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Hot spots in apolipoprotein A-II misfolding and amyloidosis in mice and men

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Abstract

ApoA-II is the second-major protein of high-density lipoproteins. C-terminal extension in human apoA-II or point substitutions in murine apoA-II cause amyloidosis. The molecular mechanism of apolipoprotein misfolding, from the native predominantly α -helical conformation to cross- β -sheet in amyloid, is unknown. We used 12 sequence-based prediction algorithms to identify two ten-residue segments in apoA-II that probably initiate β -aggregation. Previous studies of apoA-II fragments experimentally verify this prediction. Together, experimental and bioinformatics studies explain why the C-terminal extension in human apoA-II causes amyloidosis and why, unlike murine apoA-II, human apoA-II normally does not cause amyloidosis despite its unusually high sequence propensity for β -aggregation.

Keywords

High-density lipoprotein; amyloidogenic mutations; α -helix to β -sheet conversion; amphipathic α -helix; lipid surface binding proteins

INTRODUCTION

Amyloidoses are protein misfolding diseases in which a normally soluble functional protein changes its conformation to an intermolecular cross- β -pleated sheet and forms insoluble fibrillar deposits in vivo [1]. Apolipoproteins (apos) are lipid surface-binding proteins that are central to lipid transport and metabolism and are overrepresented in amyloidoses [2–4]. For example, apoE3 is an important risk factor in Alzheimer's disease and is found in most amyloid deposits; α -synuclein forms amyloid in Lewy bodies in Parkinson's disease; serum amyloid A deposits as fibrils in chronic inflammation; non-variant apoA-I deposits as amyloid in atherosclerotic plaques; also, specific mutations in apoA-I, apoA-II and other apolipoproteins can cause systemic amyloidosis in humans ([2–4] and references therein). To prevent or block apolipoprotein misfolding, it is essential to understand the molecular mechanism of this pathogenic process and its important determinants, which are largely unknown. Here, we combine bioinformatics approaches with previous in vitro and animal model studies to gain insight into misfolding of apolipoprotein A-II (apoA-II).

ApoA-II in circulation

ApoA-II (9 kDa) is the second-major protein of plasma high-density lipoproteins (HDL, or “Good Cholesterol”) that remove excess cholesterol from peripheral tissues and protect against cardiovascular disease. HDL are nanoparticles comprised of lipids and exchangeable (water-soluble) proteins, mainly apoA-I and apoA-II (70% and 20%, respectively). ApoA-II is an important modulator of HDL metabolism [5–7]. Most apoA-II circulates on mature “spherical” HDL, sHDL(A-I/A-II), that also contain apoA-I. ApoA-II is more hydrophobic and more strongly associated with sHDL than apoA-I or any other exchangeable apolipoprotein [5]. The amino acid sequence of apoA-II is predicted to form three class-A amphipathic α -helices that are the major structural and functional motif in this protein family (Fig. 1A–C) [8]. Such α -helices have large apolar faces that are optimized for lipid surface binding [9]. Two class-A α -helices, predicted in apoA-II residues 9–30 and 51–70, have particularly large apolar faces spanning $\sim 180^\circ$ (as compared to $\sim 120^\circ$ in apoA-I) (Fig. 1A, C). These large apolar faces enable apoA-II α -helices to insert more deeply into phospholipid monolayer and bind more strongly to HDL surface than other exchangeable apolipoproteins. As a result, apoA-II does not readily dissociate from sHDL during metabolism and, hence, is thought to be practically non-exchangeable. We propose that this strong association with sHDL helps protect apoA-II from misfolding.

Most human apoA-II circulates on mature sHDL(A-I/A-II), which is a major HDL species formed upon maturation and fusion of nascent discoidal HDL containing either apoA-I or apoA-II, dHDL(A-I) and dHDL(A-II); alternatively, sHDL(A-I/A-II) can form upon maturation of dHDL(A-I/A-II) [10]. Nascent dHDL containing apoA-II and/or apoA-I are formed upon efflux of cell cholesterol to these proteins, mediated by lipid transporter ABCA1 ([7, 10–12] and references therein). Nascent dHDL(A-II) is a minor species that is rapidly converted into mature sHDL(A-I/A-II) upon fusion with HDL(A-I) [11, 12]. In contrast to its particularly strong association with sHDL, apoA-II dissociates relatively readily from dHDL [13–15]; this difference is probably due to the distinct geometry of the amphipathic helix–lipid interactions on discoidal and spherical particles [6]. The dissociated apoA-II provides a potential source of transient free apoA-II in plasma. In contrast to HDL-bound apolipoproteins that are stabilized by high kinetic barriers [16, 17], free apoA-II in solution has reduced helical content, marginal thermodynamic stability, and forms a partially folded molten globule [18]. This structurally labile transient species is proposed to form the direct precursor of amyloid [19, 20].

In humans and higher primates, apoA-II forms a dimer disulfide-linked via the sole cysteine, Cys6, while in other species apoA-II lacks Cys and is monomeric. Dimer formation decelerates apoA-II transfer among phospholipid vesicles [21], increases structural stability of apoA-II on dHDL [22], decelerates apoA-II dissociation from dHDL, and increases the activation energy of this dissociation [13, 23]. We posit that S-S linked dimer formation helps protect human apoA-II from misfolding by hampering its dissociation from the lipid. The cross-linking studies of model dHDL(A-II) containing dimeric human apoA-II showed that the two molecules within the dimer form an antiparallel helical hairpin [24]. Such an antiparallel α -helical arrangement may provide additional protection from apoA-II misfolding into a parallel β -sheet, which is the major structural motif predicted and observed in amyloid fibrils formed by apolipoproteins [25–27] (discussed below) and many other proteins [28, 29].

Human apoA-II amyloidosis

In humans, apoA-II can cause hereditary amyloidosis (AApoAII), an autosomal dominant disorder that results from stop codon mutations, stop78 to Gly, Ser, Arg or Leu, leading to a 21-residue C-terminal extension ([19, 20] and references therein). Full-length mutant apoA-

II deposits as fibrils primarily in kidneys, causing renal damage and failure, and in other organs (heart, adrenal, spleen, etc.). The only available treatment for AApoAII is dialysis followed by kidney transplant [30]. It is unclear why the C-terminal extension leads to amyloidosis. One explanation is that, unlike the rest of the apoA-II molecule, this extension cannot form an amphipathic class-A α -helix with a well-defined apolar lipid-binding face (Fig. 1) and, hence, is less suited for binding to lipid surface [19]. Benson and colleagues proposed that this reduced lipid binding affinity can influence HDL metabolism and shift the population distribution from HDL-bound to labile free apoA-II [19].

We hypothesize that the C-terminal extension may also i) interfere with the conformation and dynamics of free apoA-II, and ii) increase the amyloid-forming propensity of its amino acid sequence. The former idea can only be tested once the detailed structural and dynamic information on apoA-II becomes available.* To test the latter idea, we used an established concept that protein misfolding is usually initiated by short 4- to 10-residue segments, or amyloid “hot spots”, with high propensity for β -aggregation. To identify such amyloid hot spots in apoA-II, we analyzed its amino acid sequence by using a combination of 12 sequence-based prediction methods.

To this end, we used consensus software AmylPred2 [31] that combines 11 prediction algorithms using various criteria, such as the packing density, hydrophobicity and charge distribution, β -sheet contiguity, correspondence to known β -sheet patterns, α -helix to β -sheet conformational switches, dual α -helix and β -sheet propensity, etc. [31–43]. Although individual prediction algorithms have limited accuracy, the consensus analysis can significantly improve the selectivity and specificity in predicting hot spots for amyloid [31]. Thus, prediction by a consensus of 5 out of 11 methods has approximately 85% specificity that further increases with the increasing number of hits in AmylPred2 [31]. In addition to AmylPred2, we used PASTA server that assesses free energy of segment pairings in amyloid and the probable orientation of these segments in a parallel or antiparallel β -sheet [43].

Figure 2 and supplemental Table S1 show the results of these predictions for human WT apoA-II (77 a. a.) and its AApoAII mutant containing 21-residue C-terminal extension. AmylPred2 reveals two major amyloid hot spots in WT apoA-II predicted by a consensus of at least 5 out of 11 methods. These include residues 10–18, LVSQYFQTV, predicted by up to 8 methods; and residues 60–70, LVNFLSYFVEL, predicted by up to 11 methods (Table S1). Notably, the latter hot spot is the strongest hit for any exchangeable apolipoprotein explored. Importantly, the C-terminal extension contains an additional hot spot in residues 79–87, SVQTIVFQP, predicted by nine out of 11 methods (Table S1). Each of these hot spots is rich in large hydrophobic residues, contains at least one aromatic residue in the middle, typically lacks basic groups (although an acidic group is tolerated), and has other properties of amyloidogenic sequences. The rank order for the three predicted hot spots in human apoA-II and its C-terminal extension mutant is (60–70)>(79–87)>(10–18) (Fig. 2A).

In agreement with AmylPred2, PASTA predicts three amyloid hot spots in similar locations and in the same rank order, and suggests that the hot spot near residues 60–70 is particularly amyloidogenic (Fig. 2B). PASTA energy of -8.2 is predicted for 60–68 segment pairing in a parallel in-register β -sheet, which is the largest negative (favorable) conformational energy for any exchangeable apolipoprotein explored. Parallel β -sheet is also predicted in residues 79–87 from the C-terminal extension (PASTA energy -5.9). The N-terminal residues 7–20

*X-ray crystal structures of full-length human apoA-II and other proteins previously published by H. M. Murphy have been discredited and should be withdrawn from the journals and the Protein Data Bank. For details see [58], <http://main.uab.edu/Sites/reporter/articles/71570/> and <http://classic.the-scientist.com/blog/display/56226/>.

are predicted more weakly to form an antiparallel β -sheet (energy -4.5). Because the probability of a particular conformation increases exponentially with its negative energy, these energy values predict with high probability that WT and mutant apoA-II form parallel in-register β -sheet in amyloid.

Notably, AmylPred2 and PASTA consistently show that apoA-II sequence has the strongest amyloid-forming propensity of all human exchangeable apolipoproteins, including apoE that is found in most amyloid deposits, as well as α -synuclein and serum amyloid A that form amyloid in Parkinson's disease and inflammation, respectively. This is not surprising, since amyloid-forming propensity correlates with the protein hydrophobicity, and apoA-II is most hydrophobic of all exchangeable apolipoproteins. Remarkably, the predicted amyloid-forming propensity of apoA-II is comparable to that of Alzheimer's A β peptide (prediction results not shown). Nevertheless, normal human apoA-II does not form amyloid in vivo. To explain this paradoxical finding, we note that the two amyloid hot spots predicted in residues 10–18 and 60–70 of the wild type (WT) human apoA-II are located inside the class-A α -helices, 9–30 and 51–70 (Fig. 1 A–C, Fig. 2). We propose that strong binding of these α -helices to sHDL surface protects these two hot spots from initiating protein misfolding. Additional protection is probably provided by the disulfide link in human apoA-II dimer, which decelerates protein's dissociation from the lipid [21, 22] including dHDL [13–15, 23].

In conclusion, amino acid sequence analysis of apoA-II suggests a dual effect of the 21-residue C-terminal extension explaining why it causes amyloidosis in humans. First, this extension cannot form an amphipathic α -helix with a well-defined apolar helical face that is necessary for optimal lipid surface binding (Fig. 1). Second, the extension contributes an additional amyloid hot spot that increases protein's propensity to misfold from an antiparallel highly helical conformation to a parallel in-register β -sheet (Fig. 2C). This additional hot spot in residues 79–87 can act in synergy with the strongest hot spot predicted in apoA-II residues 60–70, which also has high propensity to form a parallel in-register β -sheet (Fig. 2B). We speculate that these two adjacent hot spots can form a β -hairpin arranged in a parallel in-register β -sheet that nucleates amyloid (Fig. 2C). As a result, the lipid-binding affinity of apoA-II is expected to decrease while its intrinsic amyloid-forming propensity to increase upon the stop codon mutations. Both effects are probably more pronounced in the mutant/mutant apoA-II homodimer as compared to WT/mutant heterodimer. This helps explain why only the mutant protein is found in patient-derived AApoAII deposits, even though homo- and heterodimers containing mutant and WT apoA-II are found in circulation [19].

Murine apoA-II amyloidosis

In contrast to human AApoAII, which is a rare hereditary disorder, murine AApoAII is commonly associated with aging in many mouse strains and can be accelerated by mutations but does not require them [44, 45]. The disease is especially severe in the senescence-accelerated mice where full-length mature apoA-II or pro-apoA-II containing a five-residue propeptide, ALVKKR, deposits as fibrils in vital organs (liver, kidneys, heart, muscle, vessel wall, etc.) Pro-apoA-II was proposed to be particularly amyloidogenic and perhaps nucleate AApoAII amyloid [45]. Several types of murine apoA-II have been reported, which differ in residues at positions 5, 20, 26 and 38, and in their amyloid-forming propensity in vivo. Of those, type C apoA-II that contains Gln5 is particularly amyloidogenic [46]. Remarkably, apoA-II fibrils can be transmitted through various mechanisms (e. g. via an injection or with food [47–49]) and cause murine AApoAII in senescence-resistant strains, probably by nucleating the misfolded conformation in the amyloid-resistant type of apoA-II ([44] and references therein). We speculate that high amyloidogenic propensity of the apoA-II sequence augments such a prion-like infectious transmission of AApoAII in mice.

The molecular mechanism of murine apoA-II misfolding and the role of the pro-peptide and the substitutions in residues 5, 20, 26 and 38 in this process are unknown. Additional factors that can influence AApoAII are also not well understood. Higuchi's team showed that such factors include dietary lipids that can importantly influence not only lipoprotein metabolism but also the severity of murine AApoAII [50]. For example, increasing the degree of fatty acid unsaturation in the diet enhances murine AApoAII, an effect that may be due, at least in part, to high susceptibility of the unsaturated fatty acids to oxidation [51]. In fact, lipid peroxidation is expected to promote Met oxidation in apolipoproteins, and thereby reduce the structural stability of apoA-II in solution and on HDL [52], which is expected to augment the misfolding.

To gain better insight into the misfolding of murine apoA-II, we analyzed its amino acid sequence by using AmylPred2 and PASTA. The results show that full-length murine apoA-II is predicted to have two major amyloid hot spots in the N- and C-terminal locations (Fig. 3) similar to those predicted for human apoA-II. Importantly, this prediction has been verified experimentally: Higuchi's team showed that synthetic peptides containing residue fragments 6–16 and 48–65 of murine apoA-II, which overlap the predicted amyloid hot spots (Fig. 3A), readily form fibrils in vitro and are essential for amyloid nucleation [53, 54]. Further, in murine apoA-II, the C-terminal amyloid hot spot is predicted strongly to form parallel in-register β -sheet (PASTA energy -6.6 , weaker than -8.2 in human AApoAII), while the antiparallel β -sheet in the N-terminal hot spot is predicted very weakly (PASTA energy below the threshold of -4 , weaker than -4.5 in human apoA-II) (Fig. 2B, 3B).

Notably, the four amino acids in positions 5, 20, 26, and 38, which differentiate amyloid-prone (type C) from amyloid-resistant mice (types A and B), are located outside the amyloid hot spots (Fig. 3B). Replacements of these amino acid is not predicted to significantly influence the amyloidogenic profile of apoA-II (results not shown). Similarly, the five-residue basic pro-peptide, ALVKKR, is not predicted to increase the amyloidogenic propensity of apoA-II sequence. Consequently, the increased amyloid formation by type C apoA-II and by pro-apoA-II observed in vivo must result from other, still unknown, factors. We speculate that these factors possibly include the effects of the amino acid substitutions and the pro-peptide on the protein-lipid interactions and on the conformation and dynamics of free apolipoprotein, as proposed in our current structure-based studies of apoA-I [55].

In sum, AmylPred 2 and PASTA consistently predict two hot spots in murine apoA-II, which have been verified experimentally in peptide fragment studies [53, 54]. The locations of these hot spots are similar but their predicted amyloid-forming propensities are slightly weaker as compared to their human counterparts (Fig. 2B, 3B). Nevertheless, WT murine apoA-II commonly forms amyloid in vivo, while WT human apoA-II does not.

To explain this finding, we propose that dimerization of human but not murine apoA-II via the disulfide link confers extra protection from misfolding into amyloid. Furthermore, lower Met content in human as compared to murine apoA-II may also play a role. Apolipoprotein methionines can readily oxidize in vivo and in vitro and thereby influence protein misfolding in amyloid [56]. Specifically, the replacement of the apolar Met with polar Met sulfoxide in the apolar helical face can destabilize the free protein and reduce its affinity for lipid, as reported for human apoA-I and apoA-II [52, 57]. Human apoA-II has a single M26 at the edge of the apolar helical face (Fig. 1A) outside the amyloid hot spots. Murine apoA-II contains three Met located in the middle (M7, M68) or at the edge (M17) of the apolar helical faces, inside or adjacent to the amyloid hot spots (Fig. 3B). Oxidation of these methionines, which reduces protein's affinity for lipid, is expected to augment murine AApoAII. Finally, profound differences in the lipoprotein metabolism, including factors

such as dietary lipids, may importantly influence the development of AApoAII in mice and men [50, 51].

SUMMARY

Amino acid sequence analysis of human and murine apoA-II predicts two ~10-residue segments with high propensity to initiate protein misfolding (Figs. 2, 3), which is higher than in any other exchangeable apolipoprotein explored. This prediction has been verified experimentally for murine apoA-II whose fragments encompassing the predicted segments readily form amyloid in vitro [53, 54]. Despite its unusually high sequence propensity, normal apoA-II does not form amyloid in humans. We propose that normal apoA-II is protected from misfolding by its strong interactions with HDL surface, which is further enhanced upon dimerization of human apoA-II via Cys6-Cys6 disulfide [13–15, 21–23]. Amyloidogenic mutations in human apoA-II produce C-terminal extension that has a dual effect. First, the extension contains an additional amyloid hot spot that is predicted to act in synergy with the major hot spot and form a parallel in-register intermolecular β -sheet in amyloid (Fig. 2). Second, the extension cannot form class-A amphipathic α -helix (Fig. 1) and hence, is expected to reduce apoA-II affinity for lipid surface. In mice, unlike humans, apoA-II forms amyloid without such an extension. Perhaps this difference can result from lower protection of murine apoA-II monomer as compared to human apoA-II dimer by the lipid interactions, as well as from other factors such as the differences in HDL composition and dietary lipids [50, 51]. Identifying and targeting such factors can ultimately help combat apolipoprotein amyloidoses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

apo	apolipoprotein
AApoAII	apoA-II amyloidosis
HDL	high-density lipoproteins
sHDL	spherical high-density lipoprotein
dHDL	discoidal high-density lipoprotein
HDL(A-I)	HDL containing apoA-I as its sole protein
HDL(A-II)	HDL containing apoA-II as its sole protein
HDL(AI/A-II)	HDL containing both apoA-I and apoA-II
WT	wild type

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Highlights

- Modifications in ApoA-II and other apolipoproteins can cause systemic amyloidosis
- Two apoA-II segments are predicted to have unusually high potential to form amyloid
- Previous peptide fragment studies experimentally verify this prediction
- Disease-causing extension adds another amyloidogenic segment that acts in synergy
- Normal human apoA-II is protected by dimerization and strong lipid surface binding

Figure 1.

Helix wheel representation of normal human apoA-I (A, B, C) and its amyloidogenic C-terminal extension (D). Amphipathic class-A α -helices predicted in apoA-II residues 9–30 (A), 39–50 (B) and 51–70 (C) are shown; the C-terminal extension 78–98 does not form an amphipathic α -helix (D). Gray dotted arcs in panels A–C indicate apolar helical faces. Residues are color-coded: apolar (black letters, filled gray circles), polar (black), acidic (red), basic (blue), Gly (gray). Circles with green edge indicate residues located in the predicted amyloid hot spots (Fig. 2 and supplemental Table S1).

Amyloidogenic profile of human apoA-II by AmylPred2 Amyloidogenic profile of human apoA-II by PASTA Amphipathic class-A α -helices in human apoA-II **Figure 2.**

Amyloidogenic profiles predicted for human apoA-II with the C-terminal extension. Panel A shows the number of hits in AmylPred2 (from the total of 11) plotted versus residue number; larger number of hits suggests improved prediction specificity of the amyloidogenic segments [31]. Residue numbers at the top indicate segments predicted by at least five methods (at or above the dotted line). Panel B shows the amyloidogenic profile in PASTA units [43], with higher values corresponding to higher probability of β -strand pairing. The strongest amyloid hot spot predicted in residues 60–70 is shown in its entirety in the insert, but is only partially seen in panel B that zooms in on the more weakly predicted hot spots. The β -strand orientation predicted for each hot spot is shown by arrows. Methionine position

is indicated. Panel C shows how two adjacent hot spots, 60–70 and 79–87, can form a β -hairpin assembled in a parallel in-register intermolecular β -pleated sheet predicted for these hot spots. Such a β -hairpin-containing assembly may facilitate amyloid nucleation [59]. Different shades of gray show different protein molecules. Panel D shows amphipathic class-A α -helices predicted in residues 9–30, 39–50 and 51–70.

Amyloid profile of murine apoA-II by AmylPred2

Amyloid profile of murine apoA-II by PASTA

Figure 3.

Amyloidogenic profiles predicted for normal murine apoA-II. Panel A shows the number of hits in AmylPred2 plotted versus residue number. Segments predicted by at least five methods (at or above the dotted line) are shown at the top by residue numbers. Green bars and boxed residue numbers at the bottom indicate protein fragments that can readily form amyloid fibrils in vitro [53, 54]. Panel B shows the amyloidogenic profile in PASTA units. The strongest predicted hot spot encompassing residues 60–70 is shown in its entirety in the insert, but is only partially seen in panel B that zooms in on the more weakly predicted N-terminal hot spot. Arrow pairs show the predicted β -strand orientation. Met positions are indicated. Green bars and residue numbers at the bottom indicate single amino acid

substitutions that differentiate various types of murine apoA-II: normal (Pro5, Asp20, Met26, Ala38), type A (Pro5, Asn20, Met26, Ala38), type B (Pro5, Glu20, Val26, Val38), and type C (Gln5, Glu20, Val26, Ala38); type C readily forms amyloid in mice [46].