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Amyloid-Forming Properties of Human Apolipoproteins: Sequence Analyses and Structural Insights

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Abstract

Apolipoproteins are protein constituents of lipoproteins that transport cholesterol and fat in circulation and are central to cardiovascular health and disease. Soluble apolipoproteins can transiently dissociate from the lipoprotein surface in a labile free form that can misfold, potentially leading to amyloid disease. Misfolding of apoA-I, apoA-II, and serum amyloid A (SAA) causes systemic amyloidosis, apoE4 is a critical risk factor in Alzheimer's disease, and apolipoprotein misfolding is also implicated in cardiovascular disease. To explain why apolipoproteins are over-represented in amyloidosis, it was proposed that the amphipathic α -helices, which form the lipid surface-binding motif in this protein family, have high amyloid-forming propensity. Here, we use 12 sequence-based bioinformatics approaches to assess amyloid-forming potential of human apolipoproteins and to identify segments that are likely to initiate β -aggregation. Mapping such segments on the available atomic structures of apolipoproteins helps explain why some of them readily form amyloid while others do not. Our analysis shows that nearly all amyloidogenic segments: (i) are largely hydrophobic, (ii) are located in the lipid-binding amphipathic α -helices in the native structures of soluble apolipoproteins, (iii) are predicted in both native α -helices and β -sheets in the insoluble apoB, and (iv) are predicted to form parallel in-register β -sheet in amyloid. Most of these predictions have been verified experimentally for apoC-II, apoA-I, apoA-II and SAA. Surprisingly, the rank order of the amino acid sequence propensity to form amyloid (apoB > apoA-II > apoC-II > apoA-I, apoC-III, SAA, apoC-I > apoA-IV, apoA-V, apoE) does not correlate with the proteins' involvement in amyloidosis. Rather, it correlates directly with the strength of the protein-lipid association, which increases with increasing protein hydrophobicity. Therefore, the lipid surface-binding function and the amyloid-forming propensity are both rooted in apolipoproteins' hydrophobicity, suggesting that functional constraints make it difficult to completely eliminate pathogenic apolipoprotein misfolding. We propose that apolipoproteins have evolved protective mechanisms against misfolding, such as the sequestration of the amyloidogenic segments via the native protein-lipid and protein-protein interactions involving amphipathic α -helices and, in case of apoB, β -sheets.

Keywords

Lipid transport; Systemic amyloidosis; Atherosclerosis and Alzheimer's disease; Atomic protein structure; Sequence-based prediction algorithms

8.1 Lipoproteins and Apolipoproteins in Lipid Transport and Metabolism

Lipids in plasma, lymph and cerebrospinal fluid are transported via lipoproteins that are water-soluble heterogeneous nanoparticles comprised of specific proteins, termed apolipoproteins (apos), and hundreds or even thousands of lipid molecules. Most plasma lipoproteins are secreted by the liver or the intestine, while lipoproteins in the central nervous system are secreted largely by the glial cells (Ladu et al. 2000; Huang 2010). Mature lipoproteins contain an apolar lipid core comprised mainly of cholesterol esters and triacylglycerols (TG, or fat) and an amphipathic surface containing a monolayer of polar lipids, mainly phospholipids and unesterified cholesterol, and apolipoproteins (Fig. 8.1). Apolipoproteins form a flexible scaffold on the lipoprotein surface, which is essential for the lipoprotein structure and function. Apolipoproteins confer curvature to the phospholipid monolayer on the particle surface and thereby help encapsulate apolar lipids in its core. Such encapsulation is necessary to transport water-insoluble lipids in the aqueous environment in plasma, lymph and cerebrospinal fluid. In addition, apolipoproteins direct lipoprotein metabolism by interacting with lipophilic enzymes, lipid transfer proteins, lipid transporters, and lipoprotein receptors (Mahley et al. 1984; Goldstein and Brown 1992; Rached et al. 2014; Phillips 2014). Lipid metabolism is an extremely complex process whose defects can manifest themselves as serious diseases, most notably cardiovascular disease that is the leading cause of mortality worldwide (Castelli et al. 1992; Toth et al. 2013). Lipoproteins and lipoprotein receptors also play major roles in normal brain functions and in neurodegenerative diseases (Lane-Donovan et al. 2014).

Plasma lipoproteins form distinct classes differing in the particle size, density, biochemical composition, and function (Fig. 8.1a). Since proteins, which are denser than lipids, are located on the particle surface, the particle density decreases with increasing size, from high-density lipoproteins (HDL, diameter 8–12 nm) to low-density lipoproteins (LDL, 20–24 nm), intermediate-density lipoproteins (IDL, 25–35 nm), very low-density lipoproteins (VLDL, 40–100 nm), and chylomicrons (>100 nm). Each lipoprotein class has distinct functions (Mahley et al. 1984). Plasma HDL remove excess cholesterol from peripheral cells and deliver it to the liver for excretion via bile or to steroidogenic organs for hormone synthesis. Generally, plasma levels of HDL cholesterol (a. k. a. “good cholesterol”) and the major HDL protein, apoA-I, correlate inversely with the risk of developing atherosclerosis (Gordon et al. 1989; Toth et al. 2013), although this relationship is more complex than previously thought. LDL are the major plasma carriers of cholesterol in the form of cholesterol esters, which deliver it to peripheral tissues. Plasma levels of LDL cholesterol (“bad cholesterol”) and the major LDL protein, apoB, are the strongest causative risk factors in cardiovascular disease (Castelli et al. 1992). IDL are transient metabolic products of VLDL. VLDL, which are the major plasma carriers of TG, are metabolic precursors of LDL and a risk factor for metabolic syndrome and cardiovascular disease (Krauss 1998). Chylomicrons, which are generated in the gut to transport dietary lipids, contain one copy of a truncated form of apoB, termed B48 (N-terminal 48 %), and various exchangeable proteins.

Each class of plasma lipoprotein has distinct protein composition (Fig. 8.1a). HDL particle contains several copies of exchangeable (water-soluble) apolipoproteins, mainly apoA-I (28 kDa) and apoA-II (9 kDa) that constitute approximately 70 % and 20 % of the total HDL protein mass, respectively, along with minor proteins (apoE, apoCs, etc.) LDL particle contains one copy of the non-exchangeable (water-insoluble) apoB (550 kDa) that constitutes ~95 % of the total LDL protein mass. VLDL particle contain one copy of apoB and multiple copies of exchangeable proteins, mainly apoE (33 kDa) and apoCs (6–9 kDa). Chylomicrons contain one copy of a non-exchangeable protein apoB48, as well as exchangeable proteins (apoE, apoCs, etc.) Other lipophilic proteins including apolipoproteins (apoA-IV, apoA-V, apoD, apoF, apoH, apoJ, etc.) are also found as minor components on lipoproteins. Lipoprotein composition can significantly change during inflammation when most of apoA-I is displaced from plasma HDL by an acute-phase protein, serum amyloid A (SAA) (van der Westhuyzen et al. 2007; Eklund et al. 2012). In contrast to plasma, lipid transport in the central nervous system is mediated solely by HDL-like particles that contain apoE as their major protein (Huang 2010; Hauser et al. 2011).

Exchangeable apolipoproteins form a multigene family that has evolved from a common ancestral gene via the duplication and deletion of 11-mer codon repeats (Li et al. 1988). These proteins have similar gene structure containing 11-mer tandem repeats encoding for amphipathic α -helices that form the major structural and functional motif in this protein family (Segrest et al. 1990, 1992, 1994). Similar 11-mer α -helical repeat motifs are also found in other lipid surface-binding proteins, including parts of the non-exchangeable apoB, as well as in soluble apolipoprotein-related proteins such as SAA and α -synuclein (described by Uversky in Chap. 2 and by Colón and colleagues in Chap. 5 of this volume).

Although over 90 % of all apolipoproteins circulate on lipoproteins, a small fraction of the exchangeable proteins can transiently dissociate from the lipid surface (Fig. 8.1b). This occurs during inflammation, as well as during normal metabolic remodeling of lipoproteins which alters their surface-to-volume ratio and generates excess surface material that can dissociate in the form of apolipoproteins that carry little lipid. Such proteins are termed “lipid-poor” or “lipid-free”; in this chapter, we call them “free” for brevity. Free apolipoproteins can also be generated de novo. They form a metabolically active structurally labile species that can be rapidly recruited to the lipid surface (Rye and Barter 2004; Mulya et al. 2008; Jayaraman et al. 2012) or get degraded or misfolded (Hatters and Howlett 2002) (Fig. 8.1b). The misfolded apolipoproteins can deposit as amyloid fibers, potentially leading to disease.

8.2 Apolipoproteins in Amyloid Diseases

Exchangeable apolipoproteins are prominent players in amyloidoses (Hatters and Howlett 2002). In particular, apoE, which is an important multifunctional apolipoprotein in the central nervous system and in plasma (Getz and Reardon 2009), is found in most systemic and cerebral amyloid deposits in humans and is often co-deposited with other apolipoproteins. Human apoE4 isoform is a major causative risk factor in Alzheimer’s and other neurodegenerative diseases (Mahley et al. 2009; Huang 2010; Hauser et al. 2011). Another important plasma apolipoprotein, apoA-I, commonly deposits as fibrils in

atherosclerotic plaques in a process that probably contributes to atherosclerosis (Mucchiano et al. 2001; Röcken et al. 2006; Howlett and Moore 2006; Teoh et al. 2011a; Ramella et al. 2011). Moreover, in a rare hereditary form of apoA-I amyloidosis, the N-terminal fragments of apoA-I deposit as fibrils in vital organs (kidneys, liver, heart, etc.) and damage them (Obici et al. 2006; Rowczenio et al. 2011; Ramella et al. 2012; Gursky et al. 2012). Approximately 20 mutations in the apoA-I gene have been linked to this serious disease. A similar disease has been linked to stop codon mutations in human apoA-II, the second-major protein in plasma HDL. These rare mutations produce a C-terminal extension in apoA-II that causes renal amyloidosis and, ultimately, kidney failure in humans (Benson et al. 2001, 2011). In addition, amyloid deposits of a minor apolipoprotein, human apoA-IV, have been found in vivo (Bergström et al. 2004). To-date, no amyloid diseases involving apoCs have been reported. Nevertheless, human apoC-II readily forms amyloid fibrils in vitro in a process that was extensively studied by Howlett and colleagues, providing the most comprehensive model available to-date for understanding the misfolding of larger apos (summarized by Ryan et al. in Chap. 7 of this volume.) Human apoC-III can also form amyloid fibrils in vitro (de Messieres et al. 2014). Moreover, apolipoproteins such as apoC-III, apoA-I and apoE were implicated in binding amyloid- β ($A\beta$) peptide in circulation and were proposed as potential bio-markers or risk factors of Alzheimer's disease (Lewis et al. 2010; Shih et al. 2014).

The large heavily glycosylated non-exchangeable apoB has also been reported to bind $A\beta$ peptide (Safar et al. 2006). Moreover, apoB has been proposed to form amyloid-like structure in several in vitro studies of LDL (Stewart et al. 2005; Parasassi et al. 2008; Brunelli et al. 2014), and apoB misfolding in vivo was proposed to contribute to atherosclerosis (Ursini et al. 2002; Asatryan et al. 2005; Brunelli et al. 2014). Although aggregation of modified LDL was observed in these and other studies, including our own work (Jayaraman et al. 2007; Lu et al. 2012; Lu and Gursky 2013), none of these studies provided solid evidence for amyloid formation by apoB. In fact, electron and atomic force microscopy did not show typical amyloid fibers, fiber diffraction has not been reported, spectroscopic studies did not show a large increase in the β -sheet content of apoB upon LDL aggregation, and thioflavine T fluorescence studies were inconclusive since this dye binds not only to amyloid fibrils but also to lipid surfaces (Jayaraman et al. 2007; also see Chap. 6 by Gorbenko and colleagues in this volume). In sum, amyloid formation by apoB awaits experimental verification.

Other lipid surface-binding proteins that contain similar amphipathic α -helices are also prominent in amyloid diseases. In particular, N-terminal fragments of SAA, an acute-phase protein that displaces apoA-I from HDL during inflammation, is deposited in type-A amyloidosis and is also implicated in rheumatoid arthritis and atherosclerosis (van der Westhuyzen et al. 2007; Eklund et al. 2012). SAA in solution is an intrinsically disordered protein that acquires amphipathic α -helical structure on the lipid surface (Segrest et al. 1976) and forms cross- β -sheet in amyloid (see Chap. 5 by Colón et al. in this volume). Another apolipoprotein-related protein, α -synuclein (described by Uversky in Chap. 2 of this volume), is also intrinsically disordered in solution, acquires an amphipathic α -helical structure upon binding to a lipid bilayer, and can form extracellular β -sheet rich amyloid deposits in neurodegenerative diseases such as Parkinson's (Silva et al. 2013).

Why are apolipoproteins and related proteins overrepresented in amyloidoses? Hatters and Howlett (2002) noted that low structural stability and high conformational flexibility of free apolipoproteins, which facilitate their rapid incorporation into the lipid surface, make them labile to misfolding. Importantly, these and other authors have postulated that direct precursors of amyloid are the structurally labile free apolipoproteins, rather than their stable lipoprotein-bound counterparts (Hatters and Howlett 2002; Benson et al. 2011; Gursky et al. 2012; Hauser and Ryan 2013). This concept is supported by studies from several groups including ours showing that free apos in solution adopt a molten globule-like conformation with substantial α -helical content, flexible tertiary structure, low thermodynamic stability, low unfolding cooperativity, substantial solvent exposure of apolar groups, and high aggregating propensity (Gursky and Atkinson 1996a, b; Morrow et al. 2002; Guha et al. 2008). These properties are likely to facilitate β -aggregation.

In contrast to free apolipoproteins, lipoproteins are stabilized by high free energy barriers arising from disruption of extensive protein and lipid interactions during lipoprotein remodeling (Mehta et al. 2003; Jayaraman et al. 2005b, 2006; Guha et al. 2007; Lu et al. 2012). Such remodeling involves partial dissociation of apolipoproteins and changes in lipoprotein morphology such as fusion (Gursky 2005). Free energy barriers decelerate dissociation of apolipoproteins from the lipid surface and thereby delay their misfolding and proteolysis. In sum, strong evidence supports the idea that dissociation from the lipoprotein surface, which generates labile free apolipoproteins, is an obligatory early step in apolipoprotein misfolding and β -aggregation.

The amphipathic α -helix, which is the major lipid surface-binding motif in apolipoproteins and related proteins (Segrest et al. 1990, 1994), was proposed to have high intrinsic propensity for self-association and amyloid formation (Howlett and Hatters 2002). Apolipoprotein α -helices, termed class-A helices, are distinct from the class-G helices found in globular proteins (Segrest et al. 1990). First, class-A helices are comprised of 11-mer tandem repeats (rather than 7-mer repeats typical of globular proteins) that are optimized for lipid surface binding via the apolar helical faces. These large relatively flat apolar faces comprise 30–50 % of the total helical surface area (Fig. 8.2) as compared to ~15 % in typical globular proteins. In addition, class-A helices have a relatively large fraction of charged residues (~30 %) with most basic groups flanking the apolar face and most acidic groups located in the middle of the polar face (Fig. 8.2). Such charge distribution facilitates formation of salt bridge networks and is also thought to contribute to lipid surface binding (Segrest et al. 1994). In sum, apolipoprotein α -helices have a distinct distribution of hydrophobic and charged residues and have relatively few polar groups. This contrasts with prion proteins whose misfolding is driven by a domain that is rich in polar residues and poor in charged and hydrophobic groups (reviewed by Shewmaker et al. 2011).

Apolipoproteins readily self-associate in solution in a process that is distinct from β -aggregation. Such self-association is mediated via the apolar helical faces and, similar to lipid surface binding, preserves or even enhances the helical structure (Gursky and Atkinson 1998). Whether or not the apolipoprotein α -helices have high intrinsic propensity to convert into cross- β -sheet in amyloid is unclear. Recent advances in the bioinformatics approaches enable us to test this idea.

8.3 Recent Advances in Structural and Bioinformatic Studies

Last decade saw important progress in clinical and basic research of apolipoproteins as well as in molecular-level studies of amyloids. In this chapter, we combine recent advances in the structural studies of apolipoproteins with the newly developed amyloid prediction methods to better understand apolipoprotein misfolding.

High-resolution structural studies of apolipoproteins are notoriously difficult because of structural flexibility and high aggregating propensity of these proteins. After years of careful construct optimization, stable non-aggregating apolipoproteins amenable to high-resolution structural analyses have been produced, and atomic structures of several human apolipoproteins have been determined in lipid-free state. These include solution structure of modified full-length apoE determined by NMR (Chen and Wang 2011) and two high-resolution X-ray crystal structures, one of the N-terminal ~75 % residue fragment of apoA-I (Mei and Atkinson 2011) and another of the central domain of apoA-IV (Deng et al. 2012). These structures have provided tremendous insights into the functions of these proteins on and off the lipid surface. In addition, high-resolution structure of human amyloidogenic isoform of serum amyloid A, SAA1.1, in two crystal forms has been determined, providing the much-needed structural basis for understanding amyloid formation by this protein (Lu et al. 2014). Months later, x-ray crystal structure of murine SAA3 has been reported at 2 Å resolution (Derebe et al. 2014). The molecular structures of human SAA1.1 and murine SAA3 turned out to be very similar although the oligomeric states were different, with SAA1.1 crystallized as a dimer or a hexamer (Lu et al. 2014) and SAA3 forming a functional tetramer (Derebe et al. 2014). Notably, all these atomic structures represent free proteins that are direct precursors of amyloid (Howlett and Hatters 2002; Benson et al. 2011; Das et al. 2014).

Another area of recent advances is in the development and improvement of the amyloid prediction software (reviewed by Dovidchenko and Galzitskaya in Chap. 9 of this volume). During the last decade, it became increasingly clear that a wide variety of unrelated proteins and peptides can form amyloid in a process initiated by a limited number of 4- to 10-residue segments, or “hot spots”, that have high intrinsic propensity for β -aggregation (Ventura et al. 2004; Thompson et al. 2006; Tzotzos and Doig 2010). Approximately 20 sequence-based algorithms are currently available to predict such segments, including Tango, Waltz, Aggrescan, Zygggregator, 3D Rosetta-based method, FoldAmyloid and PASTA, to name a few. These algorithms use a variety of approaches, from the analysis of side chain distribution to molecular dynamics simulations, and apply a wide range of criteria, such as the side chain hydrophobicity, charge distribution, average packing density, dual α -helix and β -sheet propensity, hidden α -helix to β -sheet switches, β -sheet contiguity, correspondence to known amyloidogenic residue patterns, etc. (Galzitskaya et al. 2006; López de la Paz and Serrano 2004; Fernandez-Escamilla et al. 2004; Conchillo-Solé et al. 2007; Hamodrakas et al. 2007; Trovato et al. 2007; Zhang et al. 2007; Zibae et al. 2007; Kim et al. 2009; Tian et al. 2009; Maurer-Stroh et al. 2010; Garbuzynskiy et al. 2010; O'Donnell et al. 2011). This multitude of criteria and approaches reflects the fact that β -aggregation and fibril formation is a complex process that can be induced in unrelated proteins by a wide range of factors. Perhaps as a result of this immense complexity, individual algorithms have only limited

predictive power. To optimize the selectivity and specificity of the prediction, i.e. minimize the probability of false positives and negatives, a consensus method has been developed by Hamodrakas and colleagues. We use the expanded version of this method, termed AmylPred2 (Tsolis et al. 2013), which combines 11 individual algorithms.

In addition, we use amyloid prediction software PASTA (Trovato et al. 2007; Walsh et al. 2014) and its expanded version, PASTA2.0 (Walsh et al. 2014), that not only identifies the amyloidogenic segments but also estimates free energy of their pairing and thereby predicts which amino acid register and β -sheet orientation, parallel or antiparallel, is energetically favored. As detailed by Dovidchenko and Galzitskaya in Chap. 9 of this volume, AmylPred2 and PASTA2 are among the best amyloid prediction methods currently available. Following Trovato and colleagues (2007), we assumed that a reliable prediction of the β -sheet pairing corresponds to favorable (negative) PASTA energy of -4 units or lower (1 PASTA energy unit = 1.192 Kcal/mol). This threshold value corresponds to 73.8 % sensitivity and ~15 % selectivity, reflecting the respective rates of false negatives and positives. In this chapter we focus on segment pairings with PASTA2.0 energy circa -4 or lower.

Here, we combine these sequence-based predictions and, whenever possible, their experimental validations, with the available high-resolution structural information to glean new insights into the complex processes of apolipoprotein misfolding. The main focus of this chapter is on the major members of this protein family whose structures and amyloid-forming properties have been explored experimentally. Several minor apolipoproteins are also addressed. Comparison of these proteins leads to surprising new results and helps understand why some of them readily form amyloid while others do not.

8.4 New Insights into Apolipoprotein Misfolding from Sequence and Structural Analyses

8.4.1 Apolipoprotein A-I

Human apolipoprotein A-I (243 a. a.) is the major HDL protein that binds and activates lipid transporters, lipophilic enzymes, and HDL receptor, and thereby orchestrates crucial steps in the HDL biogenesis and functions (Navab et al. 2011; Philips 2013). Although plasma levels of apoA-I and HDL cholesterol (a. k. a. Good Cholesterol) have long been known to correlate inversely with the risk of cardiovascular disease (Gordon et al. 1989), recent studies revealed that this relationship is complex and involves additional factors, particularly functional properties of individual HDL subclasses (Rothblat and Philips 2010; Navab et al. 2011; Banach 2014). The role of these factors is intensely investigated in an effort to develop new HDL-based therapies to complement statins and other lipid-lowering drugs (Kingwell et al. 2014). The prevailing concept is that the protective function of HDL and apoA-I stems mainly from their central role in reverse cholesterol transport, which is the sole pathway of cholesterol removal from the body (Sorci-Thomas and Thomas 2013; Phillips 2014). Additional protection stems from anti-inflammatory, anti-oxidant and antithrombotic effects of apoA-I (Navab et al. 2011).

In plasma, apoA-I is in a dynamic equilibrium between lipid-bound and free forms, both important for reverse cholesterol transport (Rye and Barter 2004). Nearly 95 % of plasma apoA-I circulates in an HDL-bound form that is stabilized by high free energy barriers (Mehta et al. 2003; Jayaraman et al. 2006). The remaining ~5 % forms a transient monomeric lipid-poor/free apoA-I that is generated de novo or through dissociation from HDL (Mulya et al. 2008; Cavigiolio et al. 2010; Jayaraman et al. 2012). This metabolically active marginally stable state probably initiates protein misfolding in amyloid.

Human apoA-I amyloidosis takes on two distinct forms, acquired and familial. Acquired amyloidosis is the most common form where full-length non-variant apoA-I deposits as fibers in arteries and tissues (Röcken et al. 2006; Howlett and Moore 2006; Ramella et al. 2011). Such fibrils were found in aortic plaques of >50 % of patients who underwent atherectomy (Mucciano et al. 2001; Röcken et al. 2006). Although the causes and consequences of apoA-I fibril deposition in vivo are unclear, increasing evidence suggests that this process is pro-atherogenic (Ramella et al. 2011; Teoh et al. 2011a). In fact, apoA-I fibrils reportedly increase vulnerability of atherosclerotic plaques, activate arterial macrophages, and are toxic to cells (Suzuki et al. 2005; Lepedda et al. 2009; Patel et al. 2010; Adachi et al. 2013). Moreover, full-length wild-type human apoA-I can form amyloid in vitro upon methionine oxidation, suggesting a potential mechanism for apoA-I fibrillogenesis in the oxidative environment of atherosclerotic plaques (Wong et al. 2010; Chan et al. 2015).

Familial apoA-I amyloidosis (AApoAI) has entirely different clinical presentation. In this autosomal dominant disorder, fragments 1–83 to 1–93 of mutant apoA-I deposit as fibers in vital organs (kidney, liver, heart, nerves, skin, spleen, testes, etc.) causing organ damage (Obici et al. 2006; Ramella et al. 2012). The only treatment currently available for this potentially lethal disease is end-stage organ transplant (Gillmore et al. 2006). The mechanism of tissue damage and the pathogenic species (apoA-I oligomers or fibrils) are unknown. Moreover, it is unclear whether proteolytic cleavage that generates the N-terminal 9–11 kDa fragments found in amyloid deposits precedes or follows apoA-I misfolding (Wong et al. 2010). It is also unclear why different AApoAI mutations generate N-terminal fragments cleaved at different sites that are distant from the mutation site, and what proteases are responsible for it in vivo. Another conundrum is that symptoms associated with the same AApoAI mutation can range from mild to severe (Obici et al. 2006; Rowczenio et al. 2011), suggesting that additional, still unknown, factors are involved. We hypothesized that such factors include HDL lipids that can prevent or promote apoA-I dissociation, and thereby influence the population of the free protein (Das et al. 2014). Yet another puzzle is that AApoAI patients have lower than normal plasma levels of apoA-I and HDL resulting from reduced secretion or enhanced degradation of the protein (Marchesi et al. 2011; Arciello et al. 2011). Hence, unlike many other amyloid diseases, AApoAI is not due to protein overproduction. Paradoxically, despite apoA-I and HDL deficiency, AApoAI patients are not at a high risk for atherosclerosis (Obici et al. 2006; Rowczenio et al. 2011), which illustrates the complex relationship between plasma levels of HDL and cardiovascular disease.

Nearly 20 naturally occurring human AApoAI mutations have been identified, mainly point substitutions as well as some frame shifts and deletions (Rowczenio et al. 2011; Obici et al. 2006; Gursky et al. 2012, and references therein). Most of these mutations are located within the N-terminal segment found in amyloid deposits (“inside” mutations), yet several “outside” point mutations have also been found in residues 170–178. This raises a question: how can the “outside” mutations in the C-terminal half of the apoA-I molecule trigger amyloid deposition by its N-terminal part? The answers to this and other questions are suggested by a combined structural and sequence analysis of apoA-I.

Structural analysis of apoA-I has been complicated by its high conformational flexibility and aggregating propensity. Although the atomic structure of full-length apoA-I in solution or on HDL is not available, two x-ray crystal structures of lipid-free truncated human apoA-I have been determined: a low-resolution 4 Å structure of the N-terminally truncated apoA-I, (1–43)apoA-I (Borhani et al. 1997) and a high-resolution 2.2 Å structure of the C-terminally truncated apoA-I, (185–243)apoA-I (Mei and Atkinson 2011). These important breakthroughs provided tremendous insights into the structure-function relationship of apoA-I (Brouillette et al. 2001; Mei and Atkinson 2011; Gursky 2013; Phillips 2013 and references therein). Notably, the (185–243) apoA-I construct contains all sites of AApoAI mutations and all three apoA-I methionines (M86, M112, and M148), providing a useful model for understanding the effects of these mutations and Met oxidation on apoA-I misfolding (Gursky et al. 2012; Das et al. 2014).

Lipid-free (185–243)apoA-I forms a crystallographic dimer of two antiparallel largely α -helical molecules, stabilized by two four-segment bundles at its ends. The helices are kinked at Pro positions to form an arc 11 nm in diameter, commensurate with that of HDL (Fig. 8.3). This structure provides the basis for understanding the conformational ensemble of full-length apoA-I, either as a dimer on HDL surface or as a free monomer (Mei and Atkinson 2011; Gursky 2013). Domain swapping around the dimer twofold axis was proposed to convert the dimer into a monomer with a similar helix bundle structure (Mei and Atkinson 2011) (Fig. 8.4c). Mapping AApoAI mutations on this structure showed that most “inside” and all “outside” mutation sites are located in the well-ordered “bottom” half of the helix bundle (Fig. 8.3). This location helped explain the role of the “outside” mutations in structural integrity of free protein and prompted us to propose that these mutations destabilize the helix bundle and thereby promote its misfolding (Gursky et al. 2012).

The crystal structure also revealed that the residue segment 44–55 adopted an unpaired β -strand-like conformation that is unique in apoA-I (Fig. 8.4, dark red), suggesting that this segment helps initiate α -helix to β -sheet conversion (Gursky et al. 2012). Wong et al. (2012) reported that these residues are required for fibril formation. However, segment 44–55 turned out to have only modest predicted sequence propensity to form amyloid (Das et al. 2014). Since in free protein this polar segment is dynamic and solvent-exposed (Chetty et al. 2009; Mei and Atkinson 2011), its relatively low sequence propensity for β -aggregation probably helps protect free apoA-I from misfolding.

Surprisingly, experimental studies of selected naturally occurring mutants of human apoA-I, including common AApoAI variants G26R and W50R, showed that substantial global

destabilization of the free protein or its reduced affinity for HDL surface is neither necessary nor sufficient for amyloid formation ((Das et al. 2014) and references therein). This prompted us to search for additional factors driving apoA-I misfolding. We postulated that such factors involve local perturbations in sensitive protein regions, or amyloid “hot spots”. To identify such regions, we performed amino acid sequence analyses of human apoA-I by using AmylPred2 and PASTA2.0.

Figure 8.4 summarizes the results of this analysis. AmylPred2 profile shows that most amyloidogenic propensity resides in the N-terminal ~10 kDa segment that deposits in familial amyloidosis. This N-terminal part contains one major amyloid hot spot predicted by up to 10 out of 11 methods in residues 14–22 (LATVYVDVL), and two minor hot spots predicted by four to five methods in residues 53–58 (VTSTFS) and 69–72 (QEFW) (Fig. 8.4a). The only other major amyloidogenic segment is predicted in residues 227–232 (VSFLSA) that form the primary lipid binding site in apoA-I and are largely α -helical on the lipid (Borhani et al. 1997; Lyssenko et al. 2012; Nagao et al. 2014 and references therein). Notably, all predicted amyloidogenic segments are highly hydrophobic. Consistent with AmylPred2, PASTA2 analysis predicts three amyloidogenic segments, with 14–22 being the strongest. These predictions are supported by experimental studies of peptide fragments containing these segments, which readily formed fibrils in vitro (Wong et al. 2012; Adachi et al. 2014; Das et al. 2014; Mendoza-Espinosa et al. 2014).

Moreover, PASTA2 predicts that the major amyloidogenic segment, 14–22, forms parallel in-register intermolecular β -sheet in amyloid (favorable PASTA2 energy -6.1 , Fig. 8.4b). This prediction is corroborated by the observation that FTIR spectra of amyloid fibers formed by apoA-I fragment 1–43 lacked the diagnostic peak circa $1,680\text{ cm}^{-1}$ (Adachi et al. 2013) and thus, indicated parallel β -sheet. Moreover, the structural model of 1–83 fragment, which was derived on the basis of fluorescence studies described by Gorbenko et al. in Chap. 6 of this volume, also comprises a parallel in-register intermolecular β -sheet. Similar to the N-terminal segment 14–22, the C-terminal amyloidogenic segment 227–232 is predicted by PASTA to form a parallel in-register β -sheet, albeit with less favorable energy. Such a molecular orientation can facilitate synergy between the two major N- and C-terminal hot spots during amyloid formation by the full-length apoA-I.

Notably, the vast majority of known naturally occurring human apoA-I mutations (Sorci-Thomas and Thomas 2002) are located outside the predicted amyloid hot spots, with very few exceptions such as F71Y located inside a minor hot spot in residues 69–72. Such mutations are not expected to significantly alter the sequence propensity to form amyloid. In fact, our analysis suggests that AApoAI mutations have little or no effect on the proteins' amyloidogenic profiles predicted by AmylPred2 or PASTA2, suggesting that amyloid formation by these mutants in vivo results, at least in part, from factors other than their intrinsic sequence propensity to form amyloid. To identify such factors, we mapped the predicted amyloidogenic segments on the crystal structure of (185–243)apoA-I. The results were quite revealing.

The three N-terminal amyloidogenic segments form a well-ordered α -helical cluster in the “bottom” half of the four-segment bundle (Fig. 8.4c), close to the sites of most AApoAI

mutations. In wild type apoA-I, this well-ordered native helical structure is probably instrumental in protecting the amyloidogenic regions from β -aggregation. The C-terminal amyloidogenic segment, 227–238, is probably protected from misfolding by forming an amphipathic α -helix that strongly binds lipid; in free protein, this C-terminal helix probably folds back against the N-terminal bundle to pack against its apolar groove. Therefore, we propose that protein misfolding in AApoAI is triggered by perturbed native helical packing in the N-terminal amyloidogenic segments, which increases their mobility and solvent exposure and thereby facilitates β -aggregation (Das et al. 2014).

This idea is supported by the analysis of local protein structure and dynamics by hydrogen-deuterium exchange mass spectrometry, which was pioneered for apoA-I by Phillips, Englander and colleagues (Chetty et al. 2009, 2012) and is continued by our team. These H-D exchange studies showed increased mobility in the first ~100 residues in G26R mutant, which was proposed to help generate N-terminal fragments found in amyloid deposits (Chetty et al. 2012); however, it was unclear why the proteolysis was limited to just one site from this long mobile helix.

Our proposed mechanism helps explain why methionine oxidation promotes fibril formation in full-length wild-type human apoA-I (Wong et al. 2010). Two of the three apoA-I methionines, M112 and M148, are located at the top of the helix bundle, remote from amyloidogenic segments or the sites of AApoAI mutations (Fig. 8.4c). In contrast, M86 is buried in the hydrophobic cluster at the bottom of the bundle in close proximity to amyloidogenic segments and AApoAI mutation sites. Replacement of the apolar Met side chain with polar Met sulfoxide in position 86 will probably perturb this hydrophobic cluster, particularly the major amyloidogenic segment 14–22. Hence, oxidation of M86 is expected to perturb native packing in this sensitive region (Das et al. 2014). Experimental analyses by Cavigliolo, Jayaraman and colleagues (Chan et al. 2015) helped establish the role of individual Met oxidation in apoA-I fibrillogenesis.

Taken together, the existing studies suggest a unified mechanism of apoA-I misfolding in acquired and familial amyloidosis (Fig. 8.5). We hypothesize that the misfolding is initiated by perturbed amyloidogenic segments in apoA-I, particularly in residues 14–22. On HDL, such segments form amphipathic α -helices that are protected by lipid binding. Transient dissociation from HDL generates the labile free conformation in which these segments are normally protected by their packing in the native helix bundle structure. AApoAI mutations or Met oxidation oxidation perturbs this native packing, which increases the mobility and solvent accessibility of the amyloidogenic segments and triggers their β -aggregation.

This proposed mechanism provides a new explanation why AApoAI deposits contain N-terminal proteolytic fragments cleaved between residues 83 and 93, even though this region is normally protected from proteolysis by its well-ordered native α -helical structure. We hypothesize that the parallel intermolecular β -sheet, which is probably nucleated by the “hot spot” residues 14–22, readily propagates through the N-terminal 75 residues that have high amyloid-forming propensity (Fig. 8.9a). This propagation is probably impeded by the putative β -breaking motif EKETEG in residues 76–81 due to Columbic repulsion among the proximal Glu at pH~7. Hence, residues 1–75 are expected to form the amyloid core; the

polypeptide outside this core can be readily proteolysed. According to this mechanism, amyloid core formation precedes apoA-I proteolysis. Still, an alternative (or perhaps an additional) pathway in which proteolytic cleavage of the highly amyloidogenic N-terminal segment happens first is also potentially possible. Which pathway is more relevant to the in vivo conditions is still an open question that can only be resolved once we gain better understanding of apoA-I proteolysis in vivo.

Our hypothetical mechanism also helps explain why arterial deposits in acquired amyloidosis commonly contain full-length apoA-I. In anaerobic regions of deep atherosclerotic plaques, the pH can drop as low as 5.5, potentially leading to partial protonation of Glu and formation of carboxyl pairs in the EKETEG segment. As a result, the repulsion among these carboxyls is diminished or even replaced with attraction, which is expected to facilitate β -zipper propagation from the N- to the C-terminus to encompass all amyloid hot spots in apoA-I in a parallel in-register β -sheet conformation.

This hypothetical mechanism of apoA-I mis-folding in various types of human amyloidosis is supported by our on-going studies using hydrogen-deuterium exchange. It illustrates the combined power of structural, bioinformatic and biophysical approaches in dissecting protein mis-folding pathways and elucidating the role of protein-lipid interactions and proteolytic degradation in these complex processes.

8.4.2 Apolipoprotein A-II

Apolipoprotein A-II (77 a. a.), which is the second-major HDL protein, is the most hydrophobic exchangeable apolipoprotein. Human apoA-II forms a homodimer disulfide-linked via Cys6, which circulates largely on mature mid-size plasma HDL that also contain apoA-I (Gillard et al. 2009; Gao et al. 2012). In contrast to apoA-I whose cardioprotective role is well-established, the function of apoA-II is less clear. The emerging consensus is that apoA-II restricts the apoA-I conformation on HDL and thereby indirectly influences the interactions of apoA-I with its functional ligands which are critical in HDL metabolism (Silva et al. 2007; Gao et al. 2012; Maiga et al. 2014 and references therein).

Our current knowledge of the apoA-II structure is limited to low resolution. Human apoA-II is proposed to form a highly α -helical antiparallel dimer on HDL (Silva et al. 2007), yet in solution the protein is only partially folded and is marginally stable (Gursky and Atkinson 1996b). The amino acid sequence of apoA-II is predicted to form class-A amphipathic α -helices with particularly large apolar faces spanning $\sim 180^\circ$ (Fig. 8.2) as compared to $\sim 120^\circ$ typical of other exchangeable apos (Segrest et al. 1994). As a result, apoA-II α -helices insert more deeply into the phospholipid monolayer and bind more strongly to mature HDL than any other apos (Gao et al. 2012 and references therein). We propose that this strong association with mature HDL, which makes apoA-II practically non-exchangeable, helps protect it from misfolding.

In humans, apoA-II misfolding can cause hereditary amyloidosis, a rare autosomal dominant disorder resulting from stop codon mutations that produce a 21-residue C-terminal extension in residues 78–98 (Benson et al. 2001). Full-length mutant apoA-II forms amyloid deposits

primarily in kidneys, causing renal damage and failure. The only existing treatment for apoA-II amyloidosis is dialysis followed by kidney transplant (Magy et al. 2003).

Amino acid sequence analysis helps explain why mutant but not wild-type human apoA-II forms amyloid in vivo (Gursky 2014). In wild-type human apoA-II, two amyloid hot spots are predicted strongly in residues 10–18 (LVSQYFQTV) and 60–70 (LVNFLSYFVEL). These hydrophobic segments are predicted by 8–11 methods in AmylPred2 and have PASTA2 energies ranging from –4.4 to –7.1 (Fig. 8.6). This is the strongest predicted amyloidogenic propensity of all exchangeable apolipoproteins, approaching that of Alzheimer's A β (1–42) peptide (Fig. 8.7). Importantly, the two predicted amyloidogenic segments in apoA-II have been verified experimentally in peptide fragment studies of murine apoA-II that is homologous to the human protein (Sawashita et al. 2009). Similar to apoA-I, the amyloidogenic segments in apoA-II are located inside the predicted lipid-binding class-A α -helices (Fig. 8.6), which is expected to protect them from misfolding.

One likely reason why the C-terminal extension leads to amyloidosis is because, unlike the rest of the apoA-II molecule, this extension cannot form an amphipathic α -helix and hence, is not well-suited for lipid surface binding (Benson et al. 2011; Gursky 2014). As a result, the lipid binding affinity of the mutant protein is expected to decline, shifting the population distribution from HDL-bound to free apoA-II that is labile to misfolding. Another likely reason is that the C-terminal extension is predicted to form an additional amyloidogenic segment that can act in synergy with the major amyloidogenic segment in residues 60–70. In fact, both these segments are predicted strongly to form parallel in-register β -sheet in amyloid (Fig. 8.6c) (Gursky 2014).

In sum, compared to other exchangeable apos, normal human apoA-II is the most hydrophobic, binds most strongly to mature HDL, and has the highest sequence propensity to form amyloid. Two major amyloidogenic segments predicted in normal apoA-II have been verified experimentally (Sawashita et al. 2009). These segments are located inside the native amphipathic α -helices, and the strongest one (residues 60–70) is predicted to form parallel in-register β -sheet in amyloid. We propose that the reason why normal human apoA-II does not form amyloid is probably due to its particularly strong association with mature HDL, which is further enhanced by the protein dimerization via the disulfide link. This disulfide may also potentially interfere with the structural transition from an antiparallel α -helical dimer (Silva et al. 2007) into a parallel intermolecular β -sheet. C-terminal extension that causes human apoA-II amyloidosis is expected to have a dual effect: it reduces the lipid-binding affinity of the protein and also increases its propensity to form parallel in-register intermolecular β -sheet (Fig. 8.6).

8.4.3 Apolipoprotein A-IV

Human apoA-IV (376 a. a.) is the largest known exchangeable apolipoprotein. It is synthesized in the gut, is secreted on chylomicrons, and circulates in plasma in HDL-bound and in free states (Green et al. 1980). This minor apolipoprotein is proposed to regulate chylomicron assembly, modulate reverse cholesterol transport, serve as an anti-oxidant and as a satiety signal, and perform other functions in lipoprotein metabolism (Deng et al. 2012 and references therein). ApoA-IV was also implicated in clearance of A β peptide in the

brain (Cui et al. 2011). A case of human cardiac amyloidosis has been reported in which the N-terminal 70-residue fragment of wild-type apoA-IV deposited as fibrils (Bergström et al. 2004).

Similar to other exchangeable apolipoproteins, apoA-IV structure is comprised mainly of amphipathic α -helices. A distinct property of apoA-IV is that it contains a globular core domain (approximately residues 97–311) flanked by the N- and C-terminal segments whose interactions regulate lipid binding (Walker et al. 2014). The x-ray crystal structure of the core domain residues 64–335, which was determined to 2.4 Å resolution by Davidson's team (Deng et al. 2012), resembled that of the C-terminally truncated apoA-I (Mei and Atkinson, 2011). Similar to the N-terminal 75 % of apoA-I, the core domain of apoA-IV forms a four-helix bundle that dimerizes via the domain swapping of a helical segment (Fig. 8.8c) (Deng et al. 2012). In contrast to the highly curved apoA-I dimer that matches the HDL surface curvature, the apoA-IV dimer is nearly linear, which is consistent with its ability to bind much larger lipoproteins with lower surface curvature.

Compared to other exchangeable apos, apoA-IV binds lipid surface relatively poorly, consistent with its near-equal plasma distribution between lipid-bound and free states. Modest lipid binding affinity of apoA-IV is attributed to the strong interactions between its N- and C-terminal domains that hold the protein molecule in a closed conformation via the so-called “clasp mechanism” that modulates helix bundle opening on the lipid (Deng et al. 2013; Walker et al. 2014). Aromatic residues W12, Y14 and F334, F335 are implicated to be essential in “clasping” and in lipid binding (Walker et al. 2014) (Fig. 8.8d). For example, F334A substitution disrupts the “clasp” and increases lipid binding by apoA-IV (Tubb et al. 2007). Conserved Pro residues are also implicated in maintaining the “clasp” mechanism (Deng et al. 2015).

We propose that “clasping” of the N- and C-terminal parts helps protect apoA-IV from misfolding. This idea is based upon sequence analysis of human apoA-IV (Fig. 8.8). Both AmylPred2 and PASTA2 predict mild amyloidogenic propensity distributed throughout the polypeptide chain, with two major aromatic-rich amyloidogenic segments in residues 7–16 (VATVMWDYFS) and 331–336 (VNSFFS) from the N- and C-terminal parts, respectively (Fig. 8.8a, b). PASTA predicts that the N-terminal segment is likely to form parallel in-register β -sheet in amyloid. In addition, AmylPed2 predicts two weaker amyloidogenic segments around residues 40–41 of the N-domain and 221–222 of the core domain (Fig. 8.8a). Three major predicted amyloid hot spots are located in the putative helical regions (Walker et al. 2014) that are outside (residues 7–16 and 40–41) or at the edge (residues 331–336) of the crystallized construct; the fourth hot spot (residues 221–222) is located inside the α -helical region in the crystal structure (Fig. 8.8c). These amyloidogenic segments are probably protected from misfolding by their native helical structure, as well as by the tertiary interactions in free full-length apoA-IV. In fact, aromatic residues W12, Y14 and F334, F335 that are proposed to be essential for lipid binding and for “clasping” (Walker et al. 2014), as well as F223 that modulates lipid binding, are located inside or adjacent to the amyloidogenic segments (Fig. 8.8a, d). Hence, these sensitive regions are probably sequestered via the “clasp” mechanism in free protein or via the lipid binding on HDL.

In sum, we propose that in the native lipid-free apoA-IV, the major predicted amyloidogenic segments are protected from misfolding via the same “clasp” mechanism that regulates protein-lipid interactions (Fig. 8.8d). Further, the presence of two amyloid hot spots in residues 7–16 and 40–41 helps explain the deposition of the N-terminal fragment containing these residues in systemic amyloidosis involving wild type human apoA-IV (Bergström et al. 2004).

8.4.4 Apolipoprotein A-V

ApoA-V (343 a. a.) is a hydrophobic protein that is a potent reducer of plasma TG (Nilsson et al. 2011). Similar to its physiological antagonist apoC-III (described in the next section), apoA-V is secreted by hepatocytes and circulates in plasma as a minor component of VLDL and HDL. To our knowledge, no amyloid diseases resulting from the deposition of apoA-V have been reported. This is not surprising given minute concentration of this protein in plasma (~150 ng/ ml). Nevertheless, apoA-V has emerged as an important modulator of TG metabolism, which can influence secretion, processing and clearance of TG-rich lipoproteins via several mechanisms (Sharma et al. 2013). In an intracellular mechanism, a fraction of apoA-V binds to lipid droplets and thereby influences VLDL secretion. The extracellular mechanisms include synergistic action of apoA-V and lipoprotein lipase, a key enzyme in processing of TG-rich plasma lipoproteins. First, VLDL binding via apoA-V to heparan sulfate proteoglycans in the arterial wall brings together the lipoprotein and the lipase that is anchored to the arterial wall (see Chap. 10 by Leonova and Galzitskaya in this volume). Second, apoA-V can promote clearance of VLDL remnants by binding to a receptor that is essential for clearance of TG-rich lipoproteins (Nilsson et al. 2008). In these reactions, a basic stretch in apoA-V is thought to bind acidic moieties of the receptor and the heparan sulfate proteoglycans. This is reminiscent of apoE and apoB whose basic stretches are critical in binding acidic sites of the lipoprotein receptors or heparan sulfates (Mahley et al. 1984; Wilson et al. 1991).

Structural analysis of apoA-V, pioneered by Ryan and colleagues (Weinberg et al. 2003; Beckstead et al. 2007; Wong et al. 2008; Mauldin et al. 2010; Sharma et al. 2013), has been complicated by the low protein solubility. Most experiments have been performed in solution at pH 3 and have been limited to low-resolution techniques. The protein structure was inferred to contain tandem amphipathic α -helices, which in apoA-V form distinct domains (Weinberg et al. 2003; Beckstead et al. 2007; Wong et al. 2008). The N-terminal residues 1–146 are proposed to form a helix bundle (Wong et al. 2008). The central domain (residues 147–292) contains a basic stretch (residues 186–227) implicated in binding to acidic ligands. Finally, the C-terminal 51 residues are implicated in modulating lipid binding by apoA-V (Beckstead et al. 2007; Mauldin et al. 2010).

Amino acid sequence analysis using AmylPred2 predicts one strong amyloidogenic segment in residues 269–277 (TYLQIAAFT) and three weaker ones in residues 118–120, 150–151 and 204–210 (Fig. 8.9). One predicted amyloidogenic segment, 119–121, is in the N-domain, the other three are in the central domain, and all four are located inside the amphipathic α -helices predicted in the native structure (Weinberg et al. 2003). Notably, a strong amyloidogenic segment predicted in residues 204–210 forms a hydrophobic stretch

inside a larger predominantly basic segment 190–225 that is implicated in binding to the receptor and to heparan sulfate proteoglycans (Fig. 8.9). This larger segment contains eight basic residues, no acidic residues, and three structure-breaking Pro. Since stretches of similarly charged residues are disfavored in the parallel in-register amyloid β -sheet due to inter- and intramolecular repulsion, it is unlikely that this residue segment initiates β -aggregation. In fact, PASTA2 does not predict a strong pairing energy for this segment (profile not shown). Further, it predicts parallel in-register β -sheet for apoA-V, with the most favorable pairing energy of -3.9 , just below the threshold of -4 . Hence, this prediction by PASTA should be taken with a grain of salt.

In sum, apoA-V is a minor hydrophobic protein that is an important modulator of TG metabolism. The atomic structure of apoA-V is unknown. The predicted amyloidogenic potential is localized mainly to the central domain (Fig .

8.9). Similar to other apos, the amyloidogenic segments in apoA-V are hydrophobic, are located in the amphipathic α -helices in the native structure, and are predicted to form parallel in-register β -sheet in amyloid. These segments are expected to be protected from misfolding by their native protein-lipid and/or protein-protein interactions. Amyloidogenic segment 204–210 is probably also protected by its location inside a basic stretch that forms a binding site for VLDL receptor.

8.4.5 Apolipoproteins C-I, C-II and C-III

ApoC-I (6 kDa), C-II (10 kDa) and C-III (10 kDa) are the smallest human apolipoproteins that circulate mainly on VLDL and are also found as minor proteins on HDL and other lipoproteins. ApoCs are particularly important for metabolism of triglyceride-rich lipoproteins, as they modulate the activity of lipophilic enzymes, lipid transfer proteins, and other plasma factors that remodel these lipoproteins in vivo. ApoC-I inhibits cholesterol ester transfer protein, apoC-II is a physiological activator of lipoprotein lipase, while apoC-III is its inhibitor and is also implicated in VLDL production (Jong et al. 1999; Shachter 2001; Yao and Wang 2012; Kei et al. 2012). In addition, apoCs can displace larger proteins such as apoE from VLDL, and thereby inhibit apoE-mediated VLDL receptor binding (Sehayek and Eisenberg 1991).

Free in solution, apoCs have little ordered secondary structure ($\sim 30\%$ α -helix in apoC-I (Gursky and Atkinson 1998), less than that in apoC-II and apoC-III). Hence, similar to other small lipid surface-binding proteins such as apoA-II, SAA, A β and α -synuclein (described by Uversky in Chap. 2 of this volume), free apoCs are intrinsically disordered. NMR structures of apoC-I, apoC-II and apoC-III have been determined in lipid-mimetic environment on the micelles of sodium dodecyl sulphate (SDS) or dodecylphosphocholine (DPC) (Rozek et al. 1999; MacRaid et al. 2001, 2004; Gangabadage et al. 2008). These structures showed amphipathic class-A α -helices forming the lipid surface-binding motif in apoCs (Fig. 8.10, bottom panels). Compared to larger apos, apoCs make fewer contacts on the lipoprotein surface and can dissociate from it relatively easily (Jayaraman et al. 2005a). Such dissociation is expected to occur in vivo, e.g. during VLDL conversion to LDL. Despite marginal structural stability of free apoCs, their amyloid deposition in vivo has not been reported to-date, probably because of the low plasma concentrations of these minor

proteins. However, human apoC-II and, as reported recently, apoC-III can form amyloid fibrils in vitro (de Messieres et al. 2014). Extensive studies of apoC-II fibril formation by Howlett's team provided the most detailed model currently available for understanding apolipoprotein misfolding (summarized by Ryan et al. in Chap. 7).

Here, we report amyloidogenic profiles of apoCs (Fig. 8.10). AmylPred2 predicts that apoC-II has substantial amyloid forming potential, especially in the C-terminal part, while the sequences of apoC-I and apoC-III are less amyloidogenic. Similarly, PASTA2 suggests substantial amyloid-forming potential in the C-terminal part of apoC-II and predicts it to form parallel in-register β -sheet in amyloid (pairing energy of -4.9) (Fig. 8.11). Most favorable pairing energies predicted for apoC-I (-1.9) and apoC-III (-2.7) are well below the threshold of -4 and hence, are not very reliable (PASTA profiles not shown). Nevertheless, human apoC-III can form amyloid fibers in vitro (de Messieres et al. 2014).

The strongest predicted amyloidogenic segment in human apoCs is located in the C-terminal part of apoC-II (residues 62–76, TYTGIFTDQVLSVLK) from the native class-G helix. This prediction has been verified experimentally in careful studies by Wilson et al. (2007) (Fig. 8.11). These studies showed that in fibers of full-length human apoC-II, C-terminal segment is resistant to proteolysis and to H-D exchange, which is characteristic of amyloid core. The authors also showed that peptide fragments corresponding to the C-terminal segments of apoC-II readily form amyloid in vitro. Finally, structural analysis of apoC-II fibrils revealed parallel in-register β -sheets (Wilson et al. 2007), which verifies our prediction by PASTA (Fig. 8.11). In sum, extensive experimental evidence validates the prediction of the major amyloid hot spot in residue segment 62–76 of apoC-II and suggests that this segment initiates protein misfolding, from the predominantly α -helical native structure to parallel in-register intermolecular β -sheet in amyloid. We speculate that the native α -helical structure in this segment, which forms the major lipid binding site in apoC-II, anchors the protein to the lipid surface and thereby protects it against β -aggregation in vivo. Notably, this segment forms class-G helix that, in contrast to class-A helices, has random radial distribution of charged residues. Hence, amyloidogenic properties of apolipoproteins can be conferred by segments forming other than class-A α -helices in the native structure.

8.4.6 Apolipoprotein E

ApoE (299 a. a.) is arguably the most extensively studied apolipoprotein (~20,000 hits in PubMed) that is an important mediator of lipid transport in plasma and in the central nervous system. ApoE is found in most systemic and cerebral amyloid deposits and is particularly pertinent to the development of Alzheimer's disease (for an excellent recent review see Hauser and Ryan 2013). Plasma apoE circulates mostly on VLDL where it serves as a ligand for LDL-like receptor that mediates VLDL uptake and, ultimately, maturation to LDL (Hauser et al. 2011; Holtzman et al. 2012). During this process, TG in the lipoprotein core are hydrolyzed by lipoprotein lipase, generating excess surface material that can dissociate in the form of free apolipoproteins or small apoE-containing particles that join the plasma pool of HDL. Hence, in addition to VLDL, apoE also circulates as a minor protein on plasma HDL. In contrast to plasma, lipid transport in cerebrospinal fluid is mediated solely by HDL-like particles whose major protein is apoE. This explains why apoE is particularly

prominent in neurodegenerative diseases. Notably, apoE4 isoform is the strongest genetic predictor of Alzheimer's disease and is directly associated with the amyloid load in this disease. Although the exact role of apoE in the development of Alzheimer's disease is not fully understood, the consensus is that apoE interacts directly in an isoform-specific manner with A β peptide and thereby influences aggregation, accumulation and/or clearance of A β from the brain (Strittmatter et al. 1993; Ladu et al. 2000; Hauser and Ryan 2013).

Three major isoforms of human apoE differ in Cys/Arg substitutions at two sites: apoE2 (C112, C158), apoE3 (C112, R158) and apoE4 (R112, R158), resulting in differences in charge, structural stability and functional properties. Compared to apoE3, which is the most common isoform, apoE4 is a strong causative risk factor for Alzheimer's disease, while apoE2 is neuro-protective (Strittmatter et al. 1993; reviewed by Verghese et al. 2011; Suri et al. 2013). ApoE4 is linked strongly to decreased age of the late-onset Alzheimer's disease and more severe A β deposition in the brain, while apoE2 has an opposite effect (Verghese et al. 2011; Suri et al. 2013). Thus, the predisposition to Alzheimer's disease increases in the order E2<E3<E4. Although the molecular mechanism via which apoE isoforms differentially influence A β accumulation in the brain is subject of debate, the prevailing concept is that this difference resides in the isoform-specific domain interactions in apoE, which influence its interactions with A β (reviewed by Hauser and Ryan 2013).

Structurally, apoE is often described as a two-domain protein comprised of a globular 22 kDa N-terminal domain that forms a helix bundle in solution and contains a receptor and heparan binding site in residues 136–150, and a C-terminal 10 kDa domain that is less structured in solution and forms the primary binding site for lipids and A β . Four-helix bundle structure of the N-domain of lipid-free apoE3, which was determined by x-ray crystallography to 2.2 Å resolution, was the first known atomic structure of a mammalian apolipoprotein (Wilson et al. 1991). This structure is consistent with the solution conformation of the full-length lipid-free apoE3 determined 20 years later by NMR (Chen and Wang 2011). In the NMR structure, the globular N-domain (residues 1–167) forms a template for folding of the hinge region (168–205) and the C-domain (206–299) (Figs. 8.12 and 8.13).

The two residues, 112 and 158, differing among the apoE isoforms are located in the N-terminal α -helices (orange, Figs. 8.12 and 8.13). Cys to Arg substitution at either site weakens the interhelical interactions and reduces structural stability of the N-domain and of the full-length lipid-free apoE (Acharya et al. 2002; Weers et al. 2003; Nguyen et al. 2014). Hence, the rank order of free protein stability is E4<E3<E2, opposite to that of the predisposition to Alzheimer's disease. A similar trend was observed in other proteins whose reduced structural stability often correlates with enhanced amyloid formation and is thought to contribute to it (Hatters et al. 2005, 2006).

Tertiary structural stability of apoE isoforms and other free apolipoproteins in solution correlates inversely with the probability of their helix bundle opening on the lipid, which is a necessary step in lipoprotein formation. Therefore, apoE isoform stability in solution was proposed to correlate inversely with the lipid binding affinity (Morrow et al. 2000; Acharya et al. 2002; Weers et al. 2003). To our knowledge, relative dissociation rates of apoE

isoforms from HDL, which determine relative kinetic stabilities of HDL containing these isoforms, have not been reported. Furthermore, no isoform-specific effects have been observed in apoE association with HDL in cerebrospinal fluid ((Verghese et al. 2011) and references therein). Therefore, it is unclear whether or not differential affinity of apoE isoforms for lipid contributes to the isoform-specific effects in amyloid deposition.

Another potential origin of the isoform-specific effects is in the intrinsic sequence propensity of apoE to form amyloid. In fact, apoE misfolding was proposed to be a primary event that helps nucleate amyloid formation by A β (Ma et al. 1994; Wisniewski et al. 1995; Hatters et al. 2006). To test this idea, we analyzed amino acid sequence of apoE by using AmylPred2 and PASTA2 (Fig. 8.12). Surprisingly, both methods predict relatively weak amyloidogenic potential for apoE as compared to other exchangeable apolipoproteins. The major amyloidogenic segment is predicted by both methods in residues 33–42, with the PASTA2 energy of –3.9, just below the threshold of –4 (Fig. 8.12). Interestingly, the preferred orientation of this segment in amyloid is antiparallel β -sheet. Another surprise is that the predicted amyloidogenic propensity of apoE resides mainly within the N-domain, even though the C-domain is more hydrophobic, binds A β , and fragments corresponding to this domain can form fibers in vitro (Cho et al. 2001). This illustrates the ability of most polypeptides to form amyloid under certain conditions. We suggest that the absence of strong amyloidogenic segments in the C-domain of apoE, which adopts a dynamic solvent-exposed conformation in solution, helps protect free protein from misfolding in vivo.

In the N-domain of apoE3, three amyloidogenic segments are predicted in residues 33–42, 113–116 and 159–163 by five to seven methods in AmylPred2 (Fig. 8.12). Mapping these segments on the NMR structure of the modified full-length protein shows that they are located in the amphipathic α -helical regions (Fig. 8.13). The only exception is residues 41 and 42 that are located in a solvent-accessible interhelical loop (blue arrow, Fig. 8.13) and hence, are potentially labile to misfolding. Notably, sites 112 and 158 that distinguish apoE isoforms are located at the edges of the two respective amyloidogenic hot spots, 113–116 and 159–163. Cys to Arg substitutions at these sites slightly reduce the amyloid-forming potential of the adjacent hot spots (profiles not shown). This is not surprising, since the amyloid-forming potential of Arg is lower than that of free Cys in most contexts. Further, amyloidogenic segment 159–163 is close to the basic stretch in residues 136–150 that form the receptor and heparan sulfate binding site in apoE (Wilson et al. 1991). We speculate that, in the absence of polyanionic ligands such as heparan sulfate proteoglycans, this basic stretch may help block the β -zipper propagation along the polypeptide chain.

Since apoE has been previously found to directly nucleate A β aggregation in amyloid (Ma et al. 1994; Wisniewski et al. 1995; Hatters et al. 2006), we tested whether a particular segment in apoE is likely to pair with A β to nucleate an intermolecular β -sheet co-polymer. To do so, we used AmylPred2 and PASTA2 to analyze a chimeric amino acid sequence comprised of apoE and A β ₄₂ sequences. The two strongest hot spots in AmylPred2 came from A β ₄₂ (Fig. 8.7). Likewise, PASTA2 did not predict any favorable pairings between A β and apoE. This analysis suggests that apoE is unlikely to nucleate amyloid formation by A β via the segment pairing between the two proteins.

Our analysis can be summarized as follows. First, despite its prominence in amyloid diseases, apoE has weaker sequence propensity to form amyloid than many other apos. Second, this propensity decreases slightly upon Cys to Arg substitutions at sites 112 and 158 that differentiate apoE isoforms. Hence, the amyloid-forming sequence potential increases in order E4<E3<E2, which is reverse of the isoform-specific predisposition to neurodegenerative diseases. Consequently, this pre-disposition must result from factors other than sequence propensity of apoE isoforms for β -aggregation. Together, these findings suggest that apoE misfolding per se is probably not a major factor in amyloid deposition in Alzheimer's disease.

Third, the NMR structure of free full-length apoE shows that a part of the strongest predicted amyloidogenic segment 33–42 is located in the solvent-accessible loop, which makes it potentially labile to β -aggregation. This loop (residues V40, Q41, T42, L43) links N-terminal helices H1 and H1', and is packed against helix HC1 from the C-domain (Fig. 8.13). Since inter-domain interactions in apoE are isoform-specific, the protection of the major amyloid hot spot by these interactions is also expected to be isoform-specific. We hypothesize that: (i) in lipid-free full-length apoE, the major amyloid hot spot in residues 33–42 is protected from misfolding by secondary and tertiary structure, including inter-domain interactions; (ii) destabilization of the N-domain and changes in its interactions with the C-domain reduce this protection in an isoform-specific manner, from E2 (most stable and most protected) to E4 (least stable and least protected). These differences in protection of amyloid hot spots in the N-domain may potentially contribute to the isoform-specific effects in amyloid deposition.

8.4.7 Serum Amyloid A

Human SAA (104 a. a.) is an intrinsically disordered protein from a family of highly conserved acute-phase proteins whose normal function in immune response is subject of debate (van der Westhuyzen et al. 2007; Eklund et al. 2012; also see Chap. 5 by Colón et al. in this volume). SAA is synthesized predominantly by the liver, circulates mainly on plasma HDL, and is best known for its role in secondary amyloidosis linked to inflammation. In this serious complication, N-terminal fragments of SAA largely containing residues 1–76, termed amyloid A (AA), form extracellular fibrillar deposits in vital organs (kidney, liver, spleen) and damage them. AA amyloidosis can be triggered by chronic infections, inflammation, rheumatoid arthritis, and certain cancers, and is the most prevalent form of human systemic amyloidosis developed by 5–10 % of patients with chronic inflammation. Several SAA isoforms are found in humans, among which SAA 1.1 is most amyloidogenic in vivo. The reasons for the isoform-specific amyloid formation are unclear and were proposed to involve differences in the protein structure, stability and proteolysis (Srinivasan et al. 2013; van der Hilst et al. 2008). It is also unclear whether cleavage of the N-terminal AA fragment precedes or follows SAA misfolding in AA amyloidosis.

During inflammation, plasma levels of SAA can increase up to 1,000-fold, reaching 1 mg/ml or more, and making SAA the most abundant protein in plasma (Gillmore et al. 2001). As a result of mass action, SAA can displace most apoA-I from plasma HDL to form large dense SAA-rich HDL (van der Westhuyzen et al. 2007; Eklund et al. 2012). In addition, in acute

phase response, HDL surface is saturated with SAA and a substantial fraction of SAA (up to 25 %) circulates in labile free form (van der Westhuyzen et al. 2007). SAA on HDL is predominantly α -helical, yet this helical structure is unstable in free protein at near-physiologic conditions, which is thought to promote fibril formation in vivo (Wang et al. 2005). This structural instability of SAA has greatly complicated its atomic-resolution studies that culminated in 2014 with publication of the high-resolution x-ray crystal structure of human SAA1.1 in two crystal forms, one of which contains hexamers and probably depicts the structure of the hexameric SAA found in solution (Lu et al. 2014). Molecular structure of human SAA1 in these crystals is in excellent agreement with the structure of murine SAA3 that was crystallized as a tetramer (Derebe et al. 2014).

Our analysis of human SAA1.1 by using AmylPred2 and PASTA2 shows that the amyloid-forming propensity is modest and is confined largely to the N-terminal ~75 residues (Fig. 8.14a), which is consistent with the deposition of 1–76 residue fragment in AA amyloidosis. AmylPred2 predicts two major amyloid hot spots in residues 2–9 (SFFSFLGE) and 53–55 (WAA), with a minor hot spot in the nearby residues 67–70. Although these consensus predictions are not very strong (4–6 out of 11 methods), they are in excellent agreement with the sequence analysis by using ZipperDB and TANGO algorithms (Lu et al. 2014) as well as PASTA2 (Fig. 8.14b). The latter shows that the major N-terminal amyloid hot spot has weak pairing energy of -2.7 PASTA units which is comparable for the parallel and antiparallel β -sheet. Importantly, the predicted hot spots in SAA have been verified in experimental studies by using site-directed mutagenesis and peptide fragments (Patel et al. 1996; Egashira et al. 2011; Lu et al. 2014).

Compared to other major HDL proteins, the predicted amyloidogenic potential of SAA is comparable to that of apoA-I and much lower than that of apoA-II (Figs. 8.4, 8.6, and 8.14). This unexpected result suggests that AA amyloidosis is probably driven not so much by the high intrinsic propensity of SAA for β -aggregation but mainly by the protein overproduction (de Beer et al. 1982) combined with its structural instability and solvent exposure of the N-terminal amyloidogenic segment in free SAA monomer (Lu et al. 2014).

In the crystal structure, this major amyloidogenic segment in residues 2–9 is located at the end of the N-terminal amphipathic α -helix (Fig. 8.14c, blue). This segment is solvent-exposed in free SAA monomer but is sequestered in the hexamer, suggesting that hexamer dissociation is prerequisite for SAA misfolding (Lu et al. 2014). Importantly, this amyloidogenic segment overlaps the primary HDL binding site in SAA (Patel et al. 1996; Ohta et al. 2009), suggesting strongly that SAA binding via its N-terminal helix to HDL surface protects the protein from misfolding (Lu et al. 2014). The second-major amyloidogenic segment is adjacent to residues 52 and 57 that differ among the major human isoforms, SAA 1.1 (V52, A57), SAA 1.3 (A52, A57) and SAA 1.5 (A52, V57) (Fig. 8.14a, c, orange dots). Even though these isoforms are predicted to have comparable sequence propensity for β -aggregation (profiles not shown), one cannot exclude that the location of the isoform-specific mutations at the amyloidogenic segment contributes to the differences in the amyloid formation by SAA1 isoforms (Takase et al. 2014). We speculate that isoform-specific effects in SAA binding to HDL may also be a potential contributing factor in amyloidosis.

In sum, in spite of its name, serum amyloid A has only moderate intrinsic sequence propensity to form amyloid, which is comparable to that of apoA-I and significantly lower than that of apoA-II. The N-terminal end of SAA is predicted and observed to form the major amyloid hot spot that overlaps with the primary HDL binding site and forms a native amphipathic α -helix on the lipid (Ohta et al. 2009). Therefore, this segment is protected against misfolding by binding to lipid surface. In free SAA, this segment is sequestered in the hexamer, yet in the monomer it is solvent exposed and hence, labile to misfolding.

Despite structural instability of free SAA under near-physiologic conditions and its persistently high plasma concentrations in chronic inflammation, only a small percentage of patients develop AA amyloidosis ((Gillmore et al. 2001) and references therein). To explain this observation, SAA has been proposed to develop protective mechanisms against misfolding, such as the proteolysis of its amyloid-forming N-terminal part (Yamada et al. 1995) or hexamer formation (Lu et al. 2014). We hypothesize that modest intrinsic sequence propensity to form amyloid is one of such protective mechanisms.

8.4.8 Apolipoprotein B

ApoB is a 550 kDa glycoprotein synthesized by the liver. Plasma apoB contains a single chain of 4,536 amino acids, which is one of the largest known proteins and the largest apolipoprotein. One copy of full-length apoB, termed apoB100, is present as the major protein on the surface of LDL and VLDL. A shorter variant representing the first 48 % of apoB sequence, termed apoB48, is synthesized in the intestine and forms the major protein on chylomicrons (Nakajima et al. 2014). ApoB forms a flexible structural scaffold on the lipoprotein surface and an important functional ligand for binding to LDL receptor and heparan sulfate proteoglycans (Goldstein and Brown 1976; Camejo et al. 1998). In contrast to other apolipoproteins, apoB is non-exchangeable (water-insoluble) and remains associated with its host particle during metabolism. Also in contrast with the exchangeable apos, apoB is predicted and observed to contain both α -helices and β -sheets (Segrest et al. 2001).

The enormous size and insolubility of apoB have precluded its detailed structural studies. Amino acid sequence analysis suggests five large structural domains (Segrest et al. 2001). Residue segment 1–1,023, termed $\beta\alpha 1$ domain, is thought to form an autonomously folded globular domain that protrudes from the LDL surface. Homology model of this domain has been proposed based on the x-ray crystal structure of a related protein, lamprey lipovitellin (Segrest et al. 1999). Epitope labeling combined with cryo-electron microscopy have determined the overall arrangement of human apoB on the mid-size LDL and showed that the protein fully encircles LDL, with the N- and C-terminal domains located near each other to close the circle (Liu and Atkinson 2011; Kumar et al. 2011).

The involvement of apoB in amyloid formation has been suggested in several in vitro studies of modified LDL, including oxidized and electro-negative LDL (Stewart et al. 2005; Parasassi et al. 2008; Brunelli et al. 2014). Aggregation of modified LDL was evident in these and other studies (Bancells et al. 2010), including our own work (Jayaraman et al. 2007; Lu et al. 2012; Lu and Gursky 2013). However, convincing experimental evidence for amyloid formation by apoB, such as the oriented fiber diffraction and a large increase in cross- β -sheet content measured by spectroscopy, is still lacking.

Our amino acid sequence analysis reveals that apoB has unusually high amyloid-forming potential, comparable to that of A β peptide (Figs. 8.7 and 8.15; Table 8.1). Major amyloidogenic segments, which are predicted in all five apoB domains, span regions that are thought to form native α -helices or β -sheets. Figure 8.15 shows amyloidogenic profile in three representative portions of apoB including residues 300–400 (predicted to form native α -helical structure), residues 780–880 (located in a predicted β -sheet region), and residues 2,150–2,250 from the α_2 helical domain that was proposed to be involved in β -aggregation (Parasassi et al. 2008). Many amyloidogenic segments in these and other parts of apoB are predicted strongly by AmylPred2 (8–11 methods) and span 10 or more amino acids, longer than those in the exchangeable apos (Table 8.1). Likewise, PASTA2 predicts unusually strong favorable pairing energies below –9 units for many apoB segments, which is consistent with their high hydrophobicity. In comparison, the lowest PASTA2 energies are about –8.4 for A β (1–42), –7 for apoA-II and –5.5 or weaker for other exchangeable apos (Table 8.1). Furthermore, similar to most exchangeable apos, PASTA predicts parallel in-register β -sheet for all major amyloidogenic segments in apoB.

In sum, of all apolipoproteins explored, apoB has by far the strongest sequence propensity to form amyloid, which correlates with high protein hydrophobicity of this water-insoluble protein. Nevertheless, to-date there is no compelling evidence for amyloid formation by apoB in vivo or in vitro. We propose that the reason why apoB does not readily form amyloid is due to its extensive protein-lipid and protein-protein interactions on LDL and VLDL. These native interactions make this enormous protein non-exchangeable and thereby protect it from dissociation, proteolytic degradation and misfolding.

8.5 Summary

The studies outlined in this chapter show that amyloidogenic segments in human apolipoproteins (i) are largely hydrophobic, (ii) are located almost entirely in the native amphipathic α -helices in the exchangeable apos while in the non-exchangeable apoB they span both predicted α -helices and β -sheets, and (iii) are predicted to preferentially form parallel in-register β -sheet in amyloid. These amyloidogenic segments are enriched in aromatic residues that often form native interactions stabilizing the helix bundle structures of free proteins such as apoA-I, apoA-IV and apoE (Figs. 8.4, 8.8, and 8.13). Importantly, the predicted amyloidogenic segments have been verified in the experimental studies of apoC-II, apoA-I, apoA-II and SAA (Wilson et al. 2007; Wong et al. 2012; Adachi et al. 2014; Mendoza-Espinosa et al. 2014; Das et al. 2014; Sawashita et al. 2009; Lu et al. 2014). Amyloidogenic segments in many other globular proteins share similar characteristics: they tend to be hydrophobic (with a notable exception of prions), are enriched in aromatic groups, and are often protected from misfolding by forming well-ordered secondary structure such as α -helix (Tzotzos and Doig 2010; Sabate et al. 2012). Furthermore, parallel in-register β -sheet has emerged as the predominant structural motif observed by EPR and solid-state NMR in amyloid fibrils formed by many proteins (Tycko 2006; Margittai and Langen 2008; Shewmaker et al. 2011), including fibrils of apoC-II and α -synuclein (Teoh et al. 2011b; Lv et al. 2012; Der-Sarkissian et al. 2003).

A surprising conclusion emerging from our studies is that the intrinsic sequence propensity to form amyloid decreases in order apoB > apoA-II > apoC-II apoA-I, apoC-I, apoC-III, SAA > apoA-IV, apoA-V, apoE (Table 8.1). This rank order was unexpected given that apoE and SAA are prominent players in human amyloidoses such as Alzheimer's disease and inflammation-linked AA amyloidosis, while apoB and normal human apoA-II are not. What is the reason for this apparent discrepancy between the predicted and observed amyloid formation in vivo? Clearly, intrinsic sequence propensity is just one of many factors determining amyloid formation in vivo and in vitro. Other important factors include stability of the native protein structure, protein concentration, external conditions such as pH, ligand binding, proteolytic environment, etc. Moreover, the probability of protein misfolding is determined not only by the global stability of its native structure, but also by the local conformation and dynamics in sensitive regions (Chiti and Dobson 2009). We propose that sequestration of such amyloidogenic regions via the native protein-protein and protein-lipid interactions protects apos against misfolding (Das et al. 2014; Gursky 2014; Lu et al. 2014).

Strong apolipoprotein-lipid interactions are expected and observed to be particularly protective. One example is apoB, the largest and most hydrophobic of all human apos, which interacts most strongly with its host lipoprotein. These strong interactions render apoB non-exchangeable and thereby protect it from forming amyloid despite its unusually high sequence propensity to do so (Fig. 8.15), comparable to that of A β peptide (Fig. 8.7; Table 8.1). Another example is apoA-II, the most hydrophobic exchangeable apolipoprotein that is tightly bound to the surface of mature HDL. This strong binding is reinforced by the protein dimerization via the Cys6-Cys6 disulfide bond, rendering normal human apoA-II practically non-exchangeable. We propose that this strong association with mature HDL protects wild-type human apoA-II from forming amyloid despite its high sequence propensity to do so (Fig. 8.6, Table 8.1). Notably, naturally occurring mutant apoA-II with C-terminal extension that causes human amyloidosis has high amyloid-forming propensity approaching that of A β (Table 8.1).

Interestingly, except for this mutant apoA-II, human apos that commonly form amyloid in vivo are consistently predicted to be in the middle of the amyloid propensity range (SAA1.1) or near its end (apoE) (Table 8.1). These proteins can readily dissociate from the lipid surface and circulate in labile free form. We propose that these proteins have evolved protective strategies against misfolding, which explains why their primary sequences have modest propensity for β -aggregation (Figs. 8.12 and 8.14, Table 8.1). For example, the protective strategy in free apoE is high structural stability of the helix bundle in the N-domain where all major amyloidogenic segments are located, combined with the low amyloidogenic sequence propensity of the flexible hinge and C-domains (Figs. 8.12 and 8.13). The protective strategy in apoA-IV, nearly half of which circulates as free protein, is sequestration of the major amyloidogenic segments via the "clasp" mechanism (Fig. 8.8). Additional protection may also be attained via self-association of free proteins, such as the formation of an antiparallel α -helical dimer by apoA-I (Fig. 8.3) or apoA-IV (Deng et al. 2012), or sequestration of the major amyloidogenic segments in SAA hexamer (Lu et al. 2014). Location of the amyloidogenic segments next to the charged stretches, such as the basic receptor-binding sites in apoA-V and apoE (Figs. 8.9 and 8.12a), is also expected to

protect the proteins from β -aggregation. Finally, rapid proteolysis and clearance of free apos can serve as important protective mechanisms against amyloidosis.

We conclude that apolipoproteins' propensity to bind lipid surface and to form amyloid are two sides of the same coin, both rooted in proteins' hydrophobicity. This suggests that functional constraints on apolipoproteins make it difficult to eliminate their pathologic β -aggregation. Nevertheless, the primary sequences that are optimal for lipid surface binding and for amyloid formation are distinctly different, as illustrated in several examples. First, large apolar faces of the amphipathic α -helices or β -sheets found in the exchangeable and non-exchangeable apos are essential for binding to lipid surface but not for amyloid formation. This is exemplified by the naturally occurring C-terminal extension in human apoA-II (Fig. 8.6) which is highly amyloidogenic but cannot form a lipid-binding amphipathic α -helix (Gursky 2014). Other examples include apoA-V and the C-domain of apoE, which are highly lipophilic but not highly amyloidogenic (Figs. 8.9 and 8.12). Furthermore, stretches of similarly charged amino acids, such as the receptor and heparan binding sites in apoE, apoA-V and apoB, are well-tolerated in the polar faces of the lipid-binding class-A α -helices, yet such stretches are expected to disrupt amyloid structure due to their intra- and intermolecular electrostatic repulsion in a parallel in-register β -sheet. These examples illustrate various strategies potentially employed during evolution to optimize reversible binding of apolipoproteins to lipid surface while reducing the risk of amyloid formation.

In sum, the reason why apolipoproteins are overrepresented in amyloid diseases is not due to their native class-A amphipathic α -helices per se. Rather, it is rooted in increased apolipoprotein hydrophobicity that is not only required for lipid binding but also increases protein propensity for β -aggregation. In fact, class-A apolipoprotein α -helices are not necessarily more amyloidogenic than their class-G counterparts, as suggested by the highly amyloidogenic hydrophobic C-terminal segment in apoC-II that forms a native class-G helix (Figs. 8.10b and 8.11, Table 8.1; also see Chap. 7 by Ryan et al. in this volume). Furthermore, the apolipoprotein α -helices do not necessarily have higher amyloid-forming propensity than their β -sheet counterparts. This is evident from the amino acid sequence analysis of apoB, the most amyloidogenic of all apos and the only one with significant β -sheet content. Numerous amyloidogenic segments span the whole length of apoB including predicted α -helices and β -sheets (Fig. 8.15). Importantly, the amyloidogenic segments in the exchangeable apos are located in the amphipathic α -helices and overlap their apolar lipid-binding faces, which probably protects them from misfolding. Likewise, amyloidogenic regions in globular proteins often overlap with their functional interfaces, suggesting a competition between normal protein function and pathogenic β -aggregation (Castillo and Ventura 2009).

This competition implies that increasing apolipoprotein retention on the lipid surface may provide a potential therapeutic strategy against amyloidosis. For example, increased levels of plasma triacylglycerol are well-known to promote apoA-I dissociation from the HDL surface upon enzymatic lipolysis of core TG (Lamarche et al. 1999). Consistent with this observation, clinical studies show that apoA-I amyloid deposits in atherosclerotic arteries correlate directly only with patients' age and with the plasma levels of TG (Röcken et al.

2006). We propose that this correlation may reflect causation, as elevated levels of TG in HDL induce generation of free apoA-I that is the precursor of amyloid. This prompts us to speculate that TG-reducing therapies may help protect apoA-I against dissociation from the lipid, and thereby delay amyloid formation in vivo (Das et al. 2014).

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Abbreviations

AA	Amyloid A
AApoAI	Familial apoA-I amyloidosis
Apo	Apolipoprotein
Aβ	Amyloid beta peptide
DPC	Dodecylphosphocholine
EPR	Electron paramagnetic resonance
HDL	High-density lipoprotein
LDL	Low-density lipoprotein
NMR	Nuclear magnetic resonance
SAA	Serum amyloid A
SDS	Sodium dodecyl sulfate
TG	Triacylglycerol
VLDL	Very low-density lipoprotein

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Figure 8.1.

Cartoon representation of lipoproteins and their constituents. **(a)** Major classes of plasma lipoproteins, including very-low, low- and high-density lipoproteins (VLDL, LDL and HDL), are shown in cross-section. Particle diameters are indicated. Intermediate-density lipoproteins (IDL, $d = 25\text{--}35\text{ nm}$) and chylomicrons ($d > 100\text{ nm}$) are not shown. Mature lipoproteins contain a core of apolar lipids (mainly cholesterol esters and triacylglycerols, in *yellow*) surrounded by an amphipathic surface comprised of polar lipids (mainly phospholipids and cholesterol, in *gray*) and apolipoproteins. Exchangeable (water-soluble) apolipoproteins are in *red*, non-exchangeable (water-insoluble) apoB is in *blue*. **(b)** Exchangeable apolipoproteins can reversibly dissociate from the lipoprotein surface. The dissociated free apolipoprotein is structurally labile and can (i) rapidly bind to lipoprotein surface, i.e. associate with another lipoprotein or get recruited to plasma membrane, (ii) get cleared, or (ii) misfold and form amyloid

Figure 8.2.

Amphipathic class-A α -helix is the major structural and functional motif in apolipoproteins. Helix wheel representation of residues 9–30 of human apoA-II is shown as an example. Class-A α -helix has a large apolar face (*gray filled circles*) flanked by basic residues (+, *blue letters*), with acidic residues (-, *red letters*) located in the middle of the polar face. Polar face of the helix interacts with the aqueous solution (*light-blue*), while the apolar face can bind lipid surface (*beige*) or interact with apolar faces of other helices in free protein

Figure 8.3.

Amyloidogenic mutations mapped on the atomic structure of lipid-free human apoA-I. X-ray crystal structure of the C-terminally truncated protein, (185–243) apoA-I, was determined by Mei and Atkinson (2011). The protein is ~80 % α -helical and forms a crystallographic dimer stabilized by two four-segment bundles. Two anti-parallel dimer molecules are color-coded; *arrow* shows twofold symmetry axis. The dimer forms a semi-circular arc with diameter $d = 11$ nm typical of HDL, suggesting how apoA-I confers curvature to the phospholipid monolayer on HDL surface (*dotted circle*). Dimer-to-monomer conversion is proposed to occur via the domain swapping of an helical segment around the twofold axis (Mei and Atkinson 2011). All known AApoAI mutations are located in the helix bundle (Gursky et al. 2012). The mutation sites are indicated and are color-coded: point substitutions (*yellow*), deletions (*green*), frame shifts (*pink*). Most mutations are clustered at the bottom of the helix bundle (*dashed oval*), yet some are in the middle/top region (*rectangle*) (Modified from Gursky et al. 2012)

Figure 8.4.

Amyloidogenic segments in human apoA-I. AmylPred2 (a) and PASTA2.0 profiles (b). Major amyloidogenic segments predicted by five or more methods (at or above the *dotted line*) from the total of 11 methods in AmylPred2 are shown by residue numbers and color-coded: 14–22 (*blue*), 53–58 (*teal*), and 227–232 (*red*). A minor amyloidogenic segment, 69–72 (*green*, above the *dashed line*), is predicted by four methods. *Green bar* shows protein fragments found in AApoAI deposits. *Double arrow* shows the crystallized construct. Amino acid sequence of apoA-I is shown with amyloidogenic segments color-coded. (c) Amyloidogenic segments mapped on the proposed structure of free apoA-I monomer. The monomer structure was obtained from the crystallographic dimer (Fig. 8.3) by domain swapping (Mei and Atkinson 2011). C-terminal part 185–243 (*gray solid line*), which was missing from the crystallized construct, is partially unfolded in solution, largely α -helical on the lipid, and contains the primary lipid binding site that encompasses the amyloidogenic segment (residues 227–232, in *red*) that overlaps native α -helical structure (Borhani et al. 1997). The N-terminal amyloidogenic segments (in *blue*, *teal* and *green*) are located in a well-ordered α -helical cluster. The hypothetical pH-dependent switch in residues 76–81, EKETEG, is in *purple* (Modified from Das et al. 2014)

Figure 8.5.

Hypothetical molecular mechanism of apoA-I misfolding in hereditary and in acquired amyloidosis (Modified from Das et al. 2014). (a) ApoA-I on HDL surface forms an antiparallel ~80 % α -helical dimer stabilized by high kinetic barriers (Mehta et al. 2003; Jayaraman et al. 2006). (b) Dissociation from HDL surface generates a metastable apoA-I monomer that absorbs via its C-terminus to phospholipid surface. (c) Perturbed native packing in the N-terminal amyloid hot spots, which can result from AApoAI mutations or Met oxidation, initiates monomer misfolding, from native α -helical structure to parallel in-register β -sheet in amyloid. Residues 76–81, EKETEG (in *purple*), form a putative gate-keeping motif in which three charged Glu are unlikely to be located on the same side of the β -sheet due to their intra- and intermolecular repulsion. (d) At pH 7, electrostatic repulsion among these Glu is expected to block the β -zipper propagation beyond residues 76–81. Hence, the N-terminal ~75 residues form amyloid core that is resistant to proteolysis. The polypeptide located C-terminally of residue 81, which loses its helical structure upon misfolding of the N-terminal part, can be readily proteolysed. This mechanism explains why the 9–11 kDa N-terminal fragments are found in systemic apoA-I amyloidosis. (e) At acidic pH that approaches pH 5.5 in atherosclerotic plaques, Glu in the EKETEG motif 76–81 are partially protonated and may form carboxyl pairs, facilitating β -zipper propagation from the N- to the C-terminus to encompass all four amyloid hot spots. As a result, full-length apoA-I forms amyloid in atherosclerotic plaques. (f) Molecular arrangement in a parallel in-register β -sheet. Different protein molecules are in different shades of *gray*. Main chain hydrogen bonds are aligned along the fibril axis (*arrow*)

Figure 8.6.

Predicted amyloidogenic segments in human apoA-II and its C-terminal extension.

Amyloidogenic profiles obtained by AmylPred2 (**a**) and PASTA2 (**b**) for human apoA-II with C-terminal extension 78–98 (*dark red*) that causes human amyloidosis. The major amyloidogenic segments predicted by five or more methods in AmylPred2 are shown by residue numbers. *Double arrows* show the orientation of the intermolecular β -sheet predicted by PASTA2.0. The predicted native secondary structure is shown at the *bottom*; *rectangles* and residue numbers indicate α -helices. Cys6 that forms an intermolecular disulfide link in human apoA-II dimer is in *orange*. Amino acid sequence of human apoA-II with C-terminal extension is shown; amyloidogenic segments are color-coded. (**c**) Cartoon showing parallel in-register β -sheet predicted for apoA-II with amyloidogenic extension. Different protein molecules are in different shades of *gray* (Modified from Gursky 2014)

Figure 8.7.

Amyloidogenic profile of Alzheimer's A β (1–42) peptide obtained by AmylPred2 and Pasta2.0. Pairing energies exceeding -4 PASTA units are shown; residue numbers indicate amyloid hot spots. The two predicted amyloid hot spots overlap the β -sheet regions observed in the atomic model of A β (1–40) fibrils determined by solid-state NMR (Lu et al. 2013), which verifies the prediction

Figure 8.8.

Amyloidogenic profile and the structure of human apoA-IV. AmylPred2 **(a)** and PASTA2.0 **(b)** profiles of full-length apoA-IV are shown. N- and C-terminal regions flanking the core domain are indicated. Residue numbers indicate amyloidogenic segments predicted by five or more methods (above the *dotted line*). Aromatic residues implicated in lipid binding are in *purple*. *Green bar* shows N-terminal fragments found in human apoA-IV amyloidosis (Benson et al. 2001). Predicted residue pairing and PASTA energy in the major amyloidogenic segment is shown. **(c)** Helix bundle structure of the monomeric core domain of apoA-IV (residues 64–335) obtained via the domain swapping (*circular arrow*) from the X-ray crystal structure of the dimer (Deng et al. 2012). Residues in the amyloidogenic segments predicted by AmylPred2 are indicated. **(d)** Cartoon showing the molecular architecture of free full-length apoA-IV according to (Walker et al. 2014). *Circles* H1–H4 designate α -helices forming four-helix bundle in the core domain. N-terminal segment is in *blue* and C-terminal is in *red*. Filled *ovals* show amyloid hot spots. Aromatic residues involved in “clasp” formation and lipid binding are in *purple* (Walker et al. 2014)

Figure 8.9. Amyloidogenic profile of human apoA-V obtained by AmylPred2. Residue numbers indicate amyloidogenic segments predicted by five or more methods (above the *dotted line*). *Blue bar* and +++ indicate residue segment that contains eight uncompensated basic residues and three Pro and is implicated in binding to the lipoprotein receptor and to heparan sulfate proteoglycans

Figure 8.10.

Predicted amyloidogenic profiles and the structures of human apoCs. *Top*: AmylPred2 profiles of human apoC-I (**a**), apoC-II (**b**), and apoC-III (**c**). Residues predicted to form amyloidogenic segments by five or more methods (above the *dotted line*) are indicated. *Middle*: Secondary structures of apoCs that were predicted (Segrest et al. 1992) or observed by NMR on SDS or DPC micelles (Rozek et al. 1999; MacRaild et al. 2001, 2004; Gangabadage et al. 2008). *Rectangles* and residue numbers indicate helical segments. *Bottom*: NMR structures of human apoCs on SDS micelles. Predicted amyloidogenic segments are mapped on the structures and color-coded. Amino acid sequences of the proteins are shown; the amyloidogenic segments are color-coded

Figure 8.11.

PASTA2.0 profile of human apoC-II and its experimental validation. C-terminal segment 66–75 is predicted to form parallel in-register β -sheet, which is experimentally validated by EPR (Teoh et al. 2011b). Residue segments that are resistant to proteinase digestion and hydrogen-deuterium exchange and are implicated in amyloid core formation are shown in *patterned bars*. Fragments implicated in amyloid formation in vitro are in *solid bars* (Wilson et al. 2007)

Figure 8.12.

Amyloidogenic profile of human apoE3 predicted by AmylPred2 (**a**) and PASTA2.0 (**b**). Amyloidogenic segments predicted by at least five methods in AmylPred2 are marked. *Orange dots* show locations of Cys/Arg substitutions that differ among the three major isoforms: apoE2 (C112, C158), apoE3 (C112, R158), and apoE4 (R112, R158). Helical segments observed in the NMR structure of the modified full-length human apoE3 (Chen et al. 2011) are shown by *rectangles*. Color code: N-terminal helix bundle domain (*violet*), the connecting hinge (*gray*), and the C-terminal domain (*purple*). +++ show a basic stretch (residues 136–152) implicated in binding to receptor and to heparan sulfate proteoglycans. Protein amino acid sequence with predicted amyloidogenic segments color-coded

Figure 8.13.

NMR structure of the full-length modified human apoE3, determined by Chen and Wang (2011). Structural domains are color-coded as in Fig. 8.12: N-terminal helix bundle (*violet*), hinge (*gray*), and C-terminal domain (*pink*). Helices H1 and H1' with their connecting loop in the N-domain, and helix HC1 in the C-domain are indicated. Helical nomenclature follows (Chen et al. 2011). Cys/Arg substitutions at positions 112 and 158 that distinguish the apoE isoforms are in *orange*. The major predicted amyloidogenic segments are color-coded as in Fig. 8.12: residues 34–42 (*blue*), 113–116 (*teal*) and 159–163 (*green*). Residue segment 136–150 from helix H4, which encompasses the receptor and heparan sulfate binding site, is indicated (+ + + +)

Figure 8.14.

Predicted amyloidogenic profile and the structure of human serum amyloid A. AmylPred2 (a) and PASTA profiles (b) of SAA1.1 isoform that causes human amyloidosis are shown. AmylPred2 predicts one amyloidogenic segment in residues 2–9 (blue) by six methods, and another in residues 65–70 (green) by five methods; an additional minor segment in residues 67–70 is predicted by four methods. PASTA2.0 predicts that the major N-terminal amyloidogenic segment can form parallel or antiparallel β -sheet with comparable pairing energy. Green bar shows fragment 1–76 that deposits in human AA amyloidosis linked to inflammation. Rectangles show helical segments observed in the x-ray crystal structure. Amino acid sequence shows predicted amyloidogenic segments color-coded. (c) X-ray crystal structure of human SAA1.1 monomer determined to 2.2 Å resolution by Lu et al. (2014). The predicted amyloidogenic segments are mapped on the structure and color-coded. Orange dots show positions 52 and 57 of Ala/Val substitutions that distinguish the major protein isoforms, SAA 1.1 (V52, A57), SAA 1.3 (A52, A57) and SAA 1.5 (A52, V57)

Figure 8.15.

Amyloidogenic propensity of selected portions of human apoB (total protein length 4,536 a. a.) AmylPred2 and PASTA2.0 profiles are shown for 100-residue portions that are predicted to form various native secondary structures: residues 300–400 (*left*) form mainly α -helices that are a part of the so-called A-helix region; residues 780–880 (*middle*) form mainly β -sheets that are a part of the A-sheet region; residues 2,150–2,250 (*right*) form mainly α -helices that are a part of the α_2 region (nomenclature according to Segrest et al. 2001; Wang et al. 2010). Residue numbers in *top panels* indicate amyloidogenic segments predicted by five or more methods in AmylPred2 (at or above the *dotted line*). *Bottom panels* show segments with pairing energy below -4 predicted by PASTA. Other parts of apoB have comparable predicted amyloidogenic propensity (not shown)

Table 8.1
Summary of amyloid-forming propensity of human apolipoproteins discussed in this chapter

Protein	Amino acids		AmylPred2 Hot spot residue segments	Hot spot/total	PASTA2 Min energy
	Total	Hot spots			
1. apoA-II	77	20 (31)	10–18, 60–70 (78–98)	0.26 (0.38)	–7.0
2. apoC-II	79	17	16–17, 62–76	0.21	–4.9
3. apoC-III	79	10	8–13, 52–55	0.12	–2.7
4. SAA 1.1	104	11	2–9, 53–55	0.10	–2.7
5. apoC-I	57	5	24–28	0.09	–1.9
6. apoA-I	243	22	14–22, 53–58, 227–232	0.09	–6.1
7. apoA-V	343	21	118–120, 150–151, 204–210, 269–277	0.06	–3.9
8. apoE3	299	19	33–42, 113–116, 159–163	0.06	–3.9
9. apoA-IV	376	20	7–16, 40–41, 221–222, 331–336	0.053	–4.7
10. apoB (selected regions from the total of 4,536 a. a.)					
301–400	100	35	320–325, 350–357, 366–372, 378–392	0.35	–11.5
781–880	100	19	787–790, 798–807, 852–866	0.19	–8.4
2,151–2,250	100	43	2,157–60, 2174–83, 2,195–04, 2,217–27, 2,229–36	0.43	–9.3
11. Aβ	42	20	15–22, 30–42	0.46	–8.9

Parameters for the exchangeable apos (*rows 1–9*) and for selected regions of the non-exchangeable apoB (*row 10*) are listed; Aβ(1–42) is included for comparison. Numbers in brackets refer to the naturally occurring C-terminal extension in apoA-II that causes human amyloidosis. Total number of residues in each protein or polypeptide and the number of residues predicted to be in amyloidogenic segments by five or more AmylPred2 methods (major hot spots) are listed. The ratio of these values (hot spot/total) can be used to rank amyloidogenic propensity. This propensity of the exchangeable apos decreases as listed in the top box from 1 to 9. Although other criteria, such as the minimal (most favorable) segment pairing energy by PASTA2, yield somewhat different rank order, various prediction methods consistently show that apoB has the strongest amyloidogenic potential, followed by apoA-II, apoC-II and other apos, with SAA in the middle and apoE near the end of the propensity range