strittmatter et al. 1993). The mounting genetic and epidemiological evidence strongly linking the allelic dose inheritance of the apoE4 isoform to earlier onset and increased severity of AD continues to drive research aimed at elucidating the molecular mechanisms of the protein that lead to neurological and neurodegenerative disease.

1.3.3 apoE and Alzheimer’s Disease

While the genetic associations between apoE and AD are striking, the relationship between apoE protein expression and amyloid burden is not as straightforward. Loss of apoE in an amyloid mouse model background dramatically reduces amyloid burden without affecting Aβ production (Bales, Verina et al. 1997), and led to the suggestion that apoE may be contributing to the conversion of Aβ to its more toxic, fibrillar form. However, in LDL receptor null mice, apoE levels are increased by ~50% yet this has no effect on amyloid deposition (Fryer, Demattos et al. 2005). Conversely, in ABCA1 deficient mice, apoE expression levels decrease by ~75% and the apoE that is secreted is primarily lipid depleted or deficient, and this has been associated with increased amyloid burden (Hirsch-Reinshagen, Maia et al. 2005; Koldamova, Staufenbiel et al. 2005; Wahrle, Jiang et al. 2005). Interestingly, apoE4 has been shown to be preferentially degraded by astrocytes which may explain why mice have genotype dependent effects on total apoE levels in brain, CSF, and plasma, with the phenotypic strength of allelic dose in accordance with apoE2/2>apoE3/3>apoE4/4 (Riddell, Zhou et al. 2008).

Analysis of the isolated N-terminal domains of the three human isoforms of apoE showed that apoE4 is the least resistant to chemical and heat denaturation, while the apoE2 isoform is most resistant at neutral and low pH (Morrow, Segall et al. 2000; Morrow, Hatters et al. 2002; Weers, Narayanaswami et al. 2003). Lipid binding affinity is reportedly higher for apoE4 although maximal lipid binding capacity appears equivalent across the isoforms (Saito, Dhanasekaran et al. 2003; Sakamoto, Tanaka et al. 2008). While apoE4 has
a demonstrated preference for binding to VLDL sized lipoproteins in vitro (Gregg, Zech et al. 1986; Weisgraber 1990), how this affects brain physiology is unclear since only HDL sized lipoproteins have been found in brain. It has been suggested that apoE4 is more susceptible to proteolysis compared to apoE3 (Cho, Hyman et al. 2001; Huang, Liu et al. 2001), which has led to the suggestion that apoE4 digestion products contribute to amyloid plaque formation and AD pathology since these fragments can be detected in the plaques of AD-positive brains (Wisniewski, Lalowski et al. 1995). Additional evidence suggests that the isolated N-terminal domain of apoE4 is neurotoxic (Marques, Tolar et al. 1996; Tolar, Marques et al. 1997; Marques, Owens et al. 2004), though it is not known if the proteolytic cleavage occurs prior to or after interaction with Aβ. It is also known that lipided apoE is protected from proteolytic cleavage to a greater degree than lipid-free protein despite the fact that the quantity and physiological functions of lipid-free apoE in brain remains unknown (Weisgraber, Roses et al. 1994; Narayanaswami, Maiorano et al. 2004). Finally, recent studies point to a role of apoE4 in potentiating Aβ-induced lysosomal leakage (Ji, Miranda et al. 2002; Ji, Mullendorff et al. 2006) and activating the endoplasmic reticulum stress response leading to increased apoptosis (Zhong, Ramaswamy et al. 2009).

Several studies examining the structural differences between apoE isoforms have attempted to link these differences in physical properties and structural stability to their respective correlations to AD risk. Compared to apoE2 and apoE3, apoE4 preferentially exists in a relatively unstable ‘molten globule’ state and, because of the presence of arginine at position 112, displays N- and C-terminal ‘domain interaction’ as a result of a salt bridge between arginine 61 and glutamic acid 255 (Hatters, Budamagunta et al. 2005; Hatters, Peters-Libeu et al. 2006; Zhong and Weisgraber 2009). Genetically altered mice harboring a Thr61Arg mutation in murine apoE results in decreased apoE levels in the brain and synaptic and cognitive defects (Ramaswamy, Xu et al. 2005; Zhong, Scearce-Levie et al. 2008). Transgenic amyloid mice in which murine apoE is replace by each of the three human isoforms develop the predicted isoform-specific differences in amyloid deposition with apoE4>apoE3>apoE2 (Holtzman, Bales et al. 2000; Carter, Dunn et al. 2001), but, interestingly, all the human isoform substituted mice manifest delayed onset of plaque formation compared to murine apoE-containing mice (Buttini, Yu et al. 2002; Dodart, Marr et al. 2005). Finally, mice harboring the Dutch or Iowa human mutations in the amyloid precursor protein (APP) together with wild-type murine apoE caused primarily cerebrovascular amyloid deposits, whereas mice containing human apoE shifted the distribution of amyloid deposition in favor of the parenchyma (Xu, Vitek et al. 2008). This suggests that the action and distribution of apoE is controlled by a number of factors that synergize with apoE to affect its physiological activity. Better understanding of the ways that apoE interacts with Aβ, its known receptors and other binding partners in response to various conformational and lipidation states, and the type and amount of lipid cargo within the brain may provide further insight into its role in AD pathology.

1.3.4 Amyloid β and apoE Interactions

Deposition of extracellular senile plaques formed by soluble and insoluble assemblies of the amyloid-β (Aβ) peptide is a classical hallmark of AD progression and is considered the primary event in the disease pathology according to the amyloid hypothesis. Aβ is derived from APP by sequential β- and γ-secretase-dependent
intramembrane proteolysis (Selkoe 2004). Aβ is produced and secreted under normal metabolic conditions and can be found at high levels in normal CSF and plasma (Haass, Schlossmacher et al. 1992; Seubert, Vigo-Pelfrey et al. 1992). Thus, the disease pathology is hypothesized to be driven by a net imbalance between Aβ clearance and production (Hardy 2002; Golde 2003). Gradual increases in Aβ production lead to Aβ oligomerization in the brain interstitial fluid and inside neurons (Skovronsky, Doms et al. 1998; Walsh, Tseng et al. 2000) and subsequent fibrillization through a process that produces the soluble, oligomeric amyloid plaques that are hypothesized to be the primary effectors of the disease (Selkoe 2004). The dominantly-inherited, familial form of the disease is associated with either increased production of the Aβ peptide (most commonly caused by mutations in the APP gene itself or the presenilin 1 and 2 gene products that form two of the necessary components of the γ-secretase complex) (Selkoe and Kopan 2003) or increased proportion of the longer Aβ42 peptide which has been shown to have greater toxicity than the classical Aβ40 form (Hardy and Selkoe 2002). The non-familial forms of AD have been attributed to an imbalance in the relative clearance and aggregation of Aβ (Puglielli, Tanzi et al. 2003) and the only consistently associated genetic risk factor for non-familial AD is the ε4 allele of the APOE gene. Despite this mechanistic difference, the AD pathology and phenotypic display are nearly indistinguishable between the familial and non-familial forms.

The early observation that apoE bound to Aβ in the CSF prompted the study of apoE as a candidate for the acceleration of AD (Namba, Tomonaga et al. 1991; Wisniewski and Frangione 1992; Wisniewski, Golabek et al. 1993). Although the complete mechanism by which apoE (and particularly apoE4) promotes AD pathology remains incompletely understood, in vitro and in vivo evidence suggests that interactions between apoE and Aβ may be intimately associated with the disease progression. Early in vitro studies demonstrated that Aβ and delipidated apoE4 promote fibrillar formation more rapidly and with higher density that those seeded with other apoE isoforms (following an aggregation rate order of apoE4>apoE3>apoE2) (Strittmatter, Weisgraber et al. 1993; Wisniewski, Golabek et al. 1993; Sanan, Weisgraber et al. 1994). In contrast to delipidated protein, lipidated apoE has a different isoform dependent affinity for Aβ (LaDu, Pederson et al. 1995). When the affinity of lipid-bound apoE was compared using transfected eukaryotic cell lines, the level of apoE3-Aβ complex was 20-fold higher than that of apoE4-Aβ complex (LaDu, Falduto et al. 1994; Tokuda, Calero et al. 2000). It has further been shown that apoE3 rHDL complexes bind two- to three-fold more rapidly to Aβ than do apoE4 containing lipoproteins, indicating that that Aβ-apoE interaction is also affected by lipidation status (Tokuda, Calero et al. 2000). An alternative approach measuring the effect of apoE on the promotion of neurite extension demonstrated that lipidated apoE3 enhanced binding to Aβ and may facilitate clearance of the peptide and thereby prevent aggregate formation (Nathan, Bellosta et al. 1994). It has been shown that Aβ binds to apoE via the C-terminal domain, and Aβ binding has shown to abrogate apoE lipid binding (Strittmatter, Weisgraber et al. 1993; Tamamizu-Kato, Cohen et al. 2008). The results indicate that Aβ peptides interfere with the normal functioning of apoE as a lipid transport molecule in brain, which may additionally contribute to the progression of AD through alterations in lipid and cholesterol homeostasis (Fenili and McLaurin 2005; Poirier 2005; Shobab, Hsiung et al. 2005; de Chaves and Narayanaswami 2008). In a neuronal cell line
that overexpresses APP, apoE4, as compared to apoE3, was shown to increase Aβ production in vitro (Ye, Huang et al. 2005). This difference between apoE4 and apoE3 was abolished when the cells were treated with small interfering RNA’s against the LDL receptor-related protein (LRP1) or incubated with the receptor-associated protein (RAP), which is a known inhibitor of LRP1. This finding suggests that the apoE4 dependent differences in Aβ production are dependent upon the action of the LRP1 receptor, for which apoE is a known ligand. Additional in vitro studies using transfected cell lines indicate that apoE and its receptors may play a role in APP processing a Aβ production (Bu, Cam et al. 2006; Cam and Bu 2006). A possible mechanism was suggested by studies showing that the overexpression of apoE4 enhanced Aβ production through its promotion of endocytic recycling of APP (Ye, Huang et al. 2005), but how apoE4 in particular facilitates this recycling remains unclear. It is broadly accepted that clearance of Aβ is as important as its production. The two predominant pathways for clearance of Aβ are receptor mediated uptake and subsequent proteolytic degradation by microglia and astrocytes in the brain (Cole and Ard 2000; Wyss-Coray, Loike et al. 2003; Koistinaho, Lin et al. 2004) and transport across the BBB predominantly mediated by the action of LRP1 (Tanzi, Moir et al. 2004; Zlokovic 2004).

### 1.3.5 Amyloid β Transport and Clearance

Receptor-mediated clearance of Aβ in the brain is likely mediated through the action of LDL receptor family members including LDL receptor, LRP1, apoE receptor 2 (apoER2), SorLA/LR11 and VLDL receptor, which are expressed in neurons, astrocytes, and microglia of the brain parenchyma as well as endothelial cells, astrocytes, and smooth muscles cells that comprise the BBB and cerebral arteries. It has been shown that both full length and cleaved fragments of apoE (Rebeck, Reiter et al. 1993; Wisniewski, Lalowski et al. 1995), LRP1 and other LRP1 ligands (Rebeck, Reiter et al. 1993) co-localize and show immunoreactivity with amyloid plaques in the brain. Additionally, apoE receptors have been shown to bind directly to Aβ (Deane, Wu et al. 2004) and through Aβ binding partner interaction, such as apoE-Aβ. In accordance with the increased lipidated apoE3 binding to Aβ, apoE3 clears Aβ through receptor-mediated interaction more efficiently than do apoE4-Aβ complexes. In several amyloid mouse models, expression of human apoE3 resulted in less amyloid plaque deposition than did apoE4-expressing mice (Holtzman, Bales et al. 1999; Holtzman, Bales et al. 2000; DeMattos, Cirrito et al. 2004). In addition, post-mortem amyloid plaque load is increased in the brains of ε4 carriers (Schmechel, Saunders et al. 1993; Bogdanovic, Corder et al. 2002), suggesting that more efficient clearance of Aβ may ameliorate the toxic effects of amyloid formation.

Though cellular and BBB export of Aβ is most certainly receptor mediated, the responsible receptors remain controversial. Using real-time in situ microdialysis technology, Bell et al. demonstrated that Aβ42 passed more slowly across the BBB than did the rapidly transported Aβ40 peptide, though both peptides were shuttled by LRP1 (Bell, Sagare et al. 2007). Association of Aβ40 with lipid poor apoE slowed the transport and lipidated apoE complexed with Aβ blocked virtually all transport across the BBB within the 30-minute timeframe of the study. Using the same technique, an alternative study confirmed that lipidation of apoE, compared to unlipidated apoE-Aβ or free peptide alone,
dramatically slowed the transport of apoE-\(\text{A} \beta\) across the BBB and further showed that apoE disrupts the transport of \(\text{A} \beta\) in an isoform dependent manner (with a transport rate of apoE2>apoE3>apoE4) compared to free peptide (Deane, Sagare et al. 2008). Interestingly, this study demonstrated that \(\text{A} \beta\) binding to apoE4 changed the receptor-mediated clearance from LRP1 to the VLDL receptor, which was shown to export apoE4-\(\text{A} \beta\) complexes through the BBB at a slower rate than LRP1-mediated transport. Alternatively, the apoE2 and apoE3 isoforms cleared \(\text{A} \beta\) through both LRP1 and the LDL receptor at a significantly higher rate than apoE4-\(\text{A} \beta\) complexes. This apoE4-specific effect resulted in 15- and 9-fold higher brain retention of apoE4-\(\text{A} \beta\)40/42, respectively, compared to free peptide (Deane, Sagare et al. 2008). These studies show that decreases in fibril formation seen in apoE null mice may be a result of enhanced transport of free \(\text{A} \beta\) across the BBB possibly due to the absence of the isoform-specific retention of \(\text{A} \beta\) by apoE in the brain.

\(\text{A} \beta\) can also be cleared from the brain by following proteolytic cleavage of APP that requires LDL receptor or LRP1-mediated transport from neurons to astrocytes or microglia (Van Uden, Mallory et al. 2002). It has been shown that intraneuronal deposition of \(\text{A} \beta\), particularly \(\text{A} \beta\)42, is toxic to neurons, particularly when it accumulates in multivesicular bodies, late endosomes, and lysosomes and contributes to lysosomal dysfunction (Billings, Oddo et al. 2005). By contrast, receptor-mediated export and delivery of \(\text{A} \beta\) from neurons to astrocytes and microglia likely represents a more efficient and functional pathway to clear and degrade \(\text{A} \beta\) in brain (Koistinaho, Lin et al. 2004; El Khoury and Luster 2008). One attractive hypothesis that requires more testing suggests that when astrocytes, microglia, and even smooth muscle cells lining the BBB become overloaded with \(\text{A} \beta\) and exceed the capacity of this clearance pathway, the \(\text{A} \beta\) accumulates in the extracellular space and CSF leading to local \(\text{A} \beta\) aggregation, amyloid plaque formation and subsequent cellular toxicity (Bu 2009). Although the apoE4 is associated with increased risk of cerebral amyloid angiopathy among carriers and appears to alter the \(\text{A} \beta\)40/\(\text{A} \beta\)42 ratio in any amyloid mouse model (Fryer, Simmons et al. 2005), it remains unclear exactly what role apoE isoforms may have in facilitating or preventing the receptor-mediated uptake and lysosomal degradation of \(\text{A} \beta\) in glial cells.

### 1.3.6 Tau protein and apoE

Tau is a protein that is integrally bound to cellular microtubules acting to stabilize the tubules in brain tissue. Hyperphosphorylation of tau results in the formation of paired helical filaments that then lack their microtubule stabilizing property. Hyperphosphorylated tau is the primary component of pathological neurofibrillary tangles and is toxic to neurons. Interestingly, tau appears to be required for \(\text{A} \beta\)-induced neuronal dysfunction, as demonstrated by studies in which a reduction in tau in an amyloid mouse model prevented \(\text{A} \beta\)-dependent cognitive impairments in an otherwise severe AD background (Roberson, Scearce-Levie et al. 2007). However, whether hyperphosphorylated tau is a primary cause of AD-associated dementia or simply a marker for the disease remains unclear since it is equally plausible that the destabilization of microtubules by hyperphosphorylated tau interferes with cognition independent of or downstream from the pathological induction of AD. Transgenic overexpression of apoE4 in mice causes an increase in the phosphorylation of tau in neurons but not astrocytes,
suggesting a neuron specific effect (Tesseur, Van Dorpe et al. 2000; Brecht, Harris et al. 2004). However, the pathophysiological significance of this finding is unclear since neurons do not express apoE except following injury (Xu, Gilbert et al. 1999; Aoki, Uchihara et al. 2003), possibly implying that the contribution of apoE to the formation of hyperphosphorylated tau in neurons is limited to conditions of excess stress or cellular damage.

It has been shown in vitro that apoE3 readily interacts with unphosphorylated tau, whereas apoE4 binds much more weakly, but it has not been shown that either full-length isoform binds strongly to phosphorylated tau (Strittmatter, Weisgraber et al. 1994). It also remains unclear how apoE and tau, normally partitioned to distinct subcellular locations, might come to physically interact as they have been shown to do under pathological conditions. One hypothesis is that the proteolytic cleavage of apoE (observed in human and mouse AD brains) generates C-terminal truncation fragments that can more freely dissociate in the cytosol and interact with tau (Brecht, Harris et al. 2004). It should also be noted that receptor- and lipid-binding truncation fragments of apoE4 have been demonstrated to act together to cause neurotoxicity and mitochondrial dysfunction, which suggests that the proteolytic fragments may have enhanced toxicity and intracellular activity compared to the intact protein (Chang, ran Ma et al. 2005). However, the in vivo relevance of these apoE4-dependent interactions and toxicity require further investigation.

1.4 ApoE Dynamic Functions

1.4.1 Lipoprotein Stabilization

The predominant shared function among the apolipoprotein family is to stabilize lipoprotein particles by providing a barrier function to shield and solubilize the hydrophobic lipids and lipophilic payloads. Even prior to the full elucidation of the LDL receptor pathway, it was appreciated that the protein components of lipoprotein particles were important not just for the structural stability of plasma particles but also for their ability to contribute to the metabolism of lipoproteins (Skipski 1972). This biological activity inherent to apolipoproteins was very elegantly shown in Brown and Goldstein’s seminal LDL receptor binding competition assays using \(^{125\text{I}}\)-labeled lipoproteins to show that VLDL and LDL (both apoB containing lipoproteins) could efficiently compete for receptor binding while HDL could not (Goldstein and Brown 1974). Shortly thereafter, it was shown that a unique subset of HDL (termed HDLc) containing an “arginine-rich” protein but lacking apoB could effectively compete for LDL receptor binding (Mahley, Weisgraber et al. 1975; Bersot, Mahley et al. 1976). This “arginine-rich” protein would turn out to be the protein now known as apoE and the shared sequence between apoE and apoB was soon identified and would form the basis for the identification of the putative LDL receptor recognition sequence contained in the two proteins. Ever since these early studies, the factors that contribute to the interaction between apoB and apoE ligands and the LDL receptor have been vigorously investigated in order to advance the understanding of receptor-mediated cholesterol regulation. There are now several known determinants that modulate ligand-receptor interaction between apoE and the LDL receptor.

1.4.2 Interaction with Lipid
Of the two apoE domains, the C-terminal domain has a higher demonstrated affinity for binding lipid (especially residues 244-272) (Dong, Wilson et al. 1994), yet it is well established that the N-terminal domain can also bind lipid to induce a conformational change that is required for LDL receptor interaction. In early studies to characterize the “arginine rich” apoE, it was noticed that purified HDLc particles had a 10-100 fold greater receptor binding affinity compared to LDL, implicating apoE as a major LDL receptor ligand (Innerarity and Mahley 1978; Innerarity, Pitas et al. 1979). This receptor binding activity remained after partial lipid delipidation of the particles, but complete delipidation abolished all receptor interaction. This was the first suggestion that apoE required lipid or lipoprotein association to become LDL receptor-active. It has since been shown that the isolated C-terminal domain markedly increases its α-helical content upon lipid binding, whereas lipid association with the N-terminal domain is accompanied by a smaller overall change in α-helicity, suggesting instead that a tertiary structural alteration occurs to accommodate LDL receptor interaction. Additionally, it has been shown that the isolated 10 kDa C-terminus of apoE shows higher affinity binding to lipid emulsions compared to the full-length protein indicating that the C-terminal domain dominantly regulates the lipid binding of the full-length protein (Saito, Dhanasekaran et al. 2001), which is consistent with similar studies proposing that the C-terminus dictates lipoprotein association of this protein (Westerlund and Weisgraber 1993).

In an effort to understand the lipization requirements for receptor activity, Innerarity et al. created reconstituted particles by combining apoE and the phospholipid dimyristoyl phosphotidylcholine (DMPC) to create particles that were designed to mimic the isolated HDLc molecules (Innerarity, Pitas et al. 1979). These synthetic particles were efficiently taken up and degraded by cells leading to the conclusion that these particles conferred upon apoE the conformational changes necessary for receptor binding activity. Some differences in the overall structure of apoE bound to spherical versus discoidal particles have been noted including circular dichroism studies and NMR analysis following 13C-formaldehyde-induced reductive labeling of Lys residues and subsequent lipid binding (Lund-Katz, Weisgraber et al. 1993). HDLc-bound apoE showed 10% less α-helical content while the discoidal particles displayed greater Lys microenvironment heterogeneity, suggesting that the structural organization of apoE on the two particles is distinct. While it is hypothesized that the structural restraints are greater for apoE molecules that circumscribe the periphery of a discoidal particle compared to binding a spherical surface, it should be noted that the presentation of the LDL receptor recognition sequence must be only subtly different because multiple studies have noted nearly identical receptor binding affinity when comparing spherical and discoidal apoE particles (Innerarity and Mahley 1978; Innerarity, Pitas et al. 1979; Lund-Katz, Weisgraber et al. 1993).

The marked conformational flexibility of apoE has been the focus of much research as it represents a key to understanding the physiologically relevant functioning of the protein in relation to cholesterol transport, receptor binding, and disease association. Molecular dynamics studies of the apoE N-terminal domain have concluded that the domain has relatively weak tertiary compactness compared to proteins of a similar size (Prevost and Kocher 1999). This led to the conclusion that the apoE N-terminus (like other related exchangeable apolipoproteins) displays more molten globule-like characteristics.
than other globular proteins, including low cooperativity, non-two-state unfolding, high polypeptide mobility and weak tertiary stability between \( \alpha \)-helical stretches.

1.4.3 Lipid Associated Peptide Analogs

The study of isolated apoE-derived peptides has provided an efficient way to study the behavior of specified regions of the protein that are important for their specified biological activity. These studies have advanced knowledge of the structural changes required for apoE lipid-mediated conformational change as well as provided peptide analogs that mimic the ability of full-length apoE to lower plasma cholesterol levels (Mahley, Weisgraber et al. 1989). One of the first attempts to isolate peptides of apoE was by Sparrow \textit{et al.} in which the authors created four peptides of varying lengths within residues 129 and 169 and measured their ability to stably associate with DMPC lipid (Sparrow, Sparrow \textit{et al.} 1985). Only the longest peptide apoE (129-169) formed high molecular weight complexes and these particles were shown by CD to have increased \( \alpha \)-helical content (56\%) compared to unlipidated peptide.

The work of Dyer and Curtiss demonstrated that a peptide containing apoE residues 141-155 prevented the binding, internalization, and degradation of \(^{125}\text{I}\)-labeled LDL in fibroblasts and a human monocytic cell line (THP-1) (Dyer and Curtiss 1991; Dyer, Smith \textit{et al.} 1991). In both cell types, the peptide monomer was unable to prevent the degradation of LDL while a linear tandem repeat of these residues could, although a 100-200-fold molar excess was required to show the same effect as native LDL. From these studies, it was unclear whether LDL competition was due to free or LDL-associated peptide species, though the authors contended that high affinity binding was associated with the presence of multiple copies of the receptor binding region in close proximity because a linear trimer of residues 141-155 was 20 times more potent than the dimer in the competition experiment. CD analysis showed a random coil secondary structure for the monomer but nearly 70\% \( \alpha \)-helical structure for the dimer suggesting that this secondary structure is required for receptor binding (Dyer, Cistola \textit{et al.} 1995). However, when a longer linear peptide (129-162) was tested, despite showing similar \( \alpha \)-helical content to the dimer, receptor-binding activity was minimal. Additionally, it was shown that for these molecules to have a significant amount of receptor activity, either two helices in a single sequence or a dimer of receptor binding helices was required (Dyer and Curtiss 1991; Dyer, Cistola \textit{et al.} 1995). From this observation, it was proposed that the dimer was able to bind to the LDL receptor because the two tandem sequences mimic the interaction of multiple copies of apoE with the ligand binding domains of a single receptor protein. Thus, it was suggested that \( \alpha \)-helicity alone is insufficient to promote receptor binding, and that the critical positioning and density of positive charges over a specific area may be a key component of binding.

With the observation that these synthetic peptides displayed receptor binding activity that was an order of magnitude lower than that of full length apoE, modifications were made to the peptide to increase \( \alpha \)-helicity and lipid binding stability in hopes of promoting increased receptor activity (Mims, Darnule \textit{et al.} 1994). Peptide lipophilicity was increased by introducing an acyl or up to two alkyl groups on the N-terminus to increase lipid binding strength and increase the peptide \( \alpha \)-helicity. The alkylated peptide showed a
6-fold increased LDL degradation of $^{125}$I-LDL, increasing the LDL receptor binding affinity to nearly the same level as native apoE.

Because of lack of detailed structural information for the C-terminal domain and the lack of defined electron density for extremities of the N-terminal domain, peptide systems have proved useful for probing the characteristics of these regions using a simplified system. Raussens et al. utilized a purified peptide containing apoE residues 126-183 to examine the LDL receptor active sequence in isolation (Raussens, Mah et al. 2000). In the aqueous environment, the peptide was largely unstructured as expected, yet helical content increased to between 68-69% in response to titration of a lipid-mimicking cosolvent, trifluoroethanol (up to 50%) or purified dodecylphosphocholine (DPC) (up to 12.9 mM) detergent and displayed 80% helical content upon association with dimyristoyl phosphotidylglycerol (DMPG) lipid. This peptide showed high lipid binding activity in an in vitro phospholipid clearance assay and showed similar LDL receptor binding activity compared to full-length apoE NT. Solution NMR spectroscopy was used to solve the structure of the peptide in the presence of 50% TFE and micelle-forming DPC detergent (Raussens, Slupsky et al. 2002; Raussens, Slupsky et al. 2003). In the presence of 50% TFE, apoE (126-183) is predominantly helical stretching from residues Thr130 to Glu179, making the helical length 15 residues longer than that determined by the lipid-free x-ray structure. Longitudinal and transverse relaxation and heteronuclear NOE measurements by NMR confirmed the central rigidity due to helix formation with slightly more flexible regions within the flanking regions of the peptide. Backbone traces of the helical peptide showed three distinct clusters of superimposable low-energy structures, each with a different degrees of helical backbone curvature but with strong opposing hydrophobic and hydrophilic helix faces. The variable flexibility was proposed to allow apoE to bind with different radii of curvature lipoprotein particle surfaces.

Several studies have proposed that helical extension of apoE residues 153-178 upon lipid binding provides additional surface area for promoting binding interaction with the LDL receptor. In the structural analysis and elucidation of the apoE(126-183) using NMR spectroscopy, it was shown that residues 165-179 transitioned from unstructured to $\alpha$-helical to present a highly positive electrostatic surface in the presence of lipid (Raussens, Slupsky et al. 2002; Raussens, Slupsky et al. 2003). This increased charge potential upon lipid interaction was postulated to provide additional surface for interaction with LDL receptor ligand repeats (L1-L7), possibly potentiating a stronger interaction between apoE and the LDL receptor than would by hypothesized from helix bundle opening alone (Raussens, Slupsky et al. 2003). Distance estimates placed the length of lipid-bound apoE (126-183) close to the length of two LDL receptor ligand repeats separated by a short linker, suggesting that the distance between the putative LDL receptor recognition sequence (residue 136-150) and arginine 72 is structurally relevant to receptor interaction (Raussens, Slupsky et al. 2003). Comparisons of lipid-free and lipid-bound spin labeled side chains within residues 161-181 of apoE NT using electron paramagnetic resonance spectroscopy further confirmed that this region of the protein becomes more stably structured upon lipid association (Gupta, Narayanaswami et al. 2006). Although a high-resolution model of the lipid bound conformation of apoE remains unresolved, the above data are consistent with a model wherein C-terminal lipid binding initiation is followed by N-terminal reorganization such that the helix bundle unfolds and stably interacts with the
lipoprotein surface to present a highly charged surface (residues 130-183) for competent LDL receptor interaction.

1.4.4 Lipid Association on Reconstituted HDL Particles

Lipoprotein mimicking, reconstituted HDL (rHDL) particles formed by combining apoE with purified phospholipids of varying fatty acid chain length and phospholipid head group composition have become an extensively utilized model for efficient and predictable reproduction of the bioactive conformation of the protein. This model system allows for the controlled manipulation and study of the apoE conformational changes required for LDL receptor binding, making these particles a reliable tool for the study of how the protein performs its biological activity.

The canonical formulations use purified DMPC, palmitoyloleoyl phosphotidylcholine (POPC) or egg phosphotidylcholine (PC) (in which the predominant phospholipid is POPC) to create discoidal particles in which the protein is oriented around the periphery of a phospholipid bilayer (Innerarity, Pitas et al. 1979; Pitas, Innerarity et al. 1980; Sparrow, Sparrow et al. 1985). The exact conformation and orientation of apoE around the discoidal particles remains controversial yet consensus generally exists about the fact that apoE changes conformation to encircle the discoidal particle such that the hydrophobic residues of the lipid free helix bundle interact with the acyl chains of the phospholipid bilayer and shield them from the aqueous milieu (De Pauw, Vanloo et al. 1995; Raussens, Fisher et al. 1998; Lu, Morrow et al. 2000).

Several models have been proposed to explain the lipid-induced conformational change of apoE upon lipid binding (Figure 1-7). While evidence has been presented in favor of several models, the greatest support has come for two models, namely the “open conformation model” (Weisgraber 1994) and the “extended belt” conformations. In both of these models, the conformational change in apoE appears to be initiated by the bundle opening via the “hinge” region between helices 2 and 3 to stably interact with the boundary of the phospholipid disc such that the four main helices are perpendicular to the peripheral acyl chains and the hydrophobic residues face the interior of the bilayer. Fluorescence resonance energy transfer (FRET) studies have shown that interaction with DMPC results in increased separation between helices 1 and 3 that is consistent with the “hinge” hypothesis (Fisher, Narayanaswami et al. 2000). These open conformations expose the hydrophobic helical faces to directly interact with the acyl chains comprising the perimeter of the disc. This conformational change allows the surface-exposed hydrophilic residues to retain contact with the aqueous environment and effectively substitutes helix-lipid contact for the helix-helix interactions seen in the aqueous state.

The “extended belt” and “open conformation” models differ in their explanation of the overall apoE architecture upon lipid binding. In the “extended belt” conformation, the hinge between helices 2 and 3 initiates further unfolding to create a fully extended helical protein that wraps partially around the perimeter of the disc wherein a second molecule stacks in the same but opposing conformation to form a double belt around the periphery of the disc. In contrast, the “open conformation” model preferentially retains helical contact between the helix 1 and 2 and helix 3 and 4 pairs wherein the half-opened molecules wrap around the disc perimeter end to end to accommodate various diameter discs (Weisgraber 1994). Support for these models have come from a combination of FRET-based distance measurements (Fisher and Ryan 1999), Fourier transform infrared (FTIR) spectroscopy...
(Raussens, Fisher et al. 1998), and tryptophan depth penetration in lipid bilayer measures using the parallax method (Narayanaswami, Maiorano et al. 2004). An alternative, hybrid model that combines features of the “open” and “extended belt” conformation models was described using a FRET analysis. In these experiments, intermolecular FRET was observed between helix 3 of one molecule and helix 4 of a second apoE NT (and vice versa) in a model whereby two partially extended open conformation apoE molecules interlock to encircle the disc (Fisher, Narayanaswami et al. 2000). It should be noted that an alternative “picket-fence” model wherein anti-parallel 17-residue helices surround the disc and orient parallel to the lipid acyl chains has also been advanced (De Pauw, Vanloo et al. 1995). This model is difficult to reconcile with the known helical boundaries of the lipid-free crystal structure and the multiple studies that support the aforementioned conformation in which the helices are anti-parallel to the acyl chains.

It is known that alteration in the ratio of lipid to protein (ranging from 2:1 to up to 5:1 lipid to protein weight ratio) modulates the size distribution of the discoidal formulation and it has been shown that incorporation of anionic phospholipids into the discs inhibits LDL receptor binding (Yamamoto and Ryan 2007). It has been estimated that discs synthesized with a 2:1 lipid to protein weight ratio have a molecular weight of ~450-500 kDa and a protein to lipid molar ratio of ~200:1. This is consistent with a model in which the disc contains either three or four apoE NT per particle and ~600 DMPC molecules. Other hydrodynamic and electrophoretic characterizations have indicated that DMPC particles may have as many as six apoE NT molecules per particle and have a molecular weight approaching 600,000 Daltons. Both the “open” and “extended” models are consistent with a model where the total height of the disc is ~50 Å and the central helical region is ~35 Å wide. Using the “open” conformation model and assuming a helix diameter of 15-17 Å, three open molecules would cover an estimated 348 Å, which is in good agreement with electrophoresis studies that estimate disc circumference to be 345 Å.

A comprehensive study comparing particle size, apoE protein copy number and conformational parameters as they relate to changes in lipid composition and apoE size and isoform differences was reported (Schneeweis, Koppaka et al. 2005). Using purified DMPC, dimyristoylphosphotidylserine (DMPS), dipalmitoylphosphotidylcholine (DPPC), dipalmitoylphosphotidylserine (DPPS) and POPC lipids to create particles with a hydrodynamic radius of 52 Å by modifying lipid to protein ratio, Schneeweis et al. confirmed that, on average, discs contained three apoE NT or four full-length proteins with between 200-250 lipid molecules per particle. While no significant differences between particle architecture were seen between the apoE isoforms, differences in lipid order parameters and protein to lipid content were seen when comparing apoE to apoA1 (which has been shown to display a modified “belt” conformation) (Saito, Dhanasekaran et al. 2003; Schneeweis, Koppaka et al. 2005). These observations led to the conclusion that apoE can adopt one of two predominant conformations on discoidal particles, either the canonical “belt” formation around the disc perimeter or an alternative conformation where the apoE helices embed horizontally within the interfacial region of the bilayer and perturb the phospholipid head group organization (Schneeweis, Koppaka et al. 2005).

1.4.5 Lipid-Associated Crystal Structure

A model of lipid bound apoE that is distinctly different from any of the discoidal models has recently been advanced by studies examining full-length apoE4 bound to DPPC
using x-ray crystallography (Peters-Libeu, Newhouse et al. 2006; Peters-Libeu, Newhouse et al. 2007). The lipidated particles resolved to 10 Å show a markedly different “helical hairpin” conformation that displays global ellipsoidal particle architecture and contains two interlocking apoE4 molecules. Without clear delineation of the secondary structure at 10 Å resolution, a predominantly helical full length apoE4 molecule was modeled into the structure in such a way that an extended molecule doubled back on itself and folded into a curved, horseshoe-like conformation with a 310° axis of rotation. In the model, two of the horseshoe shaped proteins packed into an incomplete “toroid” containing DPPC lipid intercalated within the opposing and slightly rotated apoE molecules to form an ellipsoidal space-filling model. In further support of the model, strategically placed electron paramagnetic resonance (EPR)-sensitive labels were attached to cysteines introduced by mutagenesis and probed for their relative side chain dynamics at the various positions along the helical hairpin (Hatters, Voss et al. 2009). The use of the purified DPPC lipid was unique to this study, and despite showing that the particles were LDL receptor competent and displayed distinctly different morphology compared to DMPC bound rHDL discs by electron microscopy, no further explanation was made as to whether the unique particle size, shape, and geometry was attributable to the lipid or the apoE4 isoform.

1.5 Intein-mediated Expressed Protein Ligation

Since the report of first known protein splicing element, termed an intein (Perler, Davis et al. 1994), found within the VMA1 gene of the budding yeast Saccharomyces cerevisiae (Hirata, Ohsumi et al. 1990; Kane, Yamashiro et al. 1990), numerous other inteins have been found in eubacteria, archaebacteria, and unicellular eukaryotic organisms (Perler 2000). Inteins are protein domains that become liberated from the precursor protein following intracellular, post-translational splicing event (Davis and Jenner 1995; Perler, Olsen et al. 1997; Perler, Xu et al. 1997; Shao and Kent 1997). An intein domain is comprised of three major domains, N- and C-terminal splicing regions and an optional homing endonuclease domain (Xu and Evans 2001) that is required for the genetic mobility of the intein-containing gene (Gimble and Thorner 1992). The inteins vary in size from a few to several hundred amino acid residues and contain highly conserved flanking splice junctions (Pietrokovski 1994; Perler, Olsen et al. 1997; Pietrokovski 1998) with otherwise high variable central regions. Nearly all inteins contain Ser or Cys at the N-terminal end and commonly terminate with a His-Asn pair followed by highly conserved Ser, Thr or Cys residues at the first flanking, or extein, residue of the C-terminal splice site (Perler 2000). The conserved Ser, Thr, and Cys residues at both splice sites are directly involved in the multi-step catalysis that generates the free intein and rejoin the N- and C-extein fragments (Xu and Evans 2001). The entire splicing reaction is self-catalyzed (and does not require a cofactor or ATP) in three conserved steps, generation of a thioester by an acyl rearrangement, transesterification, and cyclization of an Asp residue, followed by a spontaneous bond rearrangement to recreate a natural peptide bond (Chong, Shao et al. 1996; Xu and Perler 1996; Chong, Mersha et al. 1997).

It was the identification of these catalysis steps and the residues directly participating in cleavage and formation of peptide bonds that led to the rapid development of in vitro applications that incorporated the intein protein chemistry. The system was
found to have significant flexibility after it was noted that inteins bear little sequence homology in the extein regions and that replacement of foreign sequence in the extein region had little bearing on splicing or cleavage efficiency (Perler 2000). These observations led to the engineering and design of inteins as fusion partners for protein purification (Chong, Mersha et al. 1997; Mathys, Evans et al. 1999; Wood, Wu et al. 1999; Xu and Evans 2003). With only slight modifications it quickly followed that proteins purified by thiol-induced, intein-mediated cleavage and containing a C-terminal thioester could be ligated to N-terminal cysteine containing protein fragments by a process known as expressed protein ligation (EPL) (Evans, Benner et al. 1998; Evans, Benner et al. 1999; Xu and Evans 2001) (or in the case of two small synthetic peptides, native chemical ligation (NCL) (Dawson, Muir et al. 1994; Tam, Lu et al. 1995; Muir, Dawson et al. 1997)). EPL methodology has now been successfully applied to systems for incorporation of non-coded amino acids into protein sequences (Ayers, Blaschke et al. 1999), synthesis of cytotoxic proteins (Evans, Benner et al. 1998; Perler and Adam 2000), segmental stable isotope labeling of proteins for NMR analysis (Xu, Ayers et al. 1999; Camarero, Shekhtman et al. 2002; Romanelli, Shekhtman et al. 2004; Muralidharan, Dutta et al. 2006), incorporation of site-specific post-translational protein modifications (Kinsland, Taylor et al. 1998; Roy, Allen et al. 1999) and the addition of strategically placed fluorescent probes to create biosensors (Cotton and Muir 2000). Additional applications include the use of trans-splicing inteins to create head-to-tail ligation polymers and cyclized proteins in living cells (Evans, Benner et al. 1999; Iwai and Pluckthun 1999) and the use of a split intein containing flanking foreign sequences that become joined upon assembly and trans-splicing of the intact intein.

1.6 Specific Aims

ApoE is an exchangeable apolipoprotein that plays a key role in lipoprotein metabolism in the brain and peripheral circulation. The primary contribution apoE makes to this process is its ability to bind cell surface receptors and promote the clearance of cholesterol-containing lipoprotein particles from the circulating pool. While apoE is a highly soluble protein, an obligate requirement of its receptor binding activity is lipid association, suggesting that a significant structural reorganization must occur prior to the adoption of this bioactive state. Elucidation of the lipid-bound structure of apoE, particularly the amino terminal domain in which the receptor binding potential resides, is likely to provide significant insight into the repertoire of structures that exchangeable apolipoproteins are capable of adopting. In order to delineate the molecular level detail of this process, an EPL methodology was adopted that provides a way to monitor the lipid-induced conformational changes in apoE using NMR spectroscopy that is conducive to the introduction of free lipid or lipid-associated protein complexes into the system. With its significant structural flexibility and high stability upon disruption and refolding, apoE NT makes for an ideal protein to study by EPL and segmental isotope labeling. The specific aims undertaken in pursuit of this thesis project are summarily outlined below:

1. Examined and tested an apoE NT helix bundle helix-to-turn (HT) mutant protein for its modulation of lipid binding properties. The modified protein was analyzed for significant
structural changes compared to wild-type protein and assessed for its structural integrity and helix bundle architecture, LDL receptor binding activity, and lipid substrate association and solubilization properties.

2. Established a robust EPL system using a novel expression and purifications system for the generation of a N-terminal cysteine-containing peptide that was necessary for competent protein ligation reactions. The reaction conditions were tested in controlled experiments to determine the optimum conditions required for all ligations that preceded these tests.

3. Refined the above ligation system for the production of an intact apoE NT protein created from two separate fragments using EPL. The independent fragment expressions and purifications were established that produced high-yield, EPL-capable protein fragments that were then ligated to produce an intact protein that was examined for its structural and functional properties as compared to wild-type protein.

4. Modified the apoE NT EPL system for the production of stable segmental isotope labeled apoE NT for examination using NMR spectroscopy. The segmental labeled protein was created and tested for functional and structural integrity and examined using NMR in lipid-free and lipid-associated conditions to probe the functional lipid-induced conformational change within the N-terminal domain of apoE.

5. Adapted the segmental isotope labeled EPL protocol for the production of triple-labeled apoE NT for use in future NMR experiments to further probe the structural detail of the changes that apoE NT undergo upon interaction with lipid and the LDL receptor.
1.7 Figures

**Figure 1-1. Amino acid sequence of apoE.** The full sequence of apoE residues 1-299 containing the N-terminal (blue) and C-terminal domains (orange). The α-helices of the N-terminal domain as defined by the x-ray structure are underlined and the isoform-specific amino acid substitutions (red) at positions 112 and 158 (apoE2=Cys112,Cys158, apoE3=Cys112, Arg158, apoE4=Arg112,Arg158) are noted.
Figure 1-2. X-ray crystal and NMR structures of apoE3 NT. Cartoon diagram of the x-ray (left) and NMR (right) structures of the lipid-free N-terminal helix bundle of apoE containing residues 23-164 and 1-183 respectively. The primary α-helical segments and the putative LDL receptor recognition sequence are noted. PDB codes 1LPE and 2KC3 for the x-ray (Wilson, Wardell et al. 1991) and NMR (Sivashanmugam and Wang 2009) structures respectively.
Figure 1-3. The LDL receptor pathway. A diagram of the cellular uptake pathway mediated by the LDL receptor elucidated by Brown and Goldstein (Brown and Goldstein 1986; Goldstein and Brown 2009).
**Figure 1-4. LDL receptor family.** Members of the LDL receptor family to which apoE is a known *in vivo* ligand. Adapted from (Rebeck, LaDu et al. 2006)
Figure 1-5. Structure of LDL receptor repeat 5. X-ray crystal structure of the isolated LDL receptor ligand binding module 5 (LA5) solved at 1.7 Å resolution. The calcium ion is coordinated by conserved acidic residues that lie at the carboxy-terminal end of the domain and. PDB code 1AJJ (Fass, Blacklow et al. 1997).
Figure 1-6. Structure of LDL receptor extracellular domain at pH=5.3. X-ray crystal structure of the soluble extracellular domain of the LDL receptor at endosomal pH (Rudenko, Henry et al. 2002). Intramolecular interaction between the β-propeller domain and the LA4-LA5 ligand binding repeat pair is hypothesized to strengthen and drive off bound ligand upon acidification of the internalized receptor-ligand complex in the endosomal compartment.
Figure 1-7. Models of lipid bound apoE NT. Models detailing the lipid-induced conformational opening of the lipid-free apoE NT helix bundle (A) into the B) “open conformation” (Weisgraber 1994), C) extended belt and D) hybrid model (combining elements of the two models) (Fisher and Ryan 1999). The primary α-helical segments of the apoE NT domain are noted.
1.8 References


CHAPTER 2: Replacement of helix 1’ enhances the lipid binding activity of apoE3 N-terminal domain
The N-terminal domain of human apolipoprotein E (apoE NT) harbors residues critical for interaction with members of the low-density lipoprotein (LDL) receptor family. Whereas lipid free apoE NT adopts a stable four-helix bundle conformation, a lipid binding induced conformational adaptation is required for manifestation of LDL receptor binding ability. To investigate the structural basis for this conformational change, the short helix connecting helix 1 and 2 in the four helix bundle was replaced by the sequence -NPNG-, introducing a β-turn. Recombinant helix-to-turn (HT) variant apoE3 NT was produced in E. coli, isolated and characterized. Stability studies revealed an unfolding transition midpoint of 1.9 M guanidine HCl for HT-apoE3 NT. Hydrodynamic studies indicated lipid free HT-apoE3 NT exists as monomers and dimers in solution. Native PAGE showed that reconstituted HDL particles prepared with HT-apoE3 NT have a diameter in the range of 9 nm and possess binding activity for the LDL receptor on cultured human skin fibroblasts. In phospholipid vesicle solubilization assays, HT-apoE3 NT was more effective than wild type apoE3 NT at inducing a time-dependent decrease in dimyristoyl phosphatidylglycerol vesicle light scattering intensity. In lipoprotein binding assays, HT-apoE3 NT protected human LDL from phospholipase C induced aggregation to a greater extent than wild type apoE3 NT. The results indicate that a mutation at one end of the apoE3 NT four-helix bundle markedly enhances the lipid binding activity of this protein. In the context of full-length apoE, increased lipid binding affinity of the NT domain may alter the balance between receptor-active and -inactive conformational states, thereby influencing the flux of apoE-dependent clearance of intermediate density lipoproteins.

2.2 Results and Discussion

2.2.1 Design of HT-apoE3 NT

On the basis of studies with structurally related helix bundle apolipoproteins (Narayanaswami, Wang et al. 1999; Wang, Sykes et al. 2002), we hypothesized that residues comprising a short connector helix in apoE3 NT functions in recognition or initiation of lipid binding, leading to helix bundle opening and formation of a stable binding interaction. Using X-ray structure data as a guide (Wilson, Wardell et al. 1991), residues comprising helix 1' (S44-E-Q-V-Q-E-L-L-S53) were deleted and replaced by a sequence predicted to adopt a β-turn, -N-P-N-G- (Wilmot and Thornton 1990). We hypothesized that the resulting “helix-to-turn” variant apoE3 NT would adopt a stable solution conformation that retains the protein’s four-helix bundle molecular architecture and LDL receptor binding capability, yet would display altered lipid binding activity.

2.2.2 Bacterial expression and characterization of HT-apoE3 NT

WT- and HT-apoE3 NT were isolated from the supernatant fraction of bacterial cultures as described by Fisher et al. (Fisher, Wang et al. 1997). SDS- PAGE analysis revealed that the HT apoE3 NT has a faster mobility than wild type apoE3 NT, consistent with the changes introduced into the amino acid sequence (Figure 2-1). MALDI-TOF mass spectrometry of HT-apoE3 NT gave rise to a mass = 20,310 (calculated mass = 20,318) versus 21,191 for WT-apoE3 NT. The self-association properties of lipid-free HT apoE3 NT
in buffer were investigated by sedimentation equilibrium experiments performed in the analytical ultracentrifuge (Figure 2-2). At loading concentrations ranging from 0.25 to 0.50 mg/ml, samples were run at 19,000, 21,000 and 23,000 rpm. Eight sets of data collected were best fit to a monomer-dimer model (63% monomer and 37% dimer), corresponding to a global average MW of 28,841.

2.2.3 Stability properties of HT-apoE3 NT
Guanidine HCl-induced unfolding of WT- and HT-apoE3 NT were monitored by fluorescence spectroscopy. In the absence of guanidine HCl, WT-apoE3 NT gave rise to a tryptophan fluorescence emission wavelength maximum of 346 nm (excitation 280 nm) while the corresponding value for HT-apoE3 NT was 348 nm. Upon exposure to increasing concentrations of guanidine HCl, however, WT- and HT-apoE3 NT unfolding resulted in an ~8 nm red shift in the wavelength of tryptophan fluorescence emission maximum.

Plots of guanidine HCl concentration versus the percent maximal change in Trp fluorescence emission wavelength maximum (Figure 2-3) revealed a transition midpoint at 2.5 M for WT- apoE3 NT and a corresponding transition at 1.9 M guanidine HCl for HT-apoE3 NT.

2.2.4 Fluorescent dye binding
To evaluate the extent to which HT-apoE3 NT manifests altered exposure of hydrophobic regions of the protein, the effect of HT-apoE3 NT and WT-apoE3 NT on ANS fluorescence emission intensity was examined (Figure 2-4). In the absence of protein, ANS has a low quantum yield with an emission wavelength maximum of 515 nm (excitation 395 nm). Introduction of WT-apoE3 NT induced a 35 nm blue shift in ANS fluorescence emission wavelength maximum together with an enhancement in quantum yield. HT-apoE3 NT induced a similar blue shift in ANS fluorescence emission wavelength maximum as well as a greater enhancement in quantum yield. Given that these incubations contained equivalent molar amounts of apolipoprotein, the data indicate that HT-apoE3 NT possesses more ANS accessible hydrophobic binding sites than WT-apoE3 NT.

2.2.5 LDL receptor binding activity of the HT-apoE3 NT
To examine if HT-apoE3 NT is a functional ligand for the LDL receptor, 125I-LDL competition binding experiments were performed. Human skin fibroblasts were grown to confluence in lipoprotein deficient serum, transferred to 4 °C and incubated with 125I-LDL in the absence or presence of competitor ligands. 125I-LDL binding in the absence of competitor (labeled LDL alone) was taken as 100% (Figure 2-5). Inclusion of a 50- fold excess of unlabeled LDL (50 x unlabeled LDL) resulted in a marked decrease in 125I-LDL binding. Likewise, WT-apoE3 NT•DMPC was shown to be an effective competitor of 125I-LDL binding. Given that the level of reduction of 125I-LDL binding observed with HT-apoE3 NT•DMPC complexes at 50 µg/ml was similar to that observed with WT-apoE3 NT•DMPC, we conclude that the HT mutation does not compromise the LDL receptor binding activity of the protein.

2.2.6 DMPG vesicle solubilization studies
A hallmark feature of exchangeable apolipoproteins is their ability to solubilize certain phospholipid bilayer vesicles, transforming them into discoidal complexes. To
determine the effect of the amino acid sequence alteration introduced into HT-apoE3 NT on the kinetics of apoE3 NT lipid solubilization activity, apolipoprotein-dependent DMPG vesicle formation was monitored as a function of time (Figure 2-6). Whereas DMPG vesicle light scattering intensity did not change upon incubation at 23 °C in buffer alone, inclusion of WT-apoE3 NT induces a time dependent reduction in light scattering intensity (T1/2 = 75 s). By comparison, HT-apoE3 NT displayed a marked enhancement in lipid binding activity, inducing clearance of the turbid vesicle substrate with a T1/2 < 10 s.

2.2.7 Interaction with lipoproteins
To examine the ability of HT-apoE3 NT to bind spherical lipoproteins, human LDL was incubated with PL-C in the presence or absence of HT-apoE3 NT or WT-apoE3 NT. PL-C induced hydrolysis of LDL phosphatidylcholine generates diacylglycerol moieties that destabilize LDL structural integrity, leading to particle aggregation and sample turbidity development. In studies of this phenomenon Liu et al. (Liu, Scraba et al. 1993) showed that exchangeable apolipoproteins bind to PL-C modified LDL, preventing lipoprotein aggregation. In control incubations lacking exogenous apolipoprotein, PL-C induces a rapid increase in LDL sample turbidity (Figure 2-7). Whereas WT-apoE3 NT showed a limited ability to protect LDL from PL-C induced turbidity development, HT-apoE3 NT conferred nearly full protection. These studies were extended by evaluating the effect of apolipoprotein concentration on their ability to protection LDL from PL-C induced aggregation (Figure 2-8). Whereas WT-apoE3 NT was unable to fully protect LDL from lipolysis-induced aggregation at any concentration examined, HT-apoE3 NT was more effective, consistent with formation of a stable binding interaction. To verify that HT-apoE3 NT associated with PL-C treated LDL, the sample was subjected to density gradient ultracentrifugation and the proteins recovered in the LDL density range analyzed by SDS-PAGE. Whereas only apoB-100 was recovered in this fraction in incubations of LDL and HT-apoE3 NT in the absence of PL-C, incubations containing PL-C resulted in recovery of HT-apoE3 NT in the LDL density range.

2.3 Summary and Conclusions
An interesting and unique aspect of apoE function relates to the fact that it manifests LDL receptor binding activity only when lipid associated. Early studies showed that apoE conformational status affects clearance of triacylglycerol-rich lipoproteins (Gianturco, Gotto et al. 1983). It was observed that, although apoE is present, certain particles were receptor inactive. Krul et al. (Krul, Tikkanen et al. 1988) employed monoclonal antibodies to show that expression of specific apoE epitopes on lipoprotein particles correlates with LDL receptor binding ability. Indeed, lipolysis of VLDL induces a conversion in apoE conformation from a receptor inactive state into a receptor active state (Sehayek, Lewin-Velvert et al. 1991). When considered in light of structural data and localization of the LDL receptor recognition sequence to helix 4 in the NT domain, these observations are consistent with the concept that the conformational status of the NT domain modulates the receptor recognition properties of apoE. More specifically, a conformational transition in
the NT domain from its receptor inactive globular four-helix bundle into an "open" lipid-bound conformation is considered to be necessary and sufficient to confer receptor-recognition properties to the protein. Whereas factors responsible for inducing or preventing this conformational transition in vivo are not known, it may be anticipated that an increase in the proportion of apoE molecules whose NT domain adopts an open conformation would increase the flux of VLDL remnants toward endocytosis and away from pro-atherogenic LDL production.

Structural and biophysical data on full-length apoE have led to the concept that the CT domain mediates initial contact with lipoprotein surfaces, effectively anchoring the NT domain at the particle surface (Narayanaswami and Ryan 2000; Saito, Dhanasekaran et al. 2001). In this manner, depending on physiological conditions, the NT domain may exist in one of two alternate conformational states. Given that the NT domain is an independently folded structural element of apoE that, when lipid associated, possesses full LDL receptor binding activity, studies of this domain in isolation may provide insight into the conformational transition that occurs upon lipid interaction as well as factors that modulate lipid surface recognition and/or initiation of the lipid binding. Two mutually exclusive models exist that describe how the apoE NT four-helix bundle initiates contact with lipid surface binding sites.

On the basis of structural characterization studies, it has been proposed that a flexible segment, connecting helix 2 and 3 (residues 79-90, termed the 80s loop), initiates apoE NT interaction with lipid surfaces (Segelke, Forstner et al. 2000). In this model it is anticipated that the helix bundle opens by helix 1 and 4 moving away from helix 2 and 3, with the loop segments connecting helix 1 and 2 and helix 3 and 4 serving as "hinges". This hypothesis is based on factors including helix bundle topology, the flexibility of this region of the protein and the high concentration of acidic amino acids in this region. It is proposed that negatively charged side chains of glutamate residues are attracted to the quaternary amino group of phosphatidylcholine at the lipid surface, while the flexibility of this region facilitates the required conformational change.

At the opposite end of the globular helix bundle there exists a ten-residue helix (helix 1’) that connects helix 1 to helix 2. In the NT domain bundle structure, helix 1’ aligns perpendicular to the long axis of helix 1 and helix 2. It is proposed that opening of the molecule at this end of the bundle would occur by helix 1 and helix 2 moving away from helix 3 and helix 4, as depicted by Weisgraber (Weisgraber 1994). Interestingly, this segment of the protein is the most highly conserved region of apoE across species. Furthermore, a similar short helix motif, present in an invertebrate helix bundle apolipoprotein (Wang, Gagne et al. 1997; Wang, Sykes et al. 2002), has been shown to play a role in lipid binding activity. Site directed mutagenesis of this helix motif resulted in a variant protein that displayed defective lipid binding (Narayanaswami, Wang et al. 1999) leading to the suggestion that this helix functions in a manner similar to the "lid" segment of lipase enzymes (Dugi, Dichek et al. 1995), reorienting to expose the helix bundle interior upon contact with a lipid surface.

We observed that replacement of helix 1’ by an unrelated sequence predicted to form a β-turn, results in a variant apoE3 NT that has a slightly lower intrinsic stability than its WT counterpart. Despite this, HT-apoE3 NT still manifests stability properties that are
higher than other exchangeable apolipoproteins, including apoA-I, apoA-II, apoA-IV (Wetterau, Aggerbeck et al. 1988), apolipoporphin III (Ryan, Oikawa et al. 1993) or apoA-V (Beckstead, Oda et al. 2003). In addition to adopting a conformation that resists guanidine HCl induced denaturation, sedimentation equilibrium studies revealed that HT-apoE3 NT exists in solution as monomers and dimers, whereas WT-apoE3 NT was previously reported to be predominantly monomeric (Aggerbeck, Wetterau et al. 1988). When associated with DMPC, HT-apoE3 NT competes with 125I-LDL for binding to the LDL receptor on cultured human skin fibroblasts. Taken together, these data indicate that the HT mutation did not compromise the ability of this domain to adopt a stable solution conformation or interfere with its ability to function as a ligand for the LDL receptor. Studies of the lipid binding properties of HT-apoE3 NT, however, revealed an increased affinity for lipid surfaces compared to WT-apoE3 NT. The observed enhanced phospholipid vesicle solubilization activity and increased binding to PL-C treated LDL compared to WT-apoE3 NT, suggests the HT mutation may have exposed otherwise less accessible lipid binding sites in the protein. Indeed, fluorescent dye binding experiments, wherein it was observed that HT-apoE3 NT induces a larger ANS fluorescence enhancement than WT apoE3 NT, are consistent with increased exposure of hydrophobic sites on the protein.

When considered in the context of the mechanism whereby apoE NT initiates contact with lipid surfaces, the data support the model proposed by Weisgraber (Weisgraber 1994). On one hand, structural changes introduced at the end of the elongated helix bundle harboring helix 1’ seem to promote increased lipid binding ability by increasing exposure of otherwise sequestered hydrophobic sites in the protein. This is likely due to the smaller size of the sequence motif introduced to replace helix 1’, which may result in greater solvent accessibility of the bundle interior and, by extension, greater attraction to hydrophobic lipid surface binding sites. On the other hand, the phospholipid vesicle solubilization studies with DMPG showed a marked enhancement in the case of HT-apoE3 NT. This result may not be expected if the opposite end of the helix bundle (the 80s loop) is the site of lipid surface recognition. The observation that vesicle solubilization rates are much faster with the anionic phospholipid, DMPG versus the zwitterionic DMPC, is not consistent with initial binding via the 80s loop, a region with a high localized concentration of acidic amino acid side chains (Weers, Narayanaswami et al. 2001). At the same time, it is evident that further work will be required to elucidate the precise mechanism whereby the NT domain initiates contact with lipid surface to undergo the conformational transition that culminates in LDL receptor recognition.

An important future goal will be to evaluate whether the increased lipid binding activity of HT-apoE3 NT is maintained in the context of full-length apoE. It is conceivable that an NT domain with increased lipid binding activity will result in a greater proportion of lipoprotein associated full-length apoE molecules to adopt a receptor-active conformation via enhanced NT domain lipid interaction. Furthermore, it may be postulated that, by increasing the proportion of apoE molecules whose NT domains adopt a lipid bound receptor-active state, the flux of lipoprotein cellular uptake via apoE-dependent receptor-mediated endocytosis may be enhanced. In the case of VLDL metabolism, if a greater percentage of IDL are removed from circulation in this manner, less will be available for catabolism to atherogenic LDL.
2.4 Materials and Methods

2.4.1 Recombinant apoE and site directed mutagenesis

Production of recombinant wild type (WT) apoE3 NT domain in *E. coli* was performed as described by Fisher et al. (Fisher, Wang et al. 1997). Polymerase chain reaction dependent site directed mutagenesis was performed as described elsewhere (Narayanaswami, Szeto et al. 2001). Human low density lipoprotein (LDL) was obtained from Intracel (Frederick, MD).

2.4.2 Analytical procedures

Protein concentrations were determined by absorbance spectroscopy (280 nm) or the bicinchoninic acid assay (Pierce Chemical Co.) with bovine serum albumin as standard. SDS-PAGE was performed on 4 - 20 % acrylamide slab gels run at a constant 30 mA for 1.5 h. Gels were stained with Gel Code (Pierce Chemical Co.) stain according to the manufacturers instructions. Mass spectrometry was performed on a Bruker Autoflex MALDI-TOF instrument equipped with a SCOUT MTP ion source. Samples were spotted onto a Scout 384 plate using a matrix of sinapinic acid saturated in 30% acetonitrile/70% water/0.1% trifluoroacetic acid. Ions were accelerated at +20 kV and masses were detected in linear mode with Protein A used as external calibrant.

2.4.3 Fluorescence spectroscopy

Fluorescence spectra were obtained using a Perkin Elmer LS 50B luminescence spectrometer. For dye binding experiments, incubations were carried out in 400 µl 10 mM sodium phosphate buffer (pH 7.0) containing 1 mM 8-anilino-1-naphthalene sulfonate (ANS) (Stryer 1965) in the absence and presence of 5 µM WT-apoE3 NT or HT-apoE3 NT. Samples were excited at 395 nm (slit width 3 nm) and emission monitored between 405 and 600 nm (3 nm slit width). For guanidine hydrochloride unfolding experiments, samples were incubated overnight at given denaturant concentrations in order to attain equilibrium. Subsequently, the samples were excited at 280 nm and scanned from 300 – 375 nm (3.0 nm slit width).

2.4.4 Analytical ultracentrifugation

Sedimentation equilibrium experiments were conducted at 20 °C in a Beckman XL-I analytical ultracentrifuge using absorbance optics, as described by Laue and Stafford (Laue and Stafford 1999). Aliquots (110 µL) of the sample solution were loaded into six sector CFE sample cells, allowing three concentrations to be run simultaneously. Runs were performed at a minimum of three different speeds and each speed was maintained until there was no significant difference in r^2/2 versus absorbance scans taken 2 h apart to ensure that equilibrium was achieved. Sedimentation equilibrium data were evaluated using the NONLIN program, which employs a nonlinear least squares curve-fitting algorithm described by Johnson et al. (Johnson, Correia et al. 1981). The data set obtained at a protein concentration of 0.25 mg/ml at 19,000 rpm was omitted due to unexplained signal noise. The protein’s partial specific volume and the solvent density were estimated using the Sednterp program (21).
2.4.5 LDL receptor binding assay

Human skin fibroblasts were grown to approximately 60% confluence in the presence of DMEM with 10% fetal bovine serum (FBS). Fibroblasts were then grown to 100% confluence in DMEM with 10% lipoprotein-deficient serum. At confluence, cells were cooled on ice for 30 min, washed twice with phosphate-buffered saline (PBS) containing 1 mg/mL fatty acid-free albumin (FAFA), then incubated with DMEM containing 1 mg/mL FAFA, 2 µg/mL \(^{125}\text{I}\)-LDL and different amounts of receptor binding competitor for 2 h at 4 °C. The medium was removed, and the cells were washed five times with chilled PBS-FAFA and two times with chilled PBS. Cells were released from the surface of the dishes by incubation with 0.1 N NaOH for 1 h at 24 °C and cell-associated radioactivity was measured on a Cobra II Auto-Gamma Counter. Cell protein was determined by the bicinchoninic acid assay using bovine serum albumin as the standard. Competitor ligands were prepared by co-sonication of dimyristoyl phosphatidylcholine (DMPC) bilayer vesicles and a given apoE3 NT, resulting in formation of disk complexes. Native PAGE analysis revealed the presence of particles with a diameter in the range of 10-11 nm.

2.4.6 DMPG vesicle solubilization studies

Dimyristoyl phosphatidylglycerol (DMPG) bilayer vesicles were prepared by extrusion through a 200 nm filter as described by Weers et al. (Weers, Narayanaswami et al. 1999). Stock solutions of protein and lipid vesicles were prepared in 20 mM sodium phosphate, pH 7.0. Six hundred nmoles DMPG was incubated at 23 °C in a thermostated cell holder in the absence or presence of 5 nmoles apolipoprotein (sample volume = 400 µl). Sample right angle light Auto-Gamma Counter. Cell protein was determined by the bicinchoninic acid assay using bovine serum albumin as the standard. Competitor ligands were prepared by co-sonication of dimyristoyl phosphatidylcholine (DMPC) bilayer vesicles and a given apoE3 NT, resulting in formation of disk complexes. Native PAGE analysis revealed the presence of particles with a diameter in the range of 10-11 nm.

2.4.7 Lipoprotein binding assay

Human LDL was incubated for 90 min at 37 °C in the presence of Bacillus cereus phospholipase C (PL-C; 0.9 U per 100 µg LDL protein). Where indicated, apolipoprotein (0 to 400 µg per 100 µg LDL protein) was included in the reaction mixture. Incubations were conducted in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 2 mM CaCl\(_2\) in a total sample volume of 200 µl. Sample absorbance at 340 nm was determined on a Spectramax 340 microtiter plate reader.
2.5 Figures

**Figure 2-1. SDS-PAGE analysis of WT- and HT-apoE3 NT.** Proteins were electrophoresed on an 8 - 25 % acrylamide SDS slab gel and stained with Coomassie Blue. Lane 1) WT-apoE3 NT; Lane 2) HT-apoE3 NT; Lane 3) Molecular weight standards.
Figure 2-2. Sedimentation equilibrium analysis of HT-apoE3 NT. Protein was dissolved in 20 mM sodium phosphate, pH 7.0, and 100 mM sodium chloride and centrifuged at 19,000 rpm (circles), 21,000 rpm; (squares) and 23,000 rpm (triangles) at 20°C. Protein concentrations used were 0.50, 0.35 and 0.25 mg/ml for Sectors A, B and C, respectively. Lower panels: $r^2/2$ versus absorbance plots. Symbols represent measured data points, and solid lines represent the fit to a monomer-dimer model. Upper panels: Residuals obtained from fitting the measured data points to a two-species model. The random, nonsystematic distribution of the residuals indicates a good fit of the data to the model.
Figure 2-3. Guanidine HCl induced unfolding of apoE3 NT. Indicated amounts of guanidine HCl were added to each apolipoprotein in buffer (20 mM sodium phosphate, pH 7.4) and at each concentration the wavelength of maximum tryptophan fluorescence emission (excitation 280 nm) was determined. The results shown here assume a model in which the change in \( \lambda_{\text{max}} \) correlates linearly with the degree of denaturation by the chaotropic salt, which, while not always an accurate assumption, should be sufficient for comparing the ‘apparent’ fraction unfolded of the two apoE NT protein populations containing the same placement and number of fluorescent side chains and a similar overall fold architecture.
Figure 2-4. Effect of apolipoproteins on ANS fluorescence emission. ANS (1 mM) in 10 mM sodium phosphate, pH 7.0, was excited at 395 nm and emission was monitored from 405 - 600 nm. Curve a) ANS in buffer at pH 7.0; curve b) ANS plus 5 µM WT-apoE3 NT; curve c) ANS plus 5 µM HT-apoE3 NT
Figure 2-5. LDL receptor binding activity of apoE3 NT. Human skin fibroblasts were incubated with DMEM containing 1 mg/mL FAFA and 2 µg/mL $^{125}$I-LDL in the absence or presence of competitors at 4 °C for 2h. $^{125}$I-LDL binding to fibroblasts treated with serum free medium in the absence of competitor ligand (corresponding to 23,742 cpm/mg cell protein) was taken as 100% (bar 1). Binding in the presence of competitors is expressed as % of control. Incubations of cells with $^{125}$I-LDL were conducted with the following competitors: a 50-fold excess of unlabeled LDL; 50 µg WT-apoE3 NT DMPC complexes and 50 µg HT-apoE3 NT DMPC complexes. Values reported are the average of 3 determinations ± SD.
Figure 2-6. Effect of apolipoproteins on DMPG vesicle light scattering intensity. DMPG vesicles (600 nmoles phospholipid) were incubated in buffer at 24 °C at pH 7.0. Sample right angle light scatter intensity was monitored as a function of time. Curve a) DMPG vesicles in buffer; curve b) DMPG vesicles plus 5 nmoles WT-apoE3 NT and Curve c) DMPG vesicles plus 5 nmoles HT-apoE3 NT.
Figure 2-7. Effect of apolipoproteins on PL-C induced aggregation of human LDL. Human LDL (100 µg protein) was incubated at 37 °C in the absence (open circles) or presence of PL-C (0.9 units) with no apolipoprotein (filled circles), 100 µg WT-apoE3 NT (open squares) or HT-apoE3 NT (filled squares). Sample absorbance at 340 nm was determined after 90 min. Values represent mean ± SD (n = 3).
Figure 2-8. ApoE3 NT concentration-dependent protection of LDL against PL-C induced aggregation. Human LDL (100 µg) and PL-C (0.9 units) were incubated at 37 °C in the presence of specified amounts of WT-apoE3 NT (open circles) or HT-apoE3 NT (filled circles). Sample absorbance at 340 nm was determined after 90 min. Values represent mean ± SD (n = 3).
2.6 References


CHAPTER 3: Expressed protein ligation using an N-terminal cysteine-containing fragment generated from a pelB fusion protein
3.1 Introduction

Advances in expressed protein ligation (EPL) methods that permit specific introduction of unique modifications into proteins have facilitated protein engineering, structure-function and protein interaction studies. An EPL-generated hybrid exchangeable apolipoprotein has been constructed from recombinant fragments of apolipoprotein E (apoE) and apolipoporhin III (apoLp-III). A recombinant fusion protein comprised of human apoE N-terminal residues 1–111, a modified Saccharomyces cerevisiae intein and a chitin binding domain was subjected to 2-mercaptoethanesulfonic acid (MESNA) induced cleavage to generate apoE(1-111)-MESNA. A second fusion protein was comprised of a bacterial pelB leader peptide fused to a variant form of Galleria mellonella apoLp-III residues 1–91. The N-terminal pelB leader sequence directed the newly synthesized fusion protein to the E. coli periplasmic space where endogenous leader peptidase cleavage generated the desired N-terminal cysteine-containing protein fragment. The resulting apoLp-III fragment, which contained no sequence tags or tails, escaped the bacteria and accumulated in the culture medium. When cultured in M9 minimal medium, Asp1 to Cys apoLp-III(1–91) was produced in high yield and was the sole major protein in the culture supernatant. Ligation reactions with apoE(1–111)-MESNA yielded an engineered hybrid apolipoprotein. The results document the utility of the pelB fusion protein system for generating active N-terminal cysteine containing proteins for EPL applications.

3.2 Results and Discussion

3.2.1 Fragment production and characterization

A diagram depicting recombinant apolipoprotein fragment generation and EPL strategy is shown in Figure 3-1. ApoE(1-111)-MESNA was generated from MESNA induced, intein-mediated cleavage of an apoE•intein•CDB fusion protein. Replacing the commonly used thiol reducing agent dithiothreitol (DTT) with MESNA resulted in a stable adduct that remained covalently bound to the carboxy terminus of alanine 111. Ultimately, the MESNA moiety serves as a leaving group during EPL (Dawson, Muir et al. 1994; Xu and Evans 2001; Muir 2003). SDS-PAGE analysis verified that the column eluate was highly enriched in apoE(1-111) (Figure 3-2, panel A) while HPLC analysis gave rise to a single major peak with an observed molecular mass = 13,193. The molecular mass increment over that calculated from the amino acid composition of this protein fragment (13,024) is consistent with the presence of a MESNA adduct (MW = 164.2 Da).

The EPL substrate fragment containing a reactive N-terminal cysteine (Asp1 to Cys apoLp-III(1-91)) was expressed in E. coli as a pelB leader peptide fusion protein. The expressed fusion protein was directed to the periplasmic space where endogenous leader peptidase cleavage generated the desired apoLp-III fragment, which escaped the bacteria and accumulated in the culture medium (Dettloff, Weers et al. 2001). ApoLp-III(1-91) was isolated from the culture supernatant by a combination of gel permeation chromatography and reversed phase HPLC (Figure 3-2, panel B). Mass spectrometry of the isolated fragment yielded a molecular mass of 10,083, in good agreement with the calculated mass of 10,091 for Asp1 to Cys apoLp-III(1-91), confirming pelB leader peptide cleavage. Final protein yield was ~30-50 mg/L.
3.2.2 Expressed protein ligation

To determine the suitability of apoE(1-111)-MESNA and Asp1 to Cys apoLp-III(1-91) protein fragments for EPL, incubations were conducted as a function of time (Figure 3-3). SDS-PAGE analysis revealed a time-dependent accumulation of the expected 23 kDa hybrid apolipoprotein product over the course of 48 h. Appearance of the hybrid apolipoprotein product correlated with disappearance of apoE(1-111)-MESNA and apoLp-III(1-91), consistent with a substrate-product relationship. Whereas EPL reactions often require inclusion of chaotropic agents or detergents to maintain substrate fragment solubility (Valiyaveetil, MacKinnon et al. 2002; Muralidharan, Cho et al. 2004; Romanelli, Shekhtman et al. 2004), apoE(1-111)-MESNA and apoLp-III(1-91) fragments (as well as the hybrid apolipoprotein product) remain fully soluble in phosphate buffered solution at the millimolar concentrations employed. This finding is in agreement with the known high solubility of intact apoE N-terminal domain and full-length apoLp-III (Wetterau, Aggerbeck et al. 1988; Wang, Sykes et al. 2002). Based on SDS-PAGE and mass spectrometry analysis, we estimate that, after 48 h, ligation product yield is ~30%. The observed time dependent increase in hybrid apolipoprotein product agreed with previous reports suggesting that EPL product formation, depending on the size and protein fragment composition, are maximal between 5 and 48 h (Hackeng, Griffin et al. 1999; Xu, Ayers et al. 1999; Romanelli, Shekhtman et al. 2004).

In an effort to optimize EPL reaction parameters using this system, further experiments were performed to assess the effect of ligation reaction temperature, pH and thiol agent concentration (Figure 3-4). While most studies suggest EPL product formation is optimal at 37 °C, isolated reports suggest that room temperature or 4 °C, with longer incubation times, improves product yield (Dawson, Muir et al. 1994; Evans, Benner et al. 1998; Xu, Ayers et al. 1999). Whereas hybrid apolipoprotein product formation was low at 4 °C, increasing the temperature to 22 °C resulted in a dramatic increase in product formation (panel A). Further increases in reaction temperature gave rise to incremental increases in hybrid apolipoprotein product formation.

Using a Tris-maleate buffer system, the effect of solution pH on EPL product formation was examined (panel B). Hybrid apolipoprotein formation was lowest at pH 6.0 and increased steadily with increasing solution pH, reaching a maximum at pH 9.0. An apparent decrease in product formation at pH 9.5 suggests a slightly basic pH may be optimal for this EPL reaction. Others have reported that pH, which affects the chemoselectivity of peptide bond formation during ligation, is optimal within a range centered at pH = 8.0 (Muralidharan and Muir 2006).

Studies reporting variable ligation efficiencies using different thiol cofactors stress that choice of thiol agent is critical for ligation reactions (Camarero, Shekhtman et al. 2002; Valiyaveetil, MacKinnon et al. 2002; Johnson and Kent 2006), independent of the thiol employed during intein-mediated fusion protein cleavage. Comparison of ligation efficiency in incubations supplemented with different thiol agents including DTT, MESNA, thiophenol and 1,2-ethanedithiol were examined for hybrid apolipoprotein EPL (panel C). As previously documented, DTT resulted in low ligation efficiency, reportedly due to instability of the DTT adduct as a function of time (Chong, Mersha et al. 1997; Evans, Benner et al. 1998). On the other hand, ligation reactions supplemented with MESNA,
thiophenol, 1,2-ethanediethiol or a combination of these agents increased hybrid apolipoprotein product formation. The effect of MESNA concentration on product formation was also evaluated (panel D). Compared to the control incubation in which added MESNA was not present, similar product yields were observed between 1 % and 5 % MESNA. The data confirm that MESNA is not only required as a chemical leaving group during EPL but also facilitates ligation reaction progress, most likely by maintaining a reducing environment and preventing unwanted oxidation or hydrolysis.

The effect of substrate concentration on ligation product yield was investigated by varying the amount of either apoE(1-111) or apoLp-III(1-91) in 25 µL reactions (Figure 3-5). As expected, in the absence of either substrate protein fragment, no reaction product was detected. When the amount of apoLp-III(1-91) substrate was increased relative to apoE(1-111)-MESNA, a positive correlation with hybrid apolipoprotein product formation was observed up to equimolar protein concentrations (panel A). However, when the amount of apoE(1-111)-MESNA in the incubation was increased relative to apoLp-III(1-91), maximal product formation was observed slightly below equimolar concentration, indicating that apoE(1-111)-MESNA concentration dependent ligation was saturable. This suggests that apoE(1-111) is partially limiting with respect to ligation efficiency, perhaps due to the presence of MESNA deficient substrate protein.

### 3.3 Summary and Conclusions

The increasing utilization of EPL methodologies for the design of specifically modified proteins has demanded new techniques for manipulating and producing thioester and N-terminal cysteine EPL reaction substrates. The present study employed a novel recombinant system for facile production of an EPL-active, N-Cys protein fragment in high yield. The system described represents an improvement over conventional in vitro protease catalyzed reactions since cleavage occurs in vivo concurrent with protein expression. Using standard protein isolation techniques, the desired apolipoprotein fragment was purified directly from a bacterial culture supernatant without the need to modify or protect the active cysteine. Although other proteins may not escape the bacteria following pelB leader peptide cleavage, localization to the periplasmic space is known to increase protein folding efficiency (Missiakas and Raina 1997; Sone, Kishigami et al. 1997). Additionally, pelB fusion protein expression is readily adaptable to growth in M9 minimal media, permitting stable isotope enrichment for high-resolution structural and biophysical studies (Valiyaveetil, MacKinnon et al. 2002; Muralidharan, Cho et al. 2004). Thus, the method described offers a valuable approach for generating EPL active, N-Cys protein fragments for use in ligation reactions, simplifying downstream processing and increasing yield. The establishment of methods to generate hybrid apolipoproteins provides new opportunities for protein engineering and structure function analysis of this and other biologically important protein families.

### 3.4 Materials and Methods

#### 3.4.1 Preparation of apoE(1-111)
Human apoE(1-111) was cloned into the pTYB1 vector (New England Biolabs) and expressed in *E. coli* ER2566 cells as an *S. cerevisiae* vacuolar ATPase subunit, intein-chitin binding domain (CBD) fusion protein. To facilitate optimal intein-mediated fusion protein cleavage (Hackeng, Griffin et al. 1999), valine 111 was mutated to alanine using the QuikChange method (Stratagene) according to the manufacturer’s instructions. Expression and purification procedures for apoE(1-111) followed standardized protocols previously established for generating intein-mediated thioester-adducted proteins (Xu and Evans 2001). Briefly, saturated overnight cultures were inoculated into 2xYT media containing 50 µg/ml ampicillin, grown to OD$_{600}$= 0.6 and induced with 1 mM isopropyl thiogalactopyranoside (IPTG). After 6 h at 30 ºC the cells were pelleted by centrifugation (8000 g for 15 min), solubilized with buffer A (20 mM Tris, 150 mM NaCl, 1 mM EDTA, pH=8.0) containing 1% Triton X-100 and stored at -20°C. Dissolved cell pellets were combined, passed through a microfluidizer, sonicated and centrifuged at 12000 g for 20 min. Isolated clarified cell extract was passed over a chitin bead column pre-equilibrated with buffer A containing 1% Triton X-100. The column was washed with 10 column volumes of detergent-free buffer A and fusion protein cleavage induced by addition of 2-mercaptoethanesulfonic acid (MESNA) to a final concentration of 60 mM. Flow was arrested for 16-24 h at 22 ºC and eluted with 2 bed volumes of buffer A containing 5 mM MESNA. The sample was dialyzed against deionized H$_2$O, lyophilized and stored at -20 ºC. ApoE(1-111)-MESNA was further purified by semi-preparative C$_8$ reversed-phase high performance liquid chromatography on a Perkin-Elmer Series 200 HPLC.

3.4.2 Preparation of apoLp-III(1-91)

The coding sequence for *G. mellonella* apoLp-III(1-91) was cloned into the pET22b plasmid (Novagen) directly adjacent to a vector encoded pelB leader sequence. Site directed mutagenesis of aspartate 1 to cysteine was performed using the QuikChange method. Expression and purification of Asp1 to Cys apoLp-III(1-91) was carried out as previously described for the wild type fragment (Dettloff, Weers et al. 2001; Dettloff, Niere et al. 2002). Briefly, saturated overnight cultures were inoculated into M9 media supplemented with 13.3 mM glucose, 0.1 mM CaCl$_2$, 2 mM MgSO$_4$ and 50 µg/ml ampicillin. At OD$_{600}$ = 0.6, the culture was induced with 2 mM IPTG. After 6 h at 30 ºC, bacteria were pelleted by centrifugation at 8000 g for 15 min and the culture supernatant collected, concentrated by ultrafiltration and chromatographed on a 2.5 x 30 cm column of Sephadex G-75. Fractions containing apoLp-III(1-91) were pooled, dialyzed against deionized H$_2$O, lyophilized and further purified by semi-preparative C$_8$ reversed-phase HPLC.

3.4.3 Analytical methods

Protein purity and/or ligation reaction progress were monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using either a 4-20% or fixed 16% acrylamide slab gel. Gels were stained with Amido Black 10B. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was performed on a Bruker Daltronics autolflex LRF as described previously (Beckstead, Block et al. 2005).

3.4.4 Expressed protein ligation

Ligation reactions employed purified apoE(1-111)-MESNA and Asp1 to Cys apoLp-III(1-91). Unless otherwise stated, fragments were dissolved in 20 mM NaH$_2$PO$_4$, pH=7.2,
150 mM NaCl (PBS) supplemented with 5 % (w/v) MESNA at a final concentration of 5 mg/mL and incubated at 37 °C for 24 h in a final volume of 50 µL. In other experiments, specified reaction parameters were varied as described in the text. Quantification of the ligation product yield was performed using ImageJ gel quantification software for Macintosh.
3.6 Figures

**Figure 3-1. Design strategy for EPL-mediated hybrid apolipoprotein production.** Substrate protein fragments were generated as recombinant fusion proteins, cleaved to generate the desired EPL active fragments and ligated to generate the product hybrid apolipoprotein. M denotes 2-mercaptoethansulfonic acid (MESNA) used to induce intein-mediated cleavage of the apoE•intein•CDB fusion protein.
Figure 3-2. Characterization of EPL reaction substrate protein fragments. Panel A) apoE(1-111)-MESNA production, purification and analysis. Left; 4-20 % acrylamide gradient SDS-PAGE analysis of fusion protein expression, cleavage and apoE(1-111)-MESNA recovery. Lane 1) molecular weight standards; lane 2) bacterial cell lysate of non-induced ER2566 E. coli cell cultures harboring the apoE-pET22b plasmid; lane 3) lysate of cells induced with 1 mM IPTG; lane 4) chitin column wash flow through following application of an induced cell culture preparation; lane 5) chitin column eluate after exposure to buffer supplemented with 60 mM MESNA. Right; Analytical reversed-phase HPLC of chitin column eluate recovered following exposure to MESNA. Inset: MALDI-TOF analysis of the protein peak with an HPLC retention time = 50 min. Panel B) N-Cys apoLp-III(1-91) production, purification and analysis. Left; 16% acrylamide SDS-PAGE analysis of pelB fusion protein expression, cleavage and Asp1 to Cys apoLp-III(1-91) recovery. Lane 1) molecular weight standards; lane 2) bacterial cell lysate of non-induced E. coli BL21 cells harboring the apoLp-III-pET22b plasmid; lane 3) lysate of cells induced with 2 mM IPTG; lane 4) M9 minimal media cell culture supernatant from induced bacterial cell cultures; Right) Analytical reversed-phase HPLC of induced bacterial cell culture supernatant following Sephadex G-75 column chromatography; Inset: MALDI-TOF analysis of the major HPLC peak (retention time = 48 min).
Figure 3-3. Effect of incubation time on EPL-mediated hybrid apolipoprotein formation. Asp1 to Cys apoLp-III(1-91) and apoE(1-111)-MESNA were dissolved in PBS supplemented with 5 % (w/v) MESNA (5 mg/mL final concentration) and incubated at 37 °C. Following incubation, hybrid apolipoprotein production was assessed by 16% acrylamide SDS-PAGE analysis. The gel was stained with Amido Black 10B.
Figure 3-4. Effect of incubation parameters on EPL-mediated hybrid apolipoprotein production. Asp1 to Cys apoLp-III(1-91) and apoE(1-111)-MESNA were incubated for 48 h at 5 mg/mL final concentration under specified conditions of temperature, buffer pH and thiol agent. Following incubation, an aliquot of the reaction mixture was subjected to SDS-PAGE and ligation product formation assessed by densitometry of the stained gel using ImageJ software.

Panel A) Incubations conducted in PBS supplemented with 5 % (w/v) MESNA at the indicated temperatures; Panel B) incubations conducted at 37 ° in Tris-maleate buffer supplemented with 5 % MESNA and adjusted to the indicated pH; Panel C) incubations as in panel A except for substitution of the specified thiol agent(s) at 10% w/v; Panel D) as in Panel A except for specified MESNA concentration. For panels C and D the control incubation was conducted without added thiol agent.
Figure 3-5. Effect of substrate protein fragment concentration on EPL product formation. Panel A) Specified concentrations of apoE(1-111)-MESNA and a fixed Asp1 to Cys apoLp-III(1-91) concentration (2.5 mg/mL final) were incubated in PBS supplemented with 5 % (w/v) MESNA at 37 ºC for 24 h (25 µL final volume). Panel B) Specified concentrations of Asp1 to Cys apoLp-III(1-91) and a fixed apoE(1-111)-MESNA concentration (2.5 mg/mL) were incubated in PBS supplemented with 5 % (w/v) MESNA at 37 ºC for 24 h (25 µL final volume). Following incubation, an aliquot of the reaction mixture was subjected to SDS-PAGE and ligation product formation assessed by densitometry of the stained gel using ImageJ software.
3.6 References


CHAPTER 4: Semisynthesis and segmental isotope labeling of apoE3 N-terminal domain using expressed protein ligation
4.1 Introduction

Apolipoprotein E (apoE) is an exchangeable apolipoprotein that functions as a ligand for members of the low-density lipoprotein (LDL) receptor family, promoting lipoprotein clearance from the circulation. Productive receptor binding requires that apoE adopt an LDL receptor-active conformation through lipid association and studies have shown that the N-terminal (NT) domain is both necessary and sufficient for receptor interaction. Using intein-mediated expressed protein ligation (EPL), a semisynthetic apoE3 NT has been generated for use in structure-function studies designed to probe the nature of the lipid-associated conformation of apoE3 NT. Circular dichroism spectroscopy of EPL-generated apoE3 NT revealed a secondary structure content similar to wild-type apoE3 NT. Likewise, lipid and LDL-receptor binding studies revealed that EPL-generated apoE3 NT is functional. Subsequently, EPL was used to construct an apoE3 NT enriched with stable isotope (\(^{15}\mathrm{N}\)) solely and specifically in residues 112 – 183. Heteronuclear nuclear magnetic resonance (NMR) spectroscopy of segmental isotope labeled apoE3 NT revealed that, compared to uniformly \(^{15}\mathrm{N}\) labeled apoE3 NT, the spectrum was greatly simplified. Two-dimensional \(^1\mathrm{H}-^{15}\mathrm{N}\) heteronuclear single quantum correlation (HSQC) spectra provided additional evidence that the ligation product is correctly folded in solution and adopts a conformation very similar to that of native apoE3 NT. The results indicate that segmental isotope labeling represents a potentially useful approach for defining the lipid bound conformation of the receptor binding element of apoE and molecular details of its interaction with the LDL receptor.

4.2 Results and Discussion

4.2.1 Semisynthesis Rationale

The goal of the present semisynthesis strategy was to generate an apoE3 NT protein specifically isotope labeled only in the receptor recognition segment of the protein (i.e. residues 130-173). The overall design strategy is outlined in Figure 4-1. Since EPL requires an N-terminal cysteine nucleophile to initiate protein ligation (Xu and Evans 2001; Muir 2003), the \(\varepsilon 3\) isoform of apoE NT, which contains a lone cysteine at position 112, is well suited for EPL. To begin, we independently generated a C-terminal thiol stabilized apoE(1-111) fragment and an apoE(112-183) fragment. This choice eliminated the need for a mutation to introduce a potentially disruptive cysteine elsewhere in the protein and employed an apoE fragment (i.e. residues 112-183) that encompasses the entire receptor binding sequence element. To generate the apoE(1-111) fragment, thiol-induced, intein-mediated cleavage of a protein chimera was performed (Hauser and Ryan 2007). CNBr cleavage of a modified intact apoE3 NT generated the apoE(112-183) fragment. Ligation of these fragments to reconstruct intact apoE3 NT, with a peptide bond between residues 111 and 112 and without extraneous tags or tails, involved incubating the fragments in the presence of excess thiol to enhance the rate-limiting transthioesterification reaction (Johnson and Kent 2006).

4.2.2 Expressed Protein Ligation
ApoE(1-111)-MESNA (~13 kDa) was produced in high yield and the isolated fragment was highly soluble and stable in a range of aqueous buffers at high concentration (Figure 4-2). The purity of the ~8 kDa apoE(112-183) ligation substrate fragment is also shown. Upon incubation of these fragments, a time-dependent appearance of a product band with electrophoretic mobility identical to that of control apoE3 NT was observed. When excess apoE(112-183) was present, ligation efficiency increased to ~70-80 % under these conditions. Similar yields have been reported for ligations that employ two similarly sized fragments greater than 8 kDa (Muralidharan, Cho et al. 2004). Ligation progress was monitored by SDS-PAGE and the reaction was quenched by dialysis. Following this the sample was processed to remove un-reacted substrate fragments. A combination of heparin affinity chromatography and reversed phase HPLC yielded a purified semisynthetic apoE3 NT (Figure 4-2, panel B).

4.2.3 Characterization of semisynthetic apoE3 NT

Mass spectrometry analysis of the ligation product (21,278 Da) and wild-type recombinant apoE3 NT (21,197 Da) agree well with each other and the calculated masses of these proteins (21,270 Da and 21,192 Da, respectively, due to a Met125Ala mutation introduced in the ligated protein to avoid cleavage by cyanogen bromide used for purification of apoE(112-183), see Materials and Methods below). To examine whether the ligation product folds in solution to adopt a conformation similar to native apoE3 NT, far-UV CD spectroscopy was performed (Figure 4-3). Consistent with earlier reports that apoE3 NT is predominantly α-helical (Aggerbeck, Wetterau et al. 1988; Fisher, Wang et al. 1997; Clement-Collin, Barbier et al. 2006), control apoE3 NT and the ligation product exhibited dual minima at 208 and 222. Guanidine HCl induced denaturation studies (Figure 4-4) revealed that control apoE3 NT and ligation product were stable in solution, yielding native to unfolded transition midpoints in the range of 2 – 2.5 M guanidine HCl. Based on this, we conclude that, following ligation, EPL generated apoE3 NT folds in solution to adopt a stable conformation characteristic of apoE3 NT.

4.2.4 LDL receptor binding activity

Control apoE3 NT and semisynthetic apoE3 NT were complexed with DMPC to generate reconstituted high density lipoprotein-sized (rHDL) particles (Raussens, Fisher et al. 1998). Native PAGE analysis revealed the WT and ligated apoE NT•DMPC rHDL particles co migrated and thus produced similarly sized complexes (Figure 4-5). To examine the relative ability of semisynthetic apoE3 NT to serve as an LDL receptor ligand, a FRET-based competition binding assay (Yamamoto, Lamoureux et al. 2006) was performed. As shown in Figure 4-6, control and semisynthetic apoE NT•DMPC complexes were nearly equivalent in their ability to compete with fluorescent-labeled Trp-null apoE3 NT•DMPC. Based on these data we conclude that semisynthetic apoE3 NT possesses the ability to form rHDL that function as ligands for the LDL receptor.

4.2.5 NMR studies of segmental isotope labeled apoE3 NT

A goal of semisynthesis of apoE3 NT is the generation of a segmental isotope labeled protein for heteronuclear multidimensional NMR spectroscopy. Previous studies in our laboratory have shown that an isolated peptide fragment of apoE3 NT corresponding to
residues 126–183 fails to adopt secondary structure in solution yet forms an extended alpha helix structure in the presence of the helix inducing cosolvent, trifluoroethanol or the micelle forming lipid dodecylphosphocholine (Raussens, Slupsy et al. 2002; Raussens, Slupsy et al. 2003). In the present approach we sought to extend this work by examining the structure of this region of the protein within the context of an intact NT domain. By culturing E. coli in minimal media containing $^{15}$NH$_4$Cl as the sole nitrogen source, efficient labeling was achieved (Fisher et al., 1997). We used $^{15}$N labeled apoE3 NT as the starting material for the generation of apoE(112-183). Subsequent ligation with MESNA-apoE(1-111) resulted in generation of a semisynthetic apoE3 NT that harbors $^{15}$N isotope only in residues 112–183. This affords a significant simplification of NMR spectra compared to uniformly $^{15}$N labeled apoE3 NT. For comparison, two-dimensional $^1$H-$^{15}$N HSQC spectra were taken for uniformly $^{15}$N-labeled and segmentally $^{15}$N-labeled apoE3 NT at pH 7.4 (Figure 4-7). In this HSQC experiment, the observed peaks correlate the chemical shift of amide protons with the amide nitrogen for each of the $^{15}$N-labeled amino acids of the polypeptide chain. From this data it can be seen that segmental isotope labeled semisynthetic apoE3 NT resonances coincide with a subset of resonance manifest by uniformly $^{15}$N-labeled apoE3 NT. The HSQC data for segmental isotope labeled protein was well dispersed with minimal spectral crowding outside of the small central region of the spectrum, a known characteristic of highly $\alpha$-helical proteins. In contrast, the uniformly $^{15}$N labeled apoE NT contained a much higher central density because of overlapping cross-peaks due to the greater number of labeled residues in a highly helical environment (Fisher, Wang et al. 1997). Given the simplified spectrum of the segmentally labeled apoE NT, assignment of the spectrum is feasible and, ultimately, should allow for detailed three-dimensional structural calculations in the lipid bound state and investigation of the protein in complex with LDLR.

4.3 Summary and Conclusions

The objective of the present study was to employ EPL methods to generate a functional apoE3 NT for structural analysis of lipid bound apoE by NMR spectroscopy. This approach complements a recent report by Weisgraber and colleagues who reported an X-ray structure of apoE bound to dipalmitoylphosphotidylcholine at 10 Å resolution (Peters-Libeu, Newhouse et al. 2006). While the apoE lipid complexes reported in the study were LDL receptor competent, the limited resolution did not afford discernable secondary structure or provide direct evidence of the detailed determinants of the receptor active conformation. The design adopted in the present study involved joining apoE fragments together via a peptide bond to create an intact NT domain that is indistinguishable from wild type apoE3 NT. Whereas one fragment (apoE residues 1-111) was conveniently generated following intein mediated cleavage of a precursor protein chimera, CNBr cleavage of a modified apoE3 NT yielded a fragment encompassing residues 112–183. In the final ligation product two amino acids substitutions were introduced to facilitate the design. Val111 was converted to Ala to facilitate intein-mediated cleavage of the precursor chimera and Met125 was converted to Ala to prevent an unwanted CNBr cleavage at that site.
Characterization studies of the ligation product provided evidence that semisynthetic apoE3 NT was folded in solution to adopt an amphipathic α-helix bundle that possesses intrinsic lipid binding activity and is able to function as a LDL receptor ligand. The yield of ligation product was up to 70%, suggesting this method can be used to generate large quantities of specifically labeled protein. The versatility of the EPL approach relates to the ability to introduce labels or modifications in specific regions or sites within a protein while the other portion remains unaffected. For example, by introducing any number of modifications including unnatural amino acids (Wang and Cole 2001; Yee, Chang et al. 2003), biophysical probes (Scheibner, Zhang et al. 2003), post-translational modifications (Zheng, Zhang et al. 2003) or isotope labels(Camarero, Shekhtman et al. 2002; Valiyaveetil, MacKinnon et al. 2002) into one fragment, EPL can be used to study structural and functional aspects of proteins that might otherwise be difficult to address. Indeed, EPL applications for the study of post-translational modifications of proteins have proved particularly useful for their ability to create large quantities of proteins with modifications that would otherwise be difficult to achieve by traditional recombinant or protein purification methods (Muir 2003). By modifying one or both fragments prior to ligation, EPL can produce proteins that contain unique characteristics that allow for the study of protein function as it pertains to alternative conformational state transitions (Roy, Allen et al. 1999; Muralidharan, Cho et al. 2004), post-translational affects (Hahn, Pellois et al. 2007), and protein-protein interactions (Scheibner, Zhang et al. 2003; Muralidharan, Cho et al. 2004). Given that the only requirements of EPL are the presence of an active cysteine at position 1 of the C-terminal fragment and a leaving group moiety on the amino-terminal fragment (Xu and Evans 2001), modifications made elsewhere in the fragments will be imparted to the ligated protein that allow for functional studies of the intact, ligated protein.

In the present study, our goal was to generate a segmental isotope labeled apoE3 NT. The ability to introduce stable isotopes specifically into the receptor-binding region of the protein permits detailed analysis of this region of the protein. Because apoE3 NT is biologically active in a lipid-associated state, characterization of its structural properties in this environment is desired. While such studies are feasible by NMR spectroscopy, the spectra of apolipoproteins become significantly crowded in the central region upon complexation with lipid (Wang, Sahoo et al. 1998). While previous NMR spectroscopy studies generated a full resonance assignment of lipid-free apoE NT (Xu, Sivashanmugam et al. 2005), spectral complication associated with lipid complexation requires an alternative approach for ascertaining details of the LDL receptor active conformation. In order to decrease spectral overlap, we adopted a strategy to specifically label the region of interest. A similar approach has been employed to investigate apoE inter-domain conformational adaptation using a stable isotope enriched C-terminal domain (Zhao, Zhang et al. 2008). This differs from the goal of the current study, which aims to investigate conformational adaptation of the receptor-binding region of the NT domain upon association with lipid to confer receptor binding activity. Earlier we studied a peptide fragment of apoE corresponding to residues 126-183. Since this peptide was not in the context of the intact NT domain, it failed to adopt a native conformation in solution. Whereas structural data was obtained in the presence of a helix inducing cosolvent (Raussens, Slupsky et al. 2002) or a lipid mimetic detergent (Raussens, Slupsky et al. 2003), it is uncertain if the absence of
the remainder of the domain influences the properties of the peptide. Thus, in order to examine the structure of this segment of the protein within the context of the intact NT domain, an EPL approach was pursued. It is evident from the characterization studies presented that the labeled portion of the protein (residues 112-183) is present in a native conformation and gives rise to an HSQC spectrum that coincides with the corresponding spectrum of uniformly $^{15}$N labeled apoE3 NT. This indicates the conformation adopted by the EPL generated protein is similar to that of wild type recombinant apoE3 NT. As such, segmental labeling offers a feasible approach to simplify NMR spectra of apoE3 NT for study of its lipid bound, receptor active conformation. Currently, efforts are underway to assign the spectra of lipid-free segmentally labeled apoE3 NT with a view to conducting studies with lipid bound protein.

4.4 Materials and Methods

4.4.1 Preparation of apoE(1-111)

Human apoE(1-111) was cloned and expressed as a S. cerevisiae-derived vacuolar ATPase intein domain and chitin binding domain (CBD) fusion protein using the pTYB1 vector (New England Biolabs) as previously reported (Hauser and Ryan 2007). To facilitate optimal intein-mediated fusion protein cleavage (Hackeng, Griffin et al. 1999), valine 111 was mutated to alanine using the QuikChange method (Stratagene) according to the manufacturer’s instructions. Expression and purification procedures for apoE(1-111) followed standardized protocols previously established for generating intein-mediated thioester-adducted proteins (Xu and Evans 2001). Briefly, saturated overnight cultures of ER2566 E. coli cells harboring the vector encoding the apoE(1-111) fusion protein were inoculated into 2xYT media containing 50 µg/ml ampicillin, grown to OD$_{600}$ = 0.6 and induced with 1 mM isopropyl thiogalactopyranoside (IPTG). After 6 h at 30 °C the cells were pelleted by centrifugation (8000 g for 15 min), solubilized with buffer A (20 mM Tris, 150 mM NaCl, 1 mM EDTA, pH=8.0) containing 1% Triton X-100 and stored at -20 °C. Dissolved cell pellets were combined, passed through a microfluidizer, sonicated and centrifuged at 12000 g for 20 min. Isolated clarified cell extract was passed over a chitin bead column pre-equilibrated with buffer A containing 1% Triton X-100. The column was washed with 10 column volumes of detergent-free buffer A and fusion protein cleavage induced by addition of 2-mercaptoethanesulfonic acid (MESNA) to a final concentration of 60 mM. Flow was arrested for 16-24 h at 22 °C and eluted with 2 bed volumes of buffer A containing 5 mM MESNA. The sample was dialyzed against deionized H$_2$O, lyophilized and stored at -20 °C. apoE(1-111)-MESNA was further purified by semi-preparative C$_8$ reversed-phase high performance liquid chromatography on a Perkin-Elmer Series 200 HPLC.

4.4.2 Preparation of apoE(112-183)

Human apoE(112-183) polypeptide was prepared from engineered apoE(1-183) starting material by CNBr-induced cleavage. Recombinant apoE(1-183) containing engineered mutations (Stratagene) at positions 111 (Val->Met) and 125 (Ala->Met) was expressed and as described elsewhere (Fisher, Wang et al. 1997). Briefly, saturated overnight cultures were inoculated into M9 media supplemented with 13.3 mM glucose, 0.1
mM CaCl₂, 2 mM MgSO₄ and 50 µg/ml ampicillin. At OD₆₀₀ = 0.6, the culture was induced with 2 mM IPTG. After 6 h at 30 °C, bacteria were pelleted by centrifugation at 8000 g for 15 min and the culture supernatant collected, concentrated by ultrafiltration, and purified by heparin affinity chromatography and semi-preparative C₈ reversed-phase HPLC. Purified apoE(1-183) was then subjected to digest by CNBr (Raussens, Mah et al. 2000). ApoE(1-183) was dissolved in 80% formic acid (5 mg/mL). CNBr was added to achieve a CNBr/methionine ratio >100 and reactions were incubated in an oxygen-free environment for 24 h in the dark. Reactions were quenched by the addition of a 10-fold excess of dH₂O and lyophilized to dryness. This procedure was repeated three times to remove residual formic acid. Complete digestion resulted in enrichment in apoE(112-183) containing the desired N-terminal cysteine (N-Cys). ApoE(112-183) was further purified by semi-preparative C₈ reversed-phase high performance liquid chromatography on a Perkin-Elmer Series 200 HPLC.

4.4.3 Analytical methods

Protein purity and/or ligation reaction progress was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using either a 10-20% acrylamide gradient tricine gel or a fixed 18% acrylamide slab gel. Gels were stained with Amido Black 10B. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was performed on a Bruker Daltronics autoflex LRF as described previously (Hauser and Ryan 2007).

4.4.4 Expressed protein ligation

Ligation reactions employed purified apoE(1-111)-MESNA and apoE(112-183) protein fragments. Typical ligation reactions were carried out in 150-250 µL incubations containing equivalent concentrations (1.5-2 mM) of apoE(1-111) and apoE(112-183) (Hauser and Ryan 2007). Protein fragments were dissolved in 20 mM NaH₂PO₄, 150 mM NaCl, 2M urea supplemented with 5 % (w/v) MESNA and 10 mM Tris(2-carboxyethyl)phosphine (TCEP) (final pH=8.5). Reactions were incubated at 37 °C for 96 h with gentle stirring. Quantification of the ligation product yield was assessed by acrylamide gel electrophoresis and quantified by densitometry using ImageJ software for Macintosh (Rasband 1997-2006; Abramoff, Magelhaes et al. 2004).

4.4.5 Circular dichroism spectroscopy

Circular dichroism (CD) measurements were performed on an AVIV 410 spectrophotometer or a Jasco 810 spectropolarimeter. Far-UV CD scans were recorded between 185 and 260 nm in 20 mM sodium phosphate (pH 7.4) using an apoE3 NT protein concentration of 0.5 mg/mL determined by the absorbance at 280 nm. The α-helical content was calculated with the self-consistent method using Dicroprot, version 2.6 (Sreerama and Woody 1993). For guanidine HCl denaturation studies, protein samples were dissolved in 20 mM sodium phosphate (pH 7.4). ApoE NT samples (0.2 mg/mL) were incubated overnight at a given denaturant concentration to attain equilibrium, and ellipticity was measured at 222 nm.

4.4.6 LDL receptor binding assay
For the LDL receptor binding assay, 2.5 mg of a soluble form of the LDL (sLDL) receptor (Yamamoto, Lamoureux et al. 2006) was resuspended in 20 mM Tris (pH 7.2), 2 mM CaCl$_2$, and 90 mM NaCl and incubated with 1.5 mg Trp-null apoE3 NT previously labeled on Cys112 with the fluorescent probe, N-(iodoacetyl)-N-(5-sulfo-1-naphthyl) ethylenediamine (AEDANS), and complexed with DMPC to form reconstituted HDL particles (Weers, Narayanaswami et al. 2001). Interaction between AEDANS–Trp null apoE3 NT•DMPC and the sLDL receptor was detected by fluorescence resonance energy transfer (FRET) between excited Trp residues in the sLDL receptor and the AEDANS moiety covalently attached to Trp null apoE3 NT•DMPC (Yamamoto, Lamoureux et al. 2006). In competition assays, the ability of a given unlabeled apoE3 NT preparation to compete with AEDANS-Trp null apoE NT•DMPC for binding to sLDL receptor was monitored as a change in AEDANS fluorescence intensity (excitation 280 nm; emission 470 nm) after 10 min incubation at 25 °C (final sample volume 300 ml). Fluorescence measurements were made on a PerkinElmer LS50b luminescence spectrometer.

4.4.7 NMR spectroscopy

NMR experiments were performed on 0.3-0.5 mM $^{15}$N-labeled apoE(1-183) or segmentally (residues 112-183) $^{15}$N-labeled apoE NT in 500 µl sodium phosphate buffer (50 mM phosphate, 50 mM NaCl, 20 mM DTT, 100 µM EDTA, pH=7.3-7.4), containing 10% D$_2$O, 0.5 mM NaN$_3$ and 0.25 mM 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal chemical shift reference. $^1$H-$^{15}$N heteronuclear single quantum correlation (HSQC) spectra were obtained at 30 °C on a Varian Unity 600-MHz NMR spectrometer. Data were processed using NMRPIPE (Delaglio, Grzesiek et al. 1995) and analyzed using NMRVIEW (Johnson and Blevins 1994).
4.5 Figures

Figure 4-1. Design strategy for EPL-mediated semisynthetic apoE NT production. ApoE NT protein fragments designed for EPL were generated as separate recombinant preproteins, cleaved by an intein-mediated reaction (residues 1-111) or CNBr chemical cleavage (residues 112-183) and ligated to produce a semisynthetic apoE NT. Using the same ligation scheme, segmentally labeled apoE NT was produced by combining the unlabeled apoE(1-111) fragment with isolated $^{15}$N labeled apoE(112-183) protein. M denotes 2-mercaptopethansulfonic acid (MESNA) used to induce intein-mediated cleavage of the apoE•intein•CDB fusion protein.
Figure 4-2. Characterization and purification of EPL reaction

**Panel A)** 4-20 % acrylamide gradient SDS-PAGE analysis of ligation precursor proteins and representative ligation conditions. Lane 1) molecular weight standards; lane 2) HPLC-purified wild-type unligated apoE NT control; lane 3) chitin column purified apoE(1-111)-MESNA protein; 4) CNBr cleaved apoE NT preprotein containing enriched apoE(112-183); lane 5) ligation mixture at 0 hr containing the apoE(1-111) and apoE(112-183) ligation competent fragments in a 1:1 protein concentration ratio; lane 6) Representative apoE NT semisynthetic EPL reaction mixture containing apoE(1-111) and apoE(112-183) in a 1:2 protein concentration ratio after 96 h incubation. **Panel B)** 4-20 % acrylamide gradient SDS-PAGE analysis of HPLC purified unligated and ligated apoE NT. Lane 1) molecular weight standards; lane 2) HPLC-purified wild-type unligated apoE NT control; lane 3) HPLC-purified semisynthetic apoE NT created from an EPL reaction.
Figure 4-3. Far-UV circular dichroism spectrum of apoE NT constructs. Far-UV CD spectra of unligated, wild-type (open circles) apoE NT and semisynthetic (closed circles) apoE NT were recorded in phosphate buffered solution at pH 7.4.
Figure 4-4. Circular dichroism guanidine-HCl denaturation curves of apoE NT proteins. CD spectra of unligated, wild-type (open circles) apoE NT and semisynthetic (closed circles) apoE NT were recorded in phosphate buffered solution at pH 7.4 following incubation with specified amounts of guanidine HCl.
Figure 4-5. Native PAGE gel electrophoresis of apoE NT•DMPC particles. 4-20 % acrylamide gradient native PAGE analysis of apoE NT•DMPC rHDL particles. Lane 1) native PAGE protein standards; lane 2) unligated apoE NT•DMPC particles; lane 3) semisynthetic apoE NT•DMPC particles.
Figure 4-6. LDL receptor binding competition studies of apoE NT•DMPC particles.
LDL receptor binding assay comparing the binding efficiency of unligated, wild-type apoE NT•DMPC particles (solid line) and segmentally constructed apoE3 NT•DMPC particles (dotted line). Binding efficiency was assessed by the measured ability of WT and ligated protein•DMPC complexes to compete with preincubated tryptophan-null, AEDANS-labeled apoE NT•DMPC particles and the soluble LDL receptor.
Figure 4-7. Two-dimensional $^1$H-$^{15}$N HSQC of uniformly and segmentally labeled apoE NT proteins. Overlaid spectra of uniformly $^{15}$N labeled apoE3 NT (black) and segmentally $^{15}$N labeled apoE3 NT (red) taken at 600 MHz (pH 7.3-7.4).
Figure 4-8. $^1$H-$^{15}$N HSQC spectra of glycine residues in apoE3 NT. Spectra of uniformly $^{15}$N labeled apoE3 NT (black) and segmentally $^{15}$N labeled apoE3 NT (red) in the region between $^1$H ppm 7.6-9.2 and $^{15}$N ppm 104-111 are shown. Glycine assignments are indicated (Xu, Sivashanmugam et al. 2005).
4.6 References


CHAPTER 5: General Discussion, Conclusions and Future Directions
5.1 Project Summary

Recently, the study of apoE has expanded into a variety of new areas as understanding of the protein’s role in normal and diseased physiological conditions ranging from macrophage cholesterol efflux, neurodegeneration and diseases of immune deficiency are being advanced. Many of these roles rely on the lipid binding and transport function of apoE. This important function has been clearly demonstrated by the critical role apoE plays in blood lipid transport and atherosclerosis. This and several other known functions of apoE appear to operate through pathways that depend on competent receptor interaction, which makes the study of apoE-LDL receptor interaction a particularly pertinent model across physiological contexts.

Despite the observation that competent receptor binding is critical to apoE function, a significant unresolved question in the understanding of apoE structure and function is the mechanism whereby the protein alters its conformation upon lipid binding to transition from a LDL receptor inactive to receptor active ligand for the uptake of lipoprotein particles. This lipid-free to lipid-bound transition requires that apoE adopt two distinct alternative conformations that are readily interconvertible yet independently stable and predictably distinct as biological effector states. To accommodate this dual existence, the apoE must readily adapt from a state where the sequestered hydrophobic residues in the aqueous helix bundle exchange their helix-helix associations for helix-lipid interactions.

Studies employing strategically placed FRET pairs (Fisher and Ryan 1999; Fisher, Narayanaswami et al. 2000; Narayanaswami, Szeto et al. 2001), FTIR spectroscopy, x-ray crystallography (Peters-Libeu, Newhouse et al. 2006) and other biophysical techniques (Gupta, Narayanaswami et al. 2006; Hatters, Voss et al. 2009) have lead to proposals for a number of differing, and sometimes contradictory, low-resolution models for how the N-terminal domain of apoE changes conformation to accommodate lipid substrates. From these studies it seems probable that this domain undergoes a dramatic conformational opening of the lipid-free four-helix bundle, though the exact mechanism of the opening has been debated, with a number of mutually exclusive models proposed. Mounting evidence suggests that helix 4 of the N-terminal domain transitions from unstructured to predominantly helical upon interaction with lipid (Raussens, Slupsky et al. 2003; Gupta, Narayanaswami et al. 2006), which has been hypothesized to provide additional positively charged surface area for interaction with a larger portion of the LDL receptor ligand binding region. However, a detailed description of how this helix extension relates to the global fold architecture upon lipid binding remains unclear. While this combined insight has dramatically enhanced understanding of the lipid-dependent apoE conformational flexibility, a more detailed model is required to understand the generalized features of competent receptor recognition sequence presentation, particularly in light of the fact that apoE is known to be associated with a wide range of lipoprotein particles, ranging from small nanometer sized HDL particles in brain to chylomicron particles of greater than one micron in diameter in the peripheral circulation. Thus, the determination of a high-resolution lipid-bound structure of apoE is likely to provide significant insight into the structural adaptation required for apoE to fulfill its function as a lipid transport molecule and high affinity receptor ligand.
5.2 Testing an apoE NT helix 1’ mutant for lipid-binding alterations

The ability of apoE to bind lipid and the degree to which this occurs is a primary determinant of the ability of apoE to modulate receptor binding. While it has been shown that the C-terminus initiates contact of apoE to lipid substrates, a small joining helix, termed helix 1’, may serve as a secondary determinant for lipid binding initiation within the N-terminal, receptor-binding domain of apoE. In an effort to expand our understanding of the lipid induced conformational “opening” of the apoE helix bundle, we created a mutagenized apoE N-terminal domain that was hypothesized to have altered lipid binding affinity owing to the substitution of helix 1’ for a short β-strand segment to test the hypothesis that helix 1’ plays an important role in driving the helix bundle towards a dramatically different steady-state, lipid-bound conformation. In the lipid free crystal structure of apoE, helix 1’ sits between helices 1 and 2 at the apex of the four helix bundle which made it an attractive candidate for possibly regulating the access of lipid substrates to otherwise buried hydrophobic residues of the lipid-free bundle.

A mutagenized apoE NT containing a predicted β-turn motif in place of the highly conserved helix 1’ was produced, characterized, and shown to maintain a high degree of structural relatedness and LDL receptor binding affinity of the intact, wild-type apoE NT. Studies of the lipid binding affinity, however, showed that the helix-turn mutant displayed a higher affinity and increased binding to lipid substrates in a number of in vitro assays, suggesting that mutant is able to more readily present hydrophobic residues for interacting with lipid substrates. This was further supported by studies showing that the mutant protein manifests increased association with a hydrophobic dye known to bind to hydrophobic residues like those found in the interior of the apoE NT helix bundle. These combined results imply that the absence of helix 1’ promotes more efficient presentation of the residues required for lipid interaction. When combined with the observation that this helix is highly conserved across species, these results suggest that the small helix acts as a “lid” to regulate the exposure of this domain to lipophilic substrates. Additional interpretation of the findings provides support for a helix bundle opening model wherein helices 1 and 2 moving away from the helix 3 and 4 pair (Weisgraber 1994) as opposed to other models supporting the helix 1 and 4 pair moving away from helices 2 and 3. Collectively, this study found that the small and highly conserved helix 1’ element of the helix bundle plays a critical role in the binding and association of the apoE NT to lipid during the transition from receptor inactive to receptor competent conformational states. Though further study is required to understand the physiological implications, these results suggest that structural changes imparted through helix 1’ modulation can affect the function of apoE in lipoprotein metabolism without dramatically changing the overall fold architecture or the mechanism of lipid-dependent apoE adaptation.

5.3 Establishing an EPL expression and purification system

Studies of the structure and function of several distinct protein systems through the aid of EPL and segmental isotope labeling provided valuable information that was otherwise difficult to obtain due to protein size constraints or the need for specific placement of labels, tags or protein modifications. What began as a posttranslational
protein splicing mechanism observed in *Saccharomyces cerevisiae* has now been utilized to provide a unique protein engineering solution for the *in vitro* and *in vivo* study of protein structure and function (Muir 2003; Muralidharan and Muir 2006). Recent expansion of the *in vivo* utility of EPL for use in tracing the biodistribution of leptin (Ceccarini, Flavell et al. 2009) provides evidence for an expanded role of EPL in tracer studies using intact proteins conjugated to fluorophore molecules for live imaging and metabolic monitoring. While technically challenging and requiring system-specific modifications, EPL affords a unique advantage in the context of structural studies using NMR spectroscopy for its ability to limit the NMR analysis to a specific set of residues within the context of a larger, functional domain or full-length protein. With our specific goal to adapt a NMR system to the study of lipid-bound apoE, segmental isotope labeling was attractive both for its reduction in apparent protein size and for its simplification of the NMR system upon addition of lipid.

The first step in developing a robust EPL system for apoE was to establish the expression systems for the two independent apoE fragments that were used to create the intact, segmental apoE NT. As a starting point, the apoE(1-111) fragment was expressed as an intein fusion protein that was cleavable under the control of a soluble thiol. This fragment expressed well and the purification proved straightforward and high-yield. Because of the inherently high charge density and insolubility of the apoE(112-183) fragment, the development of this fragment purification system was more challenging and faced setbacks from limited expression, difficulty expanding to high yield and challenges achieving high concentrations of soluble protein for promoting ligation. In an effort to initially circumvent these problems and develop a system to test the ligation system, I used a model system employing the apoE(1-111) fragment and an insect apolipophorin fragment. The apoE(1-111) and apoLpIII(1-91) fragments were used to demonstrate that the ligation system could be driven to high yield and that subsequent purification of the ligation product was possible using methods easily adapted to the desired apoE NT system. Using this robust hybrid system turned out to be particularly useful because the apoLpIII(1-91) fragment yield was higher than apoE(112-183) and thus more conducive to extensive testing and refinement for subsequent application. While this hybrid apolipoprotein is unlikely to be useful for further functional characterization, the fragment generation techniques resulted in a novel and straightforward method for producing amino-terminal cysteine containing fragments, which is one of the necessary requirements of a competent EPL system. This study provided the first evidence that the *E. coli*-derived pelB leader sequence could be used for the *in vivo* production of an EPL competent protein fragment by taking advantage of the natural endopeptidases expressed in the bacterial periplasmic space. With limited options available for facile and robust production of N-terminal cysteine containing fragments with high instability, limited solubility or cytotoxicity, this new expression technique can expand the utility of EPL to more protein systems. In the context of this thesis project, this advancement provided the methodological framework that was extensively useful for the subsequent apoE and apoA1 ligation systems described throughout this thesis.

### 5.4 EPL construction and segmental isotope labeling of apoE NT

While the hybrid apolipophorin III and apoE system was being used to establish the
appropriate ligation conditions, a apoE(112-183) fragment expression and purification scheme was found that maximized yields while also allowing for stable isotope labeling. Previous data had shown that fully stable isotope-labeled apoE NT protein displayed significant crosspeak overlap in a 2-D HSQC (Fisher, Wang et al. 1997) which indicated that the addition of lipid to the NMR system would further complicate resonance assignment. The goal of the segmental isotope system was to significantly improve the spectral crowding problem while relying on the apoE(112-183) residues to provide information about the receptor active conformation within the context of an intact N-terminal domain. After validating that the segmental labeled protein was structurally and functionally very similar to from wild-type apoE NT using standard biophysical techniques, the ligation using 15N-labeled apoE(112-183) was performed and the segmental isotope labeled apoE NT was further tested using NMR. As expected, the spectrum of the segmental apoE NT was well dispersed and simplified compared to fully labeled protein. Validation of the segmental labeled protein integrity was made using a combination of 1) HSQC spectra dispersion in accordance with expectations for a highly helical protein, 2) nearly identical crosspeak positions for residues 112-183 compared to fully labeled apoE NT protein, 3) the easily identifiable lack of tryptophan residues in the apoE(112-183) spectrum as predicted, 4) validation of the glycine assignments and the noted reduction of glycines from ten in the full length apoE NT to seven in the segmental labeled protein. From these experiments, it was established that the apoE NT ligation system was able to reproduce a functional protein that displayed the predicted properties of a fully functional four-helix bundle protein that was LDL receptor competent and structurally intact.

Several additional considerations are necessary for designing NMR experiments to define the detailed structural organization of lipidated apoE NT. Successful 13C, 15N double labeling strategies have been established using the apoE NT EPL system that, when combined with perdeuterated protein strategies, are likely to provide significantly higher NMR resolution and afford a greater variety of 3-D TOCSY, HNCA, HN(CA)CO, HN(CO)CA, and NOESY experiments for tracing the backbone and side-chains interconnectivities. Additional fast protein liquid chromatography (FPLC) purification strategies have been tested and implemented to separate the fully lipidated apoE NT from partially lipidated or unlipidated protein. While we have demonstrated that a weight ratio of 2.5:1 DMPC:apoE NT produces a uniform population of particles approximately 14 nm in diameter (Yamamoto and Ryan 2007), this step further ensures that the NMR sample is free of unlipidated protein and enriched in more homogenously sized discoidal particles. This increased homogeneity in combination with protein deuteration is predicted to provide additional improvements in resolution that will aid in defining the properties of lipid bound apoE NT using NMR.

The enhancement in signal-to-noise afforded by deuteration is likely to be significant in this system (even though the size of the labeled region may not appear to demand deuteration) because the apparent size of the protein in the NMR tube is over 20 kDa without lipid and approaching 200 kDa upon lipiddation. It has been shown convincingly that the decreased density of protons upon deuteration effectively suppresses dipolar coupled proton spin diffusion and dramatically improves signal-to-noise of the NMR signal (Sattler and Fesik 1996; Tugarinov, Ollerenshaw et al. 2005). The signal-to-noise improvement from perdeuteration would be particularly enhanced among the apoE NT residues that are held in contact with lipid or less flexible due to structural constraint.
Because these residues that remain more static under lipid bound conditions are likely to be much more numerous and central to understanding the overall protein architecture, deuteration seems a necessary future addition to the EPL system. As needed, lipid deuteration can also be introduced to prevent spin diffusion near the lipid to protein contacts. Additional deuteration dependent improvements are seen in NOESY experiments as longer NOE mixing times can be applied, allowing NOE’s from larger distance separations to be detected (Sattler and Fesik 1996). With these slight modifications currently underway, the apoE NT EPL system is poised to make significant progress toward defining the lipid bound structural restraints of apoE NT bound to discoidal particles.

5.5 Comments

Although high-resolution structures of apoE NT in the lipid free state are available, understanding of its lipid bound, LDLR-active, conformation is limited to models defined by low-resolution x-ray and biophysical measurements. The 10 Å x-ray model of apoE4 bound to DPPC depicts an α-helical hairpin conformation in the shape of a helical horseshoe in which the apex of the hairpin loop contains the residues required for LDL receptor binding. In contrast, NMR (Raussens, Slupsky et al. 2002; Raussens, Slupsky et al. 2003) and EPR experiments (Gupta, Narayanaswami et al. 2006) suggest that, upon lipid binding, this C-terminal region of helix 4 extends to form an uninterrupted, elongated helix conformation rather than a hairpin. This increased helical content compared to lipid-free apoE NT is postulated to result in alignment of key positively charged amino acids, thereby conferring receptor recognition properties to the protein. It should be noted that important differences exist between the low-resolution x-ray (Peters-Libeu, Newhouse et al. 2007) and proposed NMR apoE model systems, namely, the use of spherical versus discoidal lipid complexes, the apoE isoform employed, the lipid used to form the complexes as well as length of the apoE molecules. While it is still unclear to what extent these factors contribute to differences in the models, it suggests that apoE has a high degree of conformational flexibility that is presumably restricted primarily by the requirement of LDL receptor recognition sequence presentation.

This thesis provides evidence that an EPL system for segmental isotope labeling of apoE NT can be used to probe the lipid bound conformation of the protein using a discoidal rHDL particle model. These particles have been shown to exist as disk-shaped phospholipid bilayers wherein the apoE molecule is localized around the perimeter edge the disc. It is important to note that these complexes are similar in many respects to bicelles that have been successfully used for NMR structural analysis of membrane bound proteins (Prosser, Evanics et al. 2006), suggesting further feasibility of the approach. There are a number of questions that can be answered using an NMR spectroscopy study that combines segmental isotope labeling and lipidated apoE. A number of alternative models of lipid bound apoE have suggested different ways that the protein can be displayed around the perimeter of discoidal particles. NMR is particularly suited for resolving this question by providing detailed information about each of the residues and defining the positions of intermolecular contacts that are changed upon lipid association. With the use of the apoE discs, we expect that a highly uniform population of particles will be obtained that will provide a homogeneous system from which insight into the molecular organization of the
receptor-binding region of the protein will be deciphered with greater resolution than previously employed techniques.

The crystal structure of the entire extracellular domain of the LDL receptor at pH 5.3 provided extensive detail of the intramolecular interactions that are hypothesized to be the driving force for ligand release in the endosomal compartment of cells (Rudenko, Henry et al. 2002). After integrated this model within the context of the extensive structural information known about LDL receptor ligands such as apoE, apoB and their associated lipoproteins, it becomes clear that a major missing mechanic step in the lipoprotein-mediated cholesterol uptake pathway is a detailed understanding of the physiological interface between the LDL receptor and a lipoprotein ligand. A receptor active conformation of an LDL receptor ligand such as apoE would provide critical understanding of the molecular interactions that are likely to be induced upon surface receptor binding. With the EPL system developed in this thesis, it is plausible that segmentally labeled and lipidated apoE could also be examined in complex with a functional LDL receptor fragment. A previous report has shown that the R4-R5 LDL receptor ligand binding domain pair is both necessary and sufficient for competent apoE ligand binding (Fisher, Abdul-Aziz et al. 2004). With its small size and established *E. coli* expression system, this ligand binding pair is ideal for examining the molecular interactions between lipid bound apoE and the putative LDL receptor ligand binding domain using a combination of isothermal titration calorimetry and NMR spectroscopy.

While the focus of this thesis has been on the N-terminal domain for its possession of the LDL receptor recognition sequence, there is considerable evidence that the two domains of apoE interact. This domain interaction has been proposed to play a critical role in the isoform specific effects of apoE in a physiological context, yet many questions remain about the nature and strength of the intramolecular association. With the adaptability of the EPL methodology, it is possible to modify this system for the production of fluorophore or segmental isotope-labeled full-length apoE for studies examining domain interaction. The isoform-specific amino acid substitutions could be produced without interfering with the ligation reaction such that modifications could be alternatively introduced into the two domains for the study of structural differences relevant to the domain interaction hypothesis. Even if NMR studies do not prove feasible in this context, labeling the two domains with FRET pairs prior to ligation may provide useful structural information that can lead to further investigation of the implications of domain interaction. Application of EPL to apolipoproteins is particularly well suited because of their known ability to refold efficiently and adopt predictable structural features upon reconstruction of the intact N-terminal domain or full-length protein. While several important questions have been addressed by the findings in this thesis, the apolipoprotein EPL system that has been developed is poised to answer many of the key questions that await resolution in the field of lipid and lipoprotein transport.
5.6 References


Appendix

EPL studies of apoA-I(Milano)

There is significant and incontrovertible evidence that the lowering of LDL cholesterol through the use of HMG-CoA inhibitors, or statins, results in clinical benefit for patients with atherosclerosis by lowering plasma cholesterol and preventing its deposition in coronary arteries. Epidemiological studies also show an inverse relationship between the plasma levels of HDL cholesterol and atherosclerosis. Other means of LDL cholesterol lowering are feasible through modulation of the LDL receptor pathway but these therapies are now held to the potent efficacy standard of statins making further clinical investigation of LDL lowering therapies difficult and costly. These challenges have recently simulated a search for efficacious HDL raising therapies as a way to augment current statin therapies (Fazio and Linton 2003; Rader 2003; Sirtori 2004). Since the discovery of apoA-I Milano (apoA-I(M)) (Franceschini, Sirtori et al. 1980), a rare mutation in the human apoA-I protein, renewed focus has been paid to the benefits of HDL-based therapies for the prevention of cardiovascular disease (Chiesa and Sirtori 2003; Chiesa and Sirtori 2003; Hegele 2004; Calabresi, Sirtori et al. 2006). ApoA-I is the predominant apolipoprotein found on HDL particles, and has been shown to play a central role in reverse cholesterol transport (RCT), the pathway by which cholesterol is removed from peripheral tissues and returned to the liver fated for conversion to steroidogenic precursors, bile or subsequent secretion. Elevated HDL levels have been shown to be associated with decreased incidence of atherosclerosis and it is commonly suggested that promotion of RCT by increasing apoAI levels helps to lower plasma and whole body cholesterol levels. ApoA-I(M) was initially found in a familial population of heterozygous carriers living in Limone sul Garda (a small town near Milan, Italy) who displayed the unique phenotype of markedly reduced HDL plasma levels yet very low prevalence of atherosclerosis and increased lifespan compared to non-carriers (Franceschini, Sirtori et al. 1980). Interestingly, the apoA-I(M) protein differs from wild-type apoA-I by only a single Arg173Cys amino acid substitution. The mechanistic link between the apoA-I(M) variant and the prevention of atherosclerosis remains unclear despite many in vitro and in vivo experiments examining the protein and its phenotypic effects on cardiovascular function.

The discovery of the Milano variant of apoA-I brought on a number of in vivo and clinical trial studies to confirm and investigate the finding that apoA-I(M) could reduce atherosclerotic lesion formation despite is suppressed plasma levels compared to wild-type subjects. The first functional HDL phenotype that was identified in Milano patients was their differential HDL particle size profile compared to other subjects (Franceschini, Frosi et al. 1982). Their HDL profile was more heterogeneous than wild-type controls, with a predominant prevalence of smaller, more-dense HDL3 particles. It was also shown that the apoAI Milano protein had a shortened residence time in the plasma and caused rapid catabolism of apoAI in these carriers. Since the report of these findings, a clinical trial investigated the efficacy of apoA-I(M)-phospholipid complexes as a therapy for elevated cholesterol and blood lipids. Using a primary efficacy measure of the change in percent atheroma volume, a two-year placebo-controlled clinical trial monitoring 123 patients
found that patients receiving apoA-I(M) compared to control treatment produced significant regression of coronary atherosclerosis as measured by intravenous ultrasound (Nissen, Tsunoda et al. 2003). In a follow-up trial measuring the regression of arterial atheroma volume and changes in the lumen volume, the study found that injection of recombinant HDL containing apoA-I(M) produced significant but heterogeneous regression of coronary atherosclerosis in regions containing high plaque burden as measured by ultrasound (Nicholls, Tuzcu et al. 2006). Despite the perceived success of these trials, no further, larger-scale trials have been performed to date and recombinant HDL apoA-I(M) therapies slated for human clinical trial testing and approval have since been abandoned for undisclosed reasons.

In a transgenic murine model containing a combination of either wild-type human apoA-I, human apoA-I(M) and apoA-II or apoA-I(M) and apoA-II alone in a murine apoA-I knock-out background, mice without any wild-type apoA-I displayed significantly higher apoA-I(M) homodimer levels and the apoA-I(M)-containing HDL were restricted to small, 7.6 nm diameter particles, analogous to the human subjects (Bielicki, Forte et al. 1997). Additionally, the more extensive dimerization of apoA-I(M) severely limited cholesterol ester accumulation in the plasma HDL particles accounting for the abundance of the smaller HDL3 particles. Reconstituted HDL particles containing apoA-I(M) were also shown to preferentially exist in a dimeric state (Calabresi, Vecchio et al. 1997). This led to the suggestion that the increased dimer formation was causally linked to the 50% reduction in endogenous cholesterol esterification and reduced HDL particle size seen in mice and humans harboring the apoA-I(M) protein.

In an in vivo study where cholesterol-fed rabbits were given the apoA-I(M) protein associated with a phospholipid carrier, the animals displayed marked reduction in the size and magnitude of intimal atherosclerotic lesions including regression of existing intimas (Soma, Donetti et al. 1995). Similarly, infusion of apoA-I(M) phospholipid complexes into the carotid artery of cholesterol-fed rabbits lead to rapid removal of lipid from fatty acid streaks along the arterial wall (Chiesa, Monteggia et al. 2002). Treatment of rats in a similar manner delayed the time to thrombus formation, inhibited platelet aggregation, and reduced the weight of the thrombus (Li, Weng et al. 1999). Improved cardiovascular outcomes were consistently seen in animals given apoA-I(M) in several arterial injury models, including ischemia-reperfusion injury (Calabresi, Rossoni et al. 2003; Marchesi, Booth et al. 2004) and injury following coronary balloon angioplasty (Ameli, Hultgardh-Nilsson et al. 1994). These findings led to investigations to measure ability of apoA-I(M) to efflux cholesterol since the ability of HDL to remove cholesterol from peripheral cells and drive it to the liver for excretion was believed to explain most of the inverse correlation between plasma HDL cholesterol levels and coronary heart disease. In an in vitro model of cholesterol efflux, apoA-I(M)-containing sera from human Milano carriers and apoA-I(M) transgenic mice showed a similar ability to efflux cholesterol compared to control sera despite the nearly 70% lower apoA-I levels in the Milano mice (Franceschini, Calabresi et al. 1999). This led to the conclusion that apoA-I(M) has a significantly higher cholesterol efflux potential than wild-type apoA-I. Despite this, it has been shown that the overall amount of cholesterol efflux is not appreciably higher in an apoA-I(M) genetic background because the apoA-I(M) plasma HDL levels are so much lower than in a wild-type background, suggesting that the atheroprotection apoA-I(M) is not attributable to overall higher cellular lipid mobilization (Weibel, Alexander et al. 2007; Alexander, Weibel et al.
Study of the reduced apoA-I(M) levels in plasma has pointed to a significantly impaired secretion of apoA-I(M) protein in a cultured hepatocytes from human apoA-I(M) gene replacement mice compared to wild-type mice (Parolini, Chiesa et al. 2005). It remains unresolved how the amino acid substitution in apoA-I(M) causes the impaired hepatic secretion and whether the increased dimer formation may inhibit proper protein folding or HDL particle stabilization.

Examinations of the apoA-I(M) protein have collectively suggested that the helix bundle is less stable that wild-type apoA-I. While the mature apoA-I(M) protein is more susceptible to guanidine induced denaturation, overall α-helical content was the same as apoA-I (Zhu, Wu et al. 2005). When complexed with POPC phospholipid, apoA-I(M) forms only two distinct 12.5 and 7.8 nanometer diameter particles and displays significantly higher protease sensitivity compared to wild-type apoA-I (Calabresi, Tedeschi et al. 2001). The N-terminus of the protein is largely spared from limited proteolysis as is wild-type protein, while the C-terminus is more sensitive that apoA-I, displaying six sites in the central and carboxy-terminal portions of the protein (Calabresi, Tedeschi et al. 2001). Since the Milano mutation occurs in the N-terminus, this finding suggests that his suggests that either dimerization or intramolecular interaction via cys173 dramatically changes the presentation and conformation of the C-terminal portion of the protein upon lipid binding. An additional characterization of several amino acid substitutions at position 173 showed that three mutations destabilized the lipid-free proteins more than wild-type according to R173K>R173S>R173C (Alexander, Tanaka et al. 2009). Compared to wild-type apoA-I, apoA-I(M) had a lower affinity for lipids and the R173S apoA-I displayed intermediate affinity. Mice expressing the same R173S protein also displayed an intermediate HDL and cholesterol lowering phenotype compared to wild-type and apoA-I(M) expression. From these findings, it was suggested that the loss of the arginine and its replacement by a cysteine both contribute to the altered properties of apoA-I(M). Based on an intrahelical salt bridge observed between Arg173 and Glu169 in the x-ray crystal structure of the lipid-free apoA-I protein, these authors also suggest that disruption of this salt bridge by the combined loss of the positively charged arginine and repulsion due to the cysteine substitution causes the apoA-I(M) to be significantly destabilized and alter its normal function. Despite the mounting evidence that the Cys173 in apoA-I(M) destabilizes the protein, it remains largely unclear exactly how much of the apoA-I(M) phenotype is attributable to intermolecular dimerization, helix bundle destabilization or disruption of intramolecular interactions. Further studies are required to elucidate the mechanism by which apoA-I(M) imparts its atheroprotective effects.

**Project Design**

Using the information gained from the production of segmental labeled apoE NT, an additional application of EPL has been designed to produce the N-terminal domain of apoA-I(M) for the purpose of eventually studying the in vivo functioning and metabolism of the protein in an atherosclerotic animal model. Taking advantage of the lone amino acid substitution of apoA-I(M) at Cys173, an EPL system was developed in which the first 172 residues of apoA-I were expressed as an intein fusion and residues 173-192 were produced as a synthetic peptide. From the wild-type apoA-I X-ray crystal structure (Ajee,
Anantharamaiah et al. 2006), residues 1-192 form the boundaries of the intact N-terminal domain and are joined to the small C-terminus by a short flexible linker. While it was feasible to consider making an intact, full-length apoA-I(M) using EPL, reconstructing only the N-terminal domain afforded us the ability to construct the protein using a synthetic peptide fragment which has been shown to greatly increase ligation efficiency. Additionally, the use of a peptide as the cysteine-containing, ligation fragment allows for easy modification of a highly soluble peptide for affixing fluorescently labeled tags for application to in vivo imaging.

Preliminary ligation experiments have confirmed that the use of a smaller ligation fragment produces higher EPL yields. Compared to the EPL studies in which similarly sized 8-10 kDa apoE fragments ligated with 60-70% ligation efficiency, the apoA-I(M) ligation efficiency is closer to 90%. Initial tests were performed with untagged proteins but it has been shown that fluorescent tagging does not alter EPL ligation efficiencies in a number of other, similar systems. Because the size of the starting 1-172 apoA-I fragment and the ligated apoA-I(M) N-terminus are very similar and the proteins are untagged, separation of the ligation mixture is an important consideration. One possible solution to this problem is to rely on differential stability, solubility or lipid binding properties to devise a separation scheme. Thus far, differential stability measured by CD spectra has been observed between the 1-172 and 1-192 fragments so it is feasible that this difference can be used to segregate the two protein populations. It is also clear that the 1-172 is less stable by denaturation studies, presumably because it is not an intact and folded domain. This, too, can be used for separation using varying concentration of denaturant. Ultimately, with a clean purification scheme, studies can be undertaken to produce labeled apoA-I(M) for future in vivo imaging.

While studies have shown a number of distinguishing features between apoA-I and apoA-I(M), it is still unclear which of the differences result in the atheroprotective phenotype of the mutant protein. An in vivo monitoring system could provide significant insight into the mechanism of the apoA-I(M) cholesterol lowering and cardioprotective effects. With an EPL system designed for labeling the apoA-I(M) protein at designated positions that are unlikely to affect the in vivo functioning of the protein, such a study is now possible. The EPL system affords significant flexibility in its design, which enhances the likelihood that a bioactive apoA-I(M) probe can be produced and allows for expansion of the study should a full-length, fluorescently labeled protein be required for future studies. Using the EPL labeling techniques in combination with apoA-I-containing rHDL synthetic particles, these studies provide a biologically relevant protein delivery system that have the possibility to be used to great reward for the mechanistic study of apolipoprotein metabolism in the future.
References


