Chapter 38
Assembly of Amyloid β-Protein Variants Containing Familial Alzheimer’s Disease-Linked Amino Acid Substitutions

Aida Attar
Department of Neurology, David Geffen School of Medicine, Brain Research Institute, University of California at Los Angeles, Los Angeles, CA, USA

Derya Meral and Brigita Urbanc
Department of Physics, Drexel University, Philadelphia, PA, USA

Gal Bitan
Department of Neurology, David Geffen School of Medicine, Brain Research Institute, Molecular Biology Institute, University of California at Los Angeles, Los Angeles, CA, USA

INTRODUCTION: ‘MINOR’ CHANGES HAVE MAJOR EFFECTS

In the amino acid sequence of amyloid β-protein (Aβ), not all amino acids are equal in their importance to assembly kinetics and biologic activity. The length of the sequence itself has a major impact on Aβ assembly and bioactivity. Aβ is a product of sequential cleavage of the amyloid β-protein precursor (APP), first outside the membrane by β-secretase to release the ectodomain of APP, called soluble APPβ (sAPPβ), and then within the membrane by γ-secretase to release Aβ and the APP intracellular cytoplasmic domain (AICD; Fig. 38.1). Another enzyme, α-secretase, cleaves APP within the Aβ region, after residue 16, leading to formation of a shorter peptide, p3, which is not associated with disease. β-Secretase consistently produces Aβ starting at D1 (APP672). In contrast, γ-secretase is a promiscuous enzyme, which cleaves APP in several locations, leading to peptides ending at various C-terminal positions, most commonly from 38 to 43 (though never 41). Historically, the most studied forms of Aβ have been the most abundant ones—the 40- and 42-residue forms, but studies of the 38- and 43-residue forms are on the rise. Changes in the C-terminal length of Aβ have a major effect on the aggregation kinetics, toxicity, and role in Alzheimer’s disease (AD), even though, regardless of the exact C-terminal position, Aβ isoforms form oligomers, which are believed to be the major neurotoxic form of Aβ [1], and then go on to make fibrils [2] that are found deposited in the brain.

In the AD brain, Aβ deposits are formed in both the brain parenchyma and vasculature. The amyloid plaques found in the parenchyma are one of the pathologic hallmarks of AD. The other hallmark, neurofibrillary tangles,
comprising hyperphosphorylated Tau, is discussed in Chapter 19: Polymorphism of Tau fibrils. Classic, dense-core amyloid plaques and diffuse plaques are composed primarily of Aβ42 and Aβ43 [3–5], whereas vascular amyloid consists mainly of Aβ40 [6,7]. Levels of Aβ in the cerebrospinal fluid (CSF), which often are used as a biomarker for AD, differ between the Aβ isoforms. In non-demented persons, levels of CSF Aβ were found to be 40>38>42>39>37 [8]. In patients with AD, levels of Aβ42 and Aβ37 decreased, and levels of Aβ38 and Aβ40 increased [8] or remained unchanged [9,10]. Aβ39 concentration levels were unchanged [8]. In plasma, levels of Aβ38, Aβ40, and Aβ42 are indistinguishable between patients with AD and age-matched normal individuals [11]. Clinically, Aβ42 has been most closely associated with AD because: (1) FAD-linked mutations in the psen1 and psen2 genes, which encode the catalytic unit of γ-secretase, presenilin-1 and presenilin-2, respectively, result in increased Aβ42 levels [12,13]; and (2) Aβ42 is more prone to aggregation [3,14,15] and is more neurotoxic [16–19] than Aβ40. The two C-terminal amino acids of Aβ42, I41 and A42, induce biophysical properties distinct from those of Aβ40. Jarrett et al found that peptides of 39 or 40 residues remained kinetically soluble for days, whereas peptides of 42 or 43 residues aggregated within hours [15]. Aβ42 also forms oligomers that are different from those formed by Aβ40. For example, pentamer and hexamer ‘paranuclei’ are predominant Aβ42 oligomers whereas dimer, trimer, and tetramer are more abundantly represented in the Aβ40 oligomer population [20]. Multiple lines of evidence demonstrate that the difference in peptide length is one of the key components for controlling early oligomerization. In fact, paranuclei and several other types of oligomer form exclusively from Aβ42 and not from Aβ40 [21].

Many other regions of the Aβ sequence have been discretely studied for their unique characteristics, for example, the central hydrophobic cluster, Aβ17–21 [22,23], and M35 [24–26]. Here we focus on mutations in app that confer amino acid modifications within Aβ and the differences in biophysical and clinical properties that result from these substitutions.

**POINT MUTATIONS AFFECTING THE Aβ SEQUENCE**

**Substitutions in Positions 21–23**

**The E22Q Aβ (E693Q APP) Dutch Mutation**

The Dutch mutation was the first one discovered to cause an intra-Aβ substitution [27]. The resulting disease is called hereditary cerebral hemorrhage with amyloidosis, Dutch type (HCHWA-D). Clinically, patients with HCHWA-D have Aβ deposition predominantly in cerebral vessel walls [27], severe cerebral amyloid angiopathy (CAA), and
hemorrhages with early or diffuse plaques – but, unlike typical AD, minimal neurofibrillary tangles are observed. Likely, dementia develops as a result of the damage caused by multiple microinfarcts or hemorrhages because there is a strong correlation between hemorrhages and dementia. Because the location and degree of the infarcts are highly variable, there is a high degree of variability in the age of onset of dementia in this kindred [28,29].

Studies of the effects of wild-type (WT) Aβ40 and [E22Q]Aβ40 on cerebral microvascular endothelial cells suggest Aβ oligomer-mediated induction of apoptotic pathways through caspase signaling, with a correlation between the enhanced aggregation kinetics of the [E22Q]Aβ40 variant and enhanced apoptosis levels [30]. The Dutch mutation not only enhances the aggregation kinetics relative to WT Aβ (Fig. 38.2) [31–33], it also increases cell toxicity [34,35]. [E22Q]Aβ40 also polymerizes into protofibrils faster than WT Aβ40 [36]. In a study that did not show increased oligomerization kinetics or β-sheet levels – but did show [E22Q] Aβ40-induced apoptosis of primary human cerebral endothelial cells – the mechanism of apoptosis was attributed to the induction of the Bax mitochondrial pathway and was inhibited by the endogenous molecule tauroursodeoxycholic acid [37]. Similar to the human disease, transgenic mice expressing the Dutch mutant form of human APP develop few parenchymal plaques but exhibit prominent CAA [38] and early behavioral deficits [39].

The E22Q substitution replaces a negatively charged glutamate with a neutral glutamine, leading to a loss of negative charge. Sureshbabu et al [31] have suggested that, in the absence of other potentially confounding local environmental factors, mutations resulting in the loss of negative charge in Aβ facilitate aggregation [40] due to a decrease in the electrostatic repulsion among monomers [41]. This point is discussed in more detail in the Conclusions section, below.

The E22G Aβ (E693G APP) Arctic Mutation

Unlike some of the other cases of FAD caused by intra-Aβ substitutions leading to CAA, FAD caused by the Arctic mutation [42] is nearly indistinguishable pathologically from idiopathic AD. In Arctic FAD, Aβ is deposited mainly in the brain parenchyma and, to a lower extent, in the cerebral vasculature, resulting in few hemorrhages [43]. Interestingly, however, many of the plaques have a non-cored, ring-like character and are negative for Congo Red staining, unlike plaques from idiopathic AD brain (Fig. 38.3) [43], but are similar to FAD phenotypes linked to a deletion in exon 9 of presenilin 1 [44], or to the Dutch mutation described above [45]. Similar to sporadic AD, reduced blood flow to the parietal lobe and general brain atrophy have been observed [43]. Disease onset is around the fifth or sixth decade of life, with cognitive dysfunction similar to other familial or sporadic AD phenotypes in multiple cognitive domains, including episodic memory, attention, cognitive speed, and visuospatial functions [43,46]. Carriers of the Arctic mutation, where the polar, negatively charged glutamic acid is replaced with a non-polar, neutral glycine, have lower plasma Aβ concentration levels than patients with sporadic AD, and decreased secretion of this variant, relative to WT Aβ, also has been observed in HEK 293 cells [46].

In vitro, [E22G]Aβ40 has been shown to form fibrils at 3-times lower concentrations than WT Aβ40 and at ≥ 2-times faster rates (see e.g. Fig. 38.2) [33,47]. Similar results were observed with Aβ42, where [E22G]Aβ42 showed increased assembly into protofibrils and fibrils compared to WT Aβ42. Correspondingly, the half-maximal concentration for neurotoxicity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay for the Arctic Aβ42 isoform was ~15% that of WT Aβ42 [34]. Though the Arctic mutation causes a decrease in the

FIGURE 38.2 Most disease-associated intra-Aβ mutations increase Aβ aggregation kinetics. For continuous assessment of aggregation, Aβ variants were incubated with thioflavin T in the wells of a 96-well microtitre plate, shaken at 650 rpm, and fluorescence measurements made at regular intervals. The Q22 data clearly show a lag phase between 0 and ~40 minutes, a growth phase, 40 to 80 minutes, and a plateau phase after 80 minutes. Adapted and reprinted with permission from Betts et al. 33.
concentration levels of Aβ, both in patient plasma and in HEK 293 cell culture media, it enhances protofibril formation substantially (Fig. 38.4) [16,46,48,49], and this enhancement was proposed by Nilsberth et al [46] to be the cause for the FAD. Protofibrils are important intermediates in amyloid assembly [36], which cause selective neuronal death [50,51]. They have been shown to impair spatial learning in transgenic mice [52] and thus have been suggested to be a crucial factor in disease etiology [53].

In studies examining the rates of nucleation and elongation using thioflavin T (ThT) fluorescence, among five Aβ40 variants containing FAD-linked substitutions in residues 21–23 (E22G Dutch, E22G Arctic, E22K Italian, A21G Flemish, and D23N Iowa), the Arctic variant had the
shortest lag phase—15 minutes. The lag phase is one of three phases of growth typically measured in ThT fluorescence experiments. It is followed by growth and plateau phases (Fig. 38.2). The lag phase often is seen in the early incubation times with Aβ40 and is characterized by no ThT fluorescence increases. Presumably, nucleation events, similar to crystal nucleation, occur during this time and once they reach a critical concentration an exponential increase in fluorescence is observed during the growth phase. In comparison to the 15-minute lag phase of [E22G]Aβ40, the lag time of WT Aβ40 was 75 minutes. Similarly, the time to 50% of the maximum aggregation of Arctic Aβ40, 18 minutes, was substantially lower than that of WT Aβ40, 108 minutes, and was the shortest among the five variants studied [33]. Certain studies reported that oligomers, protofibrils, and fibrils of Arctic Aβ were indistinguishable from those of WT Aβ under free solution conditions (i.e. in the absence of a lipid surface) [54,55]. In contrast, an increase in fibril diameter of Arctic Aβ42 was observed by Dahlgren et al relative to WT Aβ42, with a corresponding decrease in the viability of neurons treated by the two Aβ42 alloforms [16]. Additionally, fibrils of [E22G] Aβ40 were found to be shorter and less rigid than those of WT Aβ40 [55], and [E22G]Aβ40 was found to give rise to higher-order oligomers than WT Aβ40 or any of the other mutant forms studied, as observed by photo-induced cross-linking of unmodified proteins (PICUP) and sodium dodecyl sulfate polyacrylamide gel electrophoresis [56].

In discrete molecular dynamics studies, the first seven amino acids in the N-terminus of [E22G]Aβ40 within oligomers (Online Video 1) were found to be more flexible and solvent-exposed than in WT Aβ40 oligomers [57]. Similarly, a flexible N-terminus has been observed in oligomers of WT Aβ42, but not in those of WT Aβ40. Thus, this is hypothesized to be important in mediating Aβ42-induced toxicity [58–60] and it may play a role in the earlier onset of disease in carriers of the Arctic mutation.

When monomers of WT Aβ40 or Aβ40 with FAD-linked mutations at positions 21–23 were incubated for 12 hours on planar lipid bilayers formed on mica by fusion of total brain lipid extract vesicles, among all the mutants, the Arctic mutant disrupted the largest area of phospholipid bilayer [55]. Human SH-SY5Y neuroblastoma cells transfected with APP containing the Arctic mutation showed enhanced sensitivity to toxic stress, as compared to cells transfected with WT APP [61]. The Arctic mutation has been shown to increase Aβ production in transfected SH-SY5Y cells by favoring cleavage by β-secretase over α-secretase. This may occur due to a change in APP cellular localization from the plasma membrane – where it is accessible to α-secretase – to intracellular locations [62]. However, HEK 293 cells, transfected with APP containing the Arctic mutation, exhibited media protein concentrations with significantly lower Aβ42 levels, yet similar
A discrete molecular dynamics trajectory showing the assembly process of [E22G]Aβ40 from a monomer into an elongated undecamer. In this animation, only a part of the simulation box with 32 peptides is visible to allow for a close-up view of the assembly into a single elongated oligomer. The purple residue depicts the N-terminal amino acid D1, and the green residue represents glycine at position 22 of the Arctic mutant [E22G]Aβ40. Distinct colors are used for individual peptide chains. Several events, in which a monomer or a smaller oligomer attaches and detaches from the oligomer under observation, can be observed during the simulation. The movie, which was created by using 400 equally-spaced time frames, spans 40 million simulation steps, equal to ~12 ms. The animation shows that the flexible N-termini play an active role in the oligomer assembly, possibly facilitating the assembly through interactions of A2 and F4 with the hydrophobic CHC and the C-terminal region.
Aβ40 levels, compared to WT APP-transfected cells [46]. The cause of the discrepancy between increased Aβ production and decreased secreted Aβ42 in these two cell culture systems requires further exploration. Mice expressing the [E693G]APP variant show increased and earlier intracellular Aβ immunoreactivity, and form parenchymal amyloid plaques more rapidly, than mice with comparable expression levels of WT APP [63,64]. These mice also show cognitive deficits in hippocampus-dependent learning [65].

The E22K Aβ (E693K APP) Italian Mutation

The Italian Aβ variant, in which a negatively charged glutamate is replaced by a positively charged lysine, leads to a disease similar to that caused by the Dutch variant. This variant causes severe CAA with a heterogeneous age of onset [28,66], CAA caused by the Italian or Dutch Aβ variants is uniquely characterized by deposition of Aβ in the smooth muscle cells surrounding the cerebral vasculature as compared to the typical Aβ pathology in sporadic AD, which is found mostly in brain parenchyma [67]. Similar to both the Dutch and Arctic mutations, HEK 293 cells transfected with APP containing the Italian mutation show decreased secretion of [E22K]Aβ42 levels, but similar levels of [E22K]Aβ40 compared to cells transfected with WT APP.

[E22K]Aβ has a greater propensity to aggregate than WT Aβ [34], which, based on molecular dynamics simulations, has been suggested to be due to an increase in α-helix formation in the region Aβ20–24 compared to WT Aβ42, potentially leading to an increase in helix–helix interactions among monomers and thus an increase in the alignment of unstructured regions near the helices to facilitate oligomerization [68]. Lin et al suggested that the increase in α-helix formation might be attributable to a loss of α-helix destabilizing electrostatic repulsion between E22 and D23 and/or to the longer aliphatic side chain of lysine relative to glutamate, which might provide a favorable hydrophobic interaction with V18 [68]. The Italian mutation in Aβ42 leads to increased toxicity in rat pheochromocytoma (PC-12) cells using the MTT assay [34] or primary cultured human cerebrovascular smooth muscle cells measured by the fluorescent Live/Dead cell assay [67] compared to WT Aβ42. There is no consensus about the rate of aggregation of [E22K]Aβ compared to other variants containing substitutions at positions 21–23. The Italian Aβ variant has been reported to be among the fastest and among the slowest aggregators [31,32]. Bets et al [33] showed that the E22K substitution resulted in a decrease in the nucleation rate but an increase in the fibril-elongation rate of [E22K]Aβ40 compared to WT Aβ40 (Fig. 38.2), which may account for these conflicting results. [E22K]Aβ40 showed larger oligomers [55,56] and shorter and less rigid fibrils relative to WT Aβ40, yet these characteristics did not correlate with the degree of disruption of the phospholipid bilayers, which were similar for the Italian and WT Aβ forms [55].

The E22 Aβ (E693 APP) Deletion – Osaka Mutation

A FAD-linked Aβ variant lacking the glutamic acid residue at position 22 was identified in 2008 in a Japanese kindred [69]. Based on their study of this Aβ variant and the affected individuals, Tomiyama et al hypothesized that the E22 deletion (ΔE22) might represent the first recessive mutation linked to AD because only homozygote carriers showed AD-type dementia. Possibly, the mutation acts in a dose-dependent manner or has incomplete penetrance [69]. ΔE22 mutation carriers showed reduced levels of retaining the amyloid-specific Pittsburgh compound-B in positron emission tomography relative to patients with sporadic AD [69].

The deletion reduced total Aβ secretion levels significantly in HEK 293 cells transfected with a mutant APP construct, compared to WT Aβ, without affecting the Aβ42/40 ratio. Synthetic [ΔE22]Aβ42 assemblies inhibited long-term potentiation more potently than WT Aβ42 [69], and induced synapse loss in mouse hippocampal slices [70], providing a clue about the mechanism by which this Aβ variant causes FAD even though Aβ levels are reduced.

In vitro, [ΔE22]Aβ40 formed β-sheet structures 400-fold faster than WT Aβ40 as measured by circular dichroism spectroscopy, and [ΔE22]Aβ42 formed β-sheet conformation so fast that it was difficult to measure before the observation of large amounts of β-sheet [71]. The effect of the [ΔE22] Aβ40 mutation on oligomer distribution, studied by PICUP, was reduced abundance of dimer, trimer, and tetramer relative to WT Aβ40 (Fig. 38.5). The oligomer size frequency distribution of [ΔE22]Aβ42 was distinct from that of WT Aβ42 and was characterized by a relatively high abundance of dodecamers and octadecamers [71]. The ΔE22 variants of both Aβ40 and Aβ42 formed short protofibrils and fibrils immediately upon solvation from lyophilizates, whereas the WT peptides only showed globular and small, string-like structures [71]. The critical concentration for fibril formation of the deletion variants was approximately half that of their corresponding WT alloforms [71]. Inayatullah et al concluded that the primary biophysical effect of the ΔE22 mutation was to accelerate and stabilize conformational changes in monomeric Aβ [71], which has been suggested to be the rate-limiting step in fibril elongation [72,73]. Additionally, this variant was found to be more resistant to degradation by neurilysin and insulin-degrading enzyme, both of which are known Aβ-degrading enzymes [74], than WT Aβ40 [69].

The A21G Aβ (A692G APP) Flemish Mutation

The Flemish mutation in APP was discovered in 1992 by Hendriks et al [75]. The mutation leads to the substitution of a nonpolar, neutral alanine residue in position 21 of Aβ by a nonpolar, neutral glycine. This substitution leads to a decrease in the hydrophobicity and loss of chirality of residue 21 with no net change in peptide charge [55]. Carriers of the Flemish mutation
develop FAD with presenile dementia characterized pathologically by unusually large plaque cores and cerebral hemorrhage due to CAA with an age of onset in the mid 40s [75–77]. Interestingly, unlike the other intra-Aβ mutation-caused substitutions, the A21G substitution was found to be associated with increased production of both Aβ40 and Aβ42 [46,78].

A 4-fold increased Aβ secretion was observed in HEK 293 cells transfected with APP harboring the Flemish mutation, compared to WT Aβ [79,80]. The Aβ(17–23) region has been found to inhibit γ-secretase cleavage by binding to an allosteric site within the γ-secretase enzyme complex. This inhibitory region is disrupted by the Flemish mutation, which reduces the inhibitory potency of the domain and leads to increased Aβ production [80]. Additionally, monomers of [A21G]Aβ40 are degraded significantly more slowly by neprilysin, though not by insulin-degrading enzyme or plasmin, compared to WT Aβ40 [33].

In cell culture experiments, the toxicity of [A21G]Aβ40 was similar to that of WT Aβ40 [34,81]. Unlike most other mutations leading to intra-Aβ substitutions, the Flemish Aβ40 variant not only does not have a greater propensity to form fibrils but actually has slower fibrillogenesis kinetics than WT Aβ40, including a longer lag phase and the longest time to half-maximal ThT fluorescence among five Aβ40 variants containing substitutions in residues 21–23 (Fig. 38.2) [33,82]. Similarly, [A21G]Aβ42 showed almost no increase in ThT fluorescence over 24 hours [34]. Experiments examining the oligomer size distribution of [A21G]Aβ42 found a narrower distribution with increased percentage of paranuclei, which have been suggested to be important in the toxicity difference between Aβ40 and Aβ42 [20], compared to WT Aβ42 [56], potentially contributing to the etiology of Flemish FAD. A different study found that the extent of phospholipid bilayer disruption by the Flemish Aβ40 variant was decreased compared to WT Aβ40 in vitro [55]. Though this difference was attributed to the increased hydrophilic character of glycine relative to alanine [55], it is unlikely that one methyl group would be entirely responsible for such a difference. Rather, the increased flexibility of the glycine likely confers conformational changes that lead to the observed reduced interaction of [A21G]Aβ40 with the membrane mimetic relative to WT Aβ40.

Though the A21G substitution does not enhance the toxicity or aggregation kinetics of Aβ, it does increase the brain levels of Aβ by both increasing production [46,78] and decreasing clearance [33]. Thus, by comparing the Flemish mutation to other mutations we may gain insight into the role of Aβ concentration versus its aggregation kinetics in AD onset.

The D23N Aβ (D694N APP) Iowa Mutation

The Iowa mutation was described first in 2001 [83]. The substitution results in severe CAA with the addition of neurofibrillary tangles and an unusually larger proportion of Aβ40 in amyloid plaques. This substitution replaces a negatively charged aspartate with a neutral asparagine, resulting in dementia around the sixth or seventh decade of life [83]. The Iowa Aβ variant leads to the formation of larger oligomers [55,56], has a greater propensity to aggregate, and is more potently toxic to PC-12 cells than WT Aβ [34]. [D23N]Aβ40 fibrils grow with a much shorter lag period and shorter growth time to half-maximal β-sheet level, second only to the Arctic mutant on both traits (Fig. 38.2) [33].
Interestingly, the D23N substitution has a dramatic effect on the quaternary structure of Aβ within amyloid fibrils. Over the last decade, multiple solid-state NMR studies showed that within fibrils, Aβ was arranged in parallel, in-register β-sheets [84–86]. The Iowa variant is the first one that has been shown to form both parallel and antiparallel β-sheet fibrils [87]. This finding was interesting not only because it was novel but because it also raised many questions about how a one-amino-acid modification could result in a major change in the quaternary structure that had been consistently observed for other variants.

**N-terminal and C-terminal Substitutions**

**The A2T Versus A2V Aβ (A673T, A673V APP) Mutations**

In 2012, a mutation that replaces an alanine at position two with threonine, which previously had been partially described in a 1993 stroke patient [88], was identified in an Icelandic population to be protective not only against AD, but also against general cognitive decline in the elderly [89]. Jonsson et al discovered that the A2T substitution resulted in a reduction of APP cleavage by β-secretase by approximately half compared to WT APP, leading to ~40% less total Aβ concentration. Interestingly, a mutation causing an A2V substitution had been identified in an Italian population, in which it causes recessive, very early onset FAD. The proband, in whom the disease was first diagnosed, was 36 years old at the onset of cognitive deficits [90]. This mutation increased Aβ production via a change in APP processing and led to enhanced formation of Aβ fibrils in vitro. Incubation of [A2V]Aβ40 with WT Aβ40 or [A2V]Aβ42 with WT Aβ42 resulted in instability of Aβ aggregates—more amorphous aggregates were observed by electron microscopy than in preparations of WT Aβ alone or [A2V]Aβ alone, and more low-molecular-weight peptide was observed by size-exclusion chromatography (SEC). In cell culture experiments, mutant:WT Aβ heteromers had diminished neurotoxicity, supporting the autosomal recessive pattern of inheritance [90].

The effect of substitutions at position 2 in Aβ, and of mutations N-terminal to the β-secretase cleavage site, i.e. outside the Aβ region (Fig. 38.1, Swedish double-mutation), which lead to increased Aβ levels, suggests that WT APP is a lower efficacy substrate for β-secretase and that at least the two residues N-terminal and the two residues C-terminal of the β-secretase cleavage site are critical for the enzymatic process [91].

**The English H6R Aβ (H677R APP) and Tottori D7N Aβ (D678N APP) Mutations**

Additional FAD-linked mutations in the N-terminal region of Aβ include the English mutation, which causes a H6R substitution [92], and the Tottori-Japanese mutation leading to a D7N substitution [93]. Both the English and the Tottori mutations have been shown to enhance fibril formation through a reduction in lag time [94] and a facilitation of the elongation phase [95]. Ono et al [94] also noticed overall accelerated kinetics in transitions of secondary structures from statistical coil to α-helix to β-sheet. Both mutations lead to increased average oligomer size, and are substantially more efficient at seeding fibril formation than WT Aβ40 or Aβ42 oligomers. The English and Tottori variants also are significantly more toxic than the WT counterparts in PC-12 cell culture as measured by the LDH assay (Fig. 38.6) [94]. Interestingly, in different experiments where [H6R] Aβ40 or [D7N] Aβ40 were seeded with WT Aβ42, in addition to eliminating the lag phase, substantial acceleration of the elongation phase also was observed [95]. When seeds made of the mutant forms or of WT Aβ40 were compared for their nucleating ability side-by-side, ThT fluorescence increased more rapidly when mutant Aβ isoform seeds

![Figure 38.6](image-url)  
**FIGURE 38.6** Toxicity of H6R and D7N measured by the lactate dehydrogenase assay. Differentiated PC-12 cells incubated for 48 hours with 25 μM un-cross-linked (UnXL) or cross-linked (XL) Aβ40 (A) and Aβ42 (B) of WT, H6R, or D7N were subjected to the lactate dehydrogenase release assay of cell toxicity. Data represent three independent experiments (*p < 0.05; **p < 0.01). Image courtesy of Drs. Kenjiro Ono and David Teplow.
were incubated with their respective monomers, suggesting that the mutant peptides have higher affinity for their homologous peptide monomers than for the WT monomers [95]. Surprisingly, the accelerated fibril formation was not accompanied by accelerated protofibril formation as measured by SEC [95]. The observation that seeding of fibrils is significantly more efficient with the English and Tottori Aβ variants suggests that the N-terminus is involved in the nucleation process [95]. This is consistent with in vitro biophysical experimental findings using scanning amino acid substitutions and PICUP, which showed that substitution of the first residue of Aβ from aspartic acid to tyrosine led to significant changes in the oligomer distribution pattern of both Aβ40 and Aβ42, possibly causing compaction of oligomer structures, and slowed assembly kinetics [96].

The K16N Aβ (K687N APP) Mutation

A mutation discovered in 2012 to result in dementia with an autosomal dominant inheritance pattern induces a lysine to asparagine substitution at position 16 [97], directly N-terminal to the cleavage site of α-secretase, which leads to the formation of the non-pathologic peptide p3 (Aβ17–x; Fig. 38.1). In experiments using HEK 293 or SH-SY5Y cells, this mutation resulted in decreased sAPPα and sAPPβ concentration levels and decreased turnover of full-length APP at the cell surface. These observations were hypothesized to be due to prolonged half-life of full-length APP caused by the reduction in α-secretase cleavage [97]. In vitro experiments demonstrated that [K16N]Aβ(11–28) was a poorer substrate for the ADAM10 α-secretase than WT Aβ(11–28). Additionally, cell culture experiments showed that α-CTF concentration levels were reduced significantly and that β-CTF increased significantly with a corresponding increase in levels of both Aβ40 and Aβ42 [97]. In SH-SY5Y cells and in rat primary hippocampal neurons, [K16N]Aβ42 was found to be less toxic than WT Aβ42 at equal concentrations. However, a mixture of the two adding up to the same concentration was equally or more toxic than WT Aβ42 alone (Fig. 38.7). The mixture of [K16N]Aβ40 and WT Aβ40 showed higher toxicity than WT Aβ40 alone. Interestingly, [K16N]Aβ40 alone showed either slightly more or equal toxicity to WT Aβ40 alone, depending on the cell type and oligomer size measured by SEC [97]. Examination of fibrillar morphology by electron microscopy suggested that neither [K16N]Aβ40 nor [K16N]Aβ42 formed rigid mature fibrils by 24 hours, and mixture of either of the mutant peptides with the corresponding WT Aβ inhibited the formation of mature fibrils and favored protofibrillar aggregates [97], providing one possible explanation for the high toxicity of these mixtures. Incubation of [K16N]Aβ42 alone with neprilysin showed decreased degradation compared to WT Aβ42 alone. Moreover, the mixture of [K16N]Aβ42 and WT Aβ42 also showed resistance to neprilysin degradation, suggesting another way by which the mutation can be linked to FAD. A mechanism proposed for the increased stability of the mixed oligomers is the potential for K16 of one monomer to form a salt bridge with N16 of another monomer, which could locally stabilize the β-sheet structure [97].

The L34V Aβ (L705V APP) Piedmont Mutation

Similar to the disease phenotype of amino acid substitutions in the 21–23 region of Aβ, the Piedmont mutation that substitutes V for L at residue 34 is characterized primarily by CAA. Interestingly, carriers of this mutation showed an absence of parenchymal Aβ deposits and neurofibrillary tangles [98]. ThT studies comparing WT Aβ40 with [L34V] Aβ40 showed little increase in fluorescence for both Aβ types up to 2 days of incubation. On day three, [L34V]Aβ40 fluorescence levels began increasing slowly but steadily and at a faster rate than WT Aβ40 [99]. Electron microscopy studies of the morphology of the Piedmont Aβ variant showed protofibrils after 3 days of incubation, compared to only small globular structures seen with WT Aβ40 [99]. In cell culture toxicity studies using immortalized human brain microvascular endothelial cells, [L34V]Aβ40 showed higher toxicity levels than WT Aβ40 and similar to those of [E22Q]Aβ40. Toxicity was measured as the number of morphologically apoptotic cells at 72 hours of incubation. With cultures of vascular smooth muscle cells, 72 hours of incubation with [L34V]Aβ40 resulted in even higher toxicity than [E22Q]Aβ40 [99].

Silent and Incomplete Penetrance Mutations

In addition to the mutations mentioned above, which lead to amino acid substitutions inside the Aβ sequence, other mutations have been identified in the Aβ-coding region of APP in AD-afflicted individuals that either do not cause an amino acid change, i.e. are silent, or do not have complete penetrance and thus are found also in non-AD individuals. Silent mutations have been found in AD-afflicted individuals at Aβ residues 34, 37, and 40 [42,100–102]. The mutation at residue 37 has been identified in several instances, including three cases with a family history of AD [42,102], one case of cerebral hemorrhage in a 41-year-old individual with no family history of AD [102], and two cases of healthy individuals, one 35-year-old with no family history of AD [102] and the other an 11-year-old child [101]. An A42V substitution has been observed in a person with schizophrenia with cognitive deficits [103] but also in a non-schizophrenia-afflicted person [100]. Finally, a double mutation that causes a substitution of A42T Aβ (A713T APP) and a silent base change at APP residue 715 also has been observed in an individual with early-onset AD, yet five relatives who carried this mutation did not present with AD [104]. It is difficult to hypothesize how silent mutations could cause disease, though the codon
usage bias or organism preference for one of many codons that encode the same amino acid, may have relevant functionalities and thus consequences. It is possible that these mutations also are random and do not correlate with AD. If this is the case, study of silent mutations may help to define the limitations of tolerable change in the Aβ sequence [104]. If the substitution-causing mutations actually are relevant to AD pathology, but also can be found in non-afflicted individuals, further exploration will be required into incomplete penetrance and co-factors that lead to disease.

**CONCLUSIONS**

Studies of mutations affecting the Aβ amino acid sequence have been critical in illuminating the regions within Aβ where structural changes may lead to disease and the molecular interactions they are involved in.

As mentioned at the end of the section on the Dutch mutation (above), one factor that can facilitate Aβ self-assembly is the loss of electrostatic repulsion among monomers [41]. This theory is supported by the increase in aggregation properties of the Dutch [E22Q], Arctic [E22G], Tottori [D7N], and Iowa [D23N] mutations, where a negatively charged amino acid is substituted by a neutral one, changing the net charge of the Aβ peptide from −3 to −2. Similarly, the E22 deletion causes the loss of a negative charge. In the case of the Italian mutation, although local repulsion between the positively charged lysine residues in position 22 still exists, the overall charge of Aβ is reduced from −3 to −1. The opposite situation happens with the K16N mutation, which leads to loss of local repulsion between the lysine residues in position 16, but an overall increase in net charge to −4. This analysis suggests that local electrostatic repulsion, global changes in peptide net charge, and other factors affecting Aβ conformation and assembly, may be linked to FAD caused by the corresponding mutations.

Another factor may be the destabilization of ‘native’ metastable structures. In the Aβ monomer, a turn region has been identified within the decapeptide Aβ(21–30), which may be one of the earliest conformations formed [105]. This turn, which was hypothesized to nucleate Aβ folding and assembly, is stabilized by hydrophobic interactions between V24 and K28 and by long-range electrostatic interactions between K28 and either E22 or D23. The destabilization of
the turn by substitutions (or deletion) at positions 22 or 23, but not 21, and the positive correlation observed between such destabilization and higher oligomerization propensity of the Dutch, Arctic, Italian, and Iowa Aβ variants, have been implicated in the causation of the resulting FAD [106].

Many of the FAD-linked mutations that affect regions of APP outside of the Aβ sequence increase Aβ levels. In contrast, the Dutch, Arctic, Italian, ΔE22, and A2T modifications cause a decrease in secreted Aβ. Because both a decrease in Aβ levels, resulting from intra-Aβ substitutions, and an increase in Aβ levels, resulting from other FAD-linked mutations, cause disease, the concentration of Aβ may be only part of the problem. This conclusion is supported by the existence of non-demented individuals with extensive Aβ plaque pathology. The studies described here suggest that Aβ sequences that differ from one another by one amino acid can have substantially distinct aggregation kinetics and degrees of toxicity. Importantly, the differences in assembly kinetics and aggregate morphology likely correlate with differences in toxicity of the Aβ variants. Interestingly, the Flemish mutation increases Aβ levels, possibly due to loss of an inhibitory domain in Aβ(17–23) [80]. In view of the decrease in Aβ levels due to changes at residue 22, it is possible that A21G causes a loss of function of the inhibitory activity whereas E22G, E22K, E22Q, and ΔE22 cause gain of function of this inhibitory domain.

Mutations discovered in the app, psen1, and psen2 genes leading to FAD suggested a causative role of Aβ in AD. The mutations affecting specifically the Aβ sequence provide particular insight into important regions, interactions, and structures involved in the way Aβ self-assembles and affects susceptible brain regions. These studies also highlight the paramount impact one amino acid change can have on multiple characteristics from protein function and folding to brain pathology and age of disease onset.

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REFERENCES

PART | IV Polymorphism of Protein Misfolding and Aggregation Processes


