Published in final edited form as:

Neurobiol Dis. 2008 September ; 31(3): 442–450. doi:10.1016/j.nbd.2008.06.001.

Aggregation and catabolism of disease-associated intra-A β mutations: reduced proteolysis of A β A21G by neprilysin

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Abstract

Five point mutations within the amyloid β -protein (A β) sequence of the APP gene are associated with hereditary diseases which are similar or identical to Alzheimer's disease and encode: the A21G (Flemish), E22G (Arctic), E22K (Italian), E22Q (Dutch) and the D23N (Iowa) amino acid substitutions. Although a substantial body of data exists on the effects of these mutations on A β production, whether or not intra-A β mutations alter degradation and how this relates to their aggregation state remain unclear. Here we report that the E22G, E22Q and the D23N substitutions significantly increase fibril nucleation and extension, whereas the E22K substitution exhibits only an increased rate of extension and the A21G substitution actually causes a decrease in the extension rate.

These substantial differences in aggregation together with our observation that aggregated wild type A β (1–40) was much less well degraded than monomeric wild type A β (1–40), prompted us to assess whether or not disease-associated intra-A β mutations alter proteolysis independent of their effects on aggregation. Neprilysin (NEP), insulin degrading enzyme (IDE) and plasmin play a major role in A β catabolism, therefore we compared the ability of these enzymes to degrade wild type and mutant monomeric A β peptides. Experiments investigating proteolysis revealed that all monomeric peptides are degraded similarly by IDE and plasmin, but that the Flemish peptide was degraded significantly more slowly by NEP than wild type A β or any of the other mutant peptides. This finding suggests that resistance to NEP-mediated proteolysis may underlie the pathogenicity associated with the A21G mutation.

Keywords

Alzheimer's disease; amyloid beta-protein; aggregation; degradation; insulin degrading enzyme; plasmin

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INTRODUCTION

Convergent evidence suggests that accumulation of the amyloid β -protein ($A\beta$) and its subsequent aggregation initiates a complex cascade that culminates in Alzheimer's disease (AD) (LaFerla and Oddo, 2005; Walsh and Selkoe, 2004). Five point mutations within the $A\beta$ sequence that are associated with hereditary diseases similar or identical to AD are clustered around the central hydrophobic core of $A\beta$ and include: the A21G Flemish mutation, E22K Italian mutation, E22G Arctic mutation, E22Q Dutch mutation and the D23N Iowa mutation (Fig. 1). These mutations have the potential to impact upon all factors known to regulate $A\beta$ monomer levels, namely production, degradation and aggregation. To date, no simple correlation between $A\beta$ production or aggregation and disease phenotype has emerged, but it, was suggested that the pathogenic effect of the Dutch, Flemish, Italian and Arctic mutations arise as a consequence of their resistance to proteolysis (Tsubuki et al., 2003).

A large number of proteases have been implicated in the catabolism of $A\beta$, but of these neprilysin (NEP, EC 3.4.24.11) and insulin-degrading enzyme (IDE, EC 3.4.22.11) are the most studied (Eckman and Eckman, 2005; Selkoe, 2001; Turner et al., 2004). NEP is a membrane-bound zinc-metallopeptidase that exists as an ectoenzyme preferentially hydrolysing extracellular oligopeptides on the amino side of hydrophobic residues (Carson and Turner, 2002). NEP has been shown by numerous investigators to be capable of degrading $A\beta$ both *in vivo* (Iwata et al., 2004) and *in vitro* (Howell et al., 1995; Kanemitsu et al., 2003; Liu et al., 2007; Shirohani et al., 2001), and its physiologic role has been demonstrated by the finding that genetic ablation of *NEP* causes elevation of endogenous $A\beta$ (Iwata et al., 2001), whereas transgenic or viral expression of *NEP* causes a lowering of cerebral $A\beta$ (El-Amouri et al., 2007; Hemming et al., 2007; Iwata et al., 2004; Leissring et al., 2003; Marr et al., 2003). IDE is a zinc-metalloprotease which shows no obvious primary amino acid sequence specificity but has been proposed to recognize a conformation that is prone to conversion to β -sheet structure (Kurochkin, 1998). As with NEP, support for the physiological importance of IDE in regulating $A\beta$ levels comes from the findings that genetic deletion of *IDE* in mice (Farris et al., 2003; Miller et al., 2003) leads to elevated levels of cerebral $A\beta$, whereas transgenic over-expression of IDE causes a decrease in brain $A\beta$ levels (Leissring et al., 2003). Unlike NEP and IDE, plasmin (EC 3.4.21.7) does not appear to contribute significantly to the normal catabolism of $A\beta$ (Tucker et al., 2004), but rather seems to play a role in the diseased brain. Plasmin is a serine protease that is produced from its inactive precursor, plasminogen, by the action of tissue-type plasminogen activator (tPA), and mice lacking either tPA or plasminogen clear injected $A\beta$ much less well than wild type mice (Melchor et al., 2003). Moreover, recent studies indicate that high levels of $A\beta$ cause a decrease in tPA activity suggesting that excessive accumulation of $A\beta$ results, in part, due to a loss of plasmin (Cacquevel et al., 2007).

In order to investigate the possibility that intra- $A\beta$ mutations mediate their effect through a common pathogenic mechanism, namely resistance to proteolysis, we examined the ability of NEP, IDE and plasmin to degrade both wild type (wt) $A\beta$ (1–40) and $A\beta$ (1–40) bearing disease-associated single amino acid substitutions (Fig. 1). Here we show that the ability of these proteases to degrade $A\beta$ is strongly retarded by aggregation but that when $A\beta$ peptides are presented in their unaggregated, monomeric state, they are efficiently degraded by NEP, IDE and plasmin, with one exception. The $A\beta$ (1–40)A21G peptide is degraded more slowly by NEP than wt $A\beta$ (1–40) or any of the other mutant peptides studied. This resistance to NEP-mediated proteolysis may represent one mechanism by which the A21G mutation causes increased cerebral accumulation of $A\beta$. On the other hand, our data suggest that resistance to proteolysis by the major known $A\beta$ degrading enzymes cannot explain the effects of the other four disease-associated intra- $A\beta$ mutations.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents

Unless otherwise stated all chemicals were purchased from Sigma-Aldrich, St. Louis, MO, USA and were of the highest purity available.

Synthetic A β peptides

The six peptide sequences shown (Fig. 1) were synthesized and purified at the Biopolymer Laboratory in UCLA. Peptide mass and quantity were determined by a combination of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Table 1), and quantitative amino acid analysis (Walsh et al., 1997). In all cases peptide purity as determined by analytical HPLC was >95%.

Aggregation studies

Aggregation was monitored using two distinct assays: (1) sedimentation of aggregates at 100,000g and visualization of the remaining soluble peptides by silver staining of samples separated on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and (2) incubation of samples with Thioflavin T to allow for continuous assessment of dye binding. For the sedimentation assay, peptides were dissolved at a concentration of 60 μ M in 0.1% NH₄OH, diluted with an equal volume of 100 mM Tris-HCl, pH 7.4, and pre-existing aggregates removed by ultracentrifugation at 100,000g and 4°C for 1h. Peptide concentrations were determined using the Micro BCA protein assay (Pierce, Rockford, IL), adjusted to 10 μ M, and 60 μ l aliquots were incubated at 37°C. At appropriate intervals, peptide solutions were ultracentrifuged at 100,000g and 4°C for 1h and 30 μ l of supernatant removed, mixed with 2 $\times$ sample buffer, boiled for 5 minutes electrophoresed on 10–20% tricine gels (Invitrogen Life Technologies Ltd, Carlsbad, CA, USA) and the A β visualized with silver stain according to the method of Shevchenko *et al.* (Shevchenko et al., 1996). A decrease in A β concentration following centrifugation is indicative of sedimentation of A β aggregates.

For continuous assessment of aggregation, A β was incubated in the presence of Thioflavin T and fibril formation estimated by increased fluorescence. Briefly, 75 μ l of a 20 μ M monomeric A β solution (prepared as described for the sedimentation assay) was added to a well of a 96 well micro-titer plate together with 75 μ l 20% DMSO and 148.5 μ l 20 mM sodium phosphate, pH 7.4 and the first reading taken immediately after addition of 1.5 μ l 2 mM Thioflavin T. Samples were incubated at 25°C and shaken at 650 rpm using a VorTemp 56™ Incubator/Shaker with an orbit of 3 mm (Labnet International, Berkshire, UK). Measurements were made at regular intervals using a Molecular Devices SpectraMax M2 microplate reader (Sunnyvale, CA, USA) with excitation and emission at 435 nm (slit width 5 nm) and 485 nm (slit width 10 nm), respectively.

Electron microscopy

Negative contrast EM was performed as described previously (Walsh et al., 1997). Briefly, sample was applied to a carbon-coated Formvar grid, fixed with glutaraldehyde, stained with uranyl acetate and then viewed using a JEOL 1200 EX or JEOL 2000 transmission electron microscope. All reagents for EM were supplied by Electron Microscopy Sciences (Hatfield, PA, USA).

Degradation studies

To prepare aggregate-free, monomeric A β solutions, peptides were dissolved in ice-cold HPLC-grade water, diluted with an equal volume of 100 mM Tris-HCl, pH 7.4, to a

concentration of 40 μM and then passed through a Microcon[®] 10 filtration device (Millipore, Bedford, MA, USA). Peptide concentrations were determined using the Micro BCA protein assay and then adjusted to 20 μM , all peptides when normalized in this way produced peaks on HPLC of similar heights, but the degree of silver staining varied somewhat between the different peptides. De-seeding of A β solutions using Microcon 10 filters has been shown to effectively produce homogenous monomeric solutions completely free of large A β assemblies (Fezoui et al., 2000). Fibril controls were prepared without de-seeding: A β (1–40) was dissolved at 1mg/ml in MilliQ water and incubated at 37°C for 1 week. To generate homogenize suspensions of fibrils the sample was sonicated at 5 watts for 30 sec using a Microson ultrasonic cell disruptor (Misonix, Farmingdale, NY). The presence of amyloid fibrils was confirmed by Congo red binding and electron microscopy.

Irrespective of the assembly form used, peptide solutions (250 μl) were incubated at 37°C with one tenth volume of protease to give a final concentration of 70 nM IDE, 80 μM NEP or 156 nM plasmin. Recombinant human IDE was prepared as described previously (Walsh et al., 2002), recombinant human soluble NEP was a gift from Drs. C. Adessi and F. Grueninger (F. Hoffman-La Roche Ltd, Basel, Switzerland) and plasmin was obtained from Calbiochem (San Diego, CA). Protease inhibitors (2 mM 1,10 Phenanthroline, 1 mM PEFA, 5 mM EDTA, 200 μM leupeptin, 1.5 μM aprotinin, 15 μM pepstatin, final concentrations) were added at the times indicated, and the extent of degradation was assessed by reverse-phase HPLC and/or by densitometric analysis of silver-stained samples separated by SDS-PAGE. Briefly, samples (100 μl) were chromatographed on a Discovery[®] C18 column (4.6 mm $\times$ 25 cm) (Supelco, Bellefonte, PA, USA) using a 0–70% acetonitrile gradient generated by a Waters 600 HPLC Controller (Waters Ltd, Milford, MA, USA), and peptides detected by absorbance at 214 nm using a Waters 996 Photodiode Array Detector. The degree of degradation was determined by the change in the peak area of intact peptide. For SDS-PAGE, samples (10 μl) were mixed with 4 $\times$ sample buffer, boiled for 5 minutes and electrophoresed on 10–20% tris-tricine gels. Gels were silver-stained, and the degree of degradation estimated by densitometric analysis using the Scion Image for Windows Beta 3 program which is available at <http://www.scioncorp.com>.

Congo Red binding assay

The presence of fibrillar aggregates in the 7-day incubated A β solutions was assessed essentially as described previously (Walsh et al., 1999). Sample (25 μl) was mixed with 20 μM Congo red in 20 mM potassium phosphate, pH 7.4, containing 0.15 M sodium chloride (225 μl) and incubated for 30 min at room temperature. The absorbance of the resulting solution was then measured at 480 and 540 nm using a Molecular Devices SpectraMax M2 microplate reader (Sunnyvale, CA, USA). All samples were assessed in triplicate and the amount of Congo red bound was calculated using the formula:

$$\text{Crb } (\mu\text{M}) = (A_{540}/25,295) - (A_{480}/46,306).$$

Mass spectroscopy

A β peptides were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Samples were prepared using the thin-layer method of Beavis and Chait (Beavis and Chait, 1996) using α -cyano-4-hydroxycinnamic acid as matrix. A β solutions were first diluted with 50% acetonitrile containing 0.1% TFA in 1:1 ratio and then further diluted 5 times with matrix solution containing saturated matrix in formic acid, water and isopropanol (1:4:4, v/v/v). An aliquot (1 μL) of peptide-matrix solution was spotted onto a sample plate with a preformed ultra thin layer of matrix. Sample spots were washed briefly with ice-cold aqueous TFA (0.1%) and then loaded into the mass spectrometer. Spectra were collected on a Voyager DE-STR time-of-flight mass

spectrometer (Applied Biosystems, Foster City, CA) in linear mode with 500 laser shots. Mass calibration was done using bovine insulin as either internal or external mass calibrant.

Statistical Analysis

For comparison of the onset of aggregation, the Tukey-Kramer Multiple Comparisons Test was used to assess the differences between the initial Thioflavin T fluorescence and the fluorescence at each subsequent time point thereafter (GraphPad Prism™ software, GraphPad Inc., San Diego, CA).

RESULTS

All known disease-associated intra-A β mutations except the A21G substitution increase aggregation propensity

To our knowledge, this is the first report to directly compare the aggregation kinetics of wt A β (1–40) and A β (1–40) peptides bearing each of the five point mutations associated with familial disorders linked to AD. For this study, we used 2 independent methods to monitor the aggregation process and electron microscopy to confirm the presence of amyloid fibrils. Initially, aggregation was monitored by incubating peptides at 37°C and measuring the loss of peptide from the supernate following 100,000 g centrifugation. We have used this protocol extensively, and it allows for detection of sedimentable fibrillar assemblies by monitoring their removal from solution (Clements et al., 1996; Clements et al., 1993; Walsh et al., 1997). When incubated at 10 μ M, neither wt A β (1–40) nor A β (1–40)A21G showed any sign of fibril formation over a four day period (Fig. 2A). Indeed, even at 20 μ M, these peptides showed no measurable aggregation, and it was only when they were incubated at 100 μ M that appreciable loss of sedimentable aggregates was detected (not shown); at 100 μ M, both peptides took more than 3 days to form sedimentable aggregates. In contrast, the rate of aggregation for the other mutant peptides was greatly accelerated compared to that of the wt peptide (Fig. 2A). At 10 μ M, peptides bearing the G22 and Q22 mutations readily aggregated and thus disappeared from the supernate within 12 h, whereas A β (1–40)D23N essentially disappeared from solution by 24 h. A β (1–40)E22K remained in solution for at least 1 day but was completely aggregated into sedimentable assemblies by 48 h. The experiment shown in Fig. 2A is representative of 3 separate experiments, and in each study the order of peptide aggregation was closely similar. However, due to the stochastic nature of protein aggregation of unagitated samples we did observe some variations both between and within experiments. For instance, the duplicates for the 6 h time points for A β (1–40)E22G and A β (1–40)E22Q are different, with one duplicate showing a substantial decrease at 6 h and the other showing no decrease. Nonetheless, this method clearly shows that A β (1–40)E22G and A β (1–40)E22Q form sedimentable aggregates most readily, with D23N next fastest, then A β (1–40)E22K and finally wt and A β (1–40)A21G the slowest. As expected, all peptides studied formed typical amyloid fibrils which were readily detected by EM in both unspun samples and in pellets produced by centrifugation (not shown).

To confirm the veracity of the results obtained using the sedimentation assay and to obtain a more quantitative comparison of aggregation kinetics we employed a Thioflavin T (ThT) binding assay in which samples could be continuously monitored. This approach is particularly useful as it limits the amount of peptide used and provides multiple data points around the time intervals of most interest. Using traditional static ThT binding assays we have previously observed wide fluctuations in the ThT binding of aggregates (Walsh et al., 2001), probably because of variations in the size of the aggregates. Therefore in this study we were careful to vigorously agitate samples throughout the course of the reaction. Such agitation results in faster aggregate formation, but more importantly, the resultant ThT binding data is more consistent among replicate samples. Using this assay, the A β (1–

40)E22G showed the shortest lag phase, taking only 15 min before the ThT signal increased significantly above the t=0 control ($P<0.001$, Fig. 2B). In this sort of experiment, the lag phase corresponds to the time taken to form nuclei that can then elongate by addition of monomer (Naiki and Nakakuki, 1996). The lag phase for A β (1–40)D23N was somewhat irregular but showed a significant increase at 30 min and all time points thereafter ($P<0.01$, Fig. 2B). The A β (1–40)E22Q had a longer lag phase, being elevated from 60 min ($P<0.01$, Fig. 2B). The lag phases for wt and A β (1–40)A21G were highly similar, with both peptides producing the first sign of appreciable ThT binding at 70 and 80 min ($P<0.05$ and $P<0.0001$, Fig. 2B), respectively. Surprisingly the lag phase of A β (1–40)E22K was the longest, with this peptide only producing a significant increase in ThT binding at 90 min. The discrepancies between the initiation of aggregation as measured by the lag time for aggregation to be detected by ThT (Fig. 2B) and differences measured by our sedimentation assay (Fig. 2A) probably reflect the ThT assay being much more sensitive, in that it can detect soluble aggregates that are not readily sedimented (Walsh et al., 1999). Both wt and A β (1–40)A21G have a shorter lag until aggregation is detected by ThT binding than does A β (1–40)E22K, but the time required for A β (1–40)E22K to achieve 50% of its aggregation maximum is much shorter (~70 min) than that of either wt (~110 min) or A β (1–40)A21G (~140 min, Table 2). It is reasonable to assume that the intensity of the ThT signal is related to both the size and number of aggregates formed and that the G22, Q22 and N23 mutations each decrease the lag time required for initial aggregation while also forming larger sedimentable aggregates at a much faster rate than wt. The K22 mutation slightly increases the lag time required to initiate aggregation, but this peptide can form larger, more readily sedimentable aggregates considerably faster than wt. The lag time for wt and A β (1–40)A21G are highly similar, but the time required to achieve maximal aggregation is longer for A β (1–40)A21G than wt (Fig. 2B). These results are consistent with prior reports in which aggregation of A β (1–40)A21G was found to be similar or slightly slower to that of wt peptide (Clements et al., 1996; Walsh et al., 2001; Walsh et al., 1997) whereas, A β (1–40)E22G, A β (1–40)E22Q, A β (1–40)E22K and A β (1–40)D23N have all been shown to aggregate faster than wt A β (1–40) (Clements et al., 1996; Melchor et al., 2000; Nilsberth et al., 2001; Sian et al., 2000; Van Nostrand et al., 2001; Walsh et al., 1997) (Fig. 2A and B).

Aggregated wt A β is degraded less well than monomeric wt A β

Some studies have reported that A β peptides bearing disease-associated mutations are resistant to degradation by NEP and IDE (Morelli et al., 2003; Tsubuki et al., 2003). But since these mutations also alter the aggregation properties of A β (Fig. 2) and it has been suggested that aggregated A β is less susceptible to degradation (Tucker et al., 2000), we sought to determine if the reported differences in proteolysis resulted from the changes in primary structure *per se* or simply reflected differences in the peptides' propensity to aggregate. To this end, we compared the ability of IDE, NEP and plasmin to degrade both aggregated and monomeric A β (1–40). Monomeric A β was prepared as described previously (Fezoui et al., 2000) and assessed for aggregates by negative contrast electron microscopy, Congo red binding (Crb) and sedimentation at 100,000 g (see Experimental Procedures). All three tests confirmed that the monomeric preparation was free of detectable aggregates (Supplementary Fig. 1).

Using these well-defined preparations, we examined the ability of IDE, NEP and plasmin to degrade both monomeric and aggregated A β . In agreement with prior studies (Howell et al., 1995; Iwata et al., 2000; McDermott and Gibson, 1997; Qiu et al., 1998; Tucker et al., 2000) all three enzymes degraded wt A β monomer in a time-dependent manner (Fig. 3, upper panels), whereas wt aggregated A β was resistant to degradation over the time course studied (Fig. 3, lower panels). Reduced rates of degradation of oligomeric or aggregate forms of A β have been observed for certain proteases. For instance, Morelli *et al.* have shown that the

E22Q peptide is degraded by IDE at a significantly reduced rate compared to wt and they hypothesised that the high degree of beta structure in the E22Q peptide was responsible for the reduced degradation (Morelli et al., 2003). Similarly, Tucker *et al.*, have reported that the degradation kinetics of aggregated A β by plasmin were much slower than degradation of monomeric A β . Using 40 μ M A β (1–40) and plasmin concentrations more than four-fold greater than those used in our experiments, Tucker *et al.* observed only a ~30% degradation of aggregated A β in a 1 h period (Tucker et al., 2000).

IDE, NEP and Plasmin are capable of degrading A β peptides bearing disease-associated amino acid substitutions

Using the filtration procedure employed above, we prepared aggregate-free monomer solutions of A β peptides bearing each of the 5 known disease-associated mutations and asked the simple question can IDE, NEP and plasmin degrade these peptides? All A β peptides studied were susceptible to degradation by IDE, NEP and plasmin (Fig. 4A–F). Indeed, the extent of degradation, whether measured by SDS-PAGE/silver stain or HPLC was similar for all peptides, with one notable exception, the A21G peptide. This peptide consistently showed decreased degradation by NEP compared to either wt peptide or the other mutant peptides (compare the G21 lane in Fig. 4B versus the other lanes). We have already demonstrated that the aggregation state of A β can influence its degradation (Fig. 3), the aggregation kinetics of A21G are very similar to that of wt A β (Fig. 2B) and are much slower than those of the other mutant peptides so the decreased catabolism of A21G by NEP (Fig. 4B) is not due to increased aggregation. Moreover, the fact that the same A21G showed no resistance to proteolysis by IDE or plasmin supports the conclusion that the A21G did not aggregate during incubation with proteases.

To preclude any anomalies resulting from a single means of measuring degradation, we carried out a separate set of experiments in which we used HPLC to monitor the degradation of the wt and mutant peptides. In addition to monitoring the loss of the intact A β substrate, HPLC simultaneously detects the appearance of its proteolytic products (Fig. 5D). As with our SDS-PAGE/silver detection procedure (Fig. 4A–C), HPLC (Fig. 4D–F) revealed that all peptides examined were degraded by each of the proteases used, and it also confirmed that the A21G peptide was less degraded by NEP (Fig. 4E). These results are in conflict with the findings of Tsubuki *et al.*, who reported that the A21G, E22Q, E22K and E22G peptides were completely resistant to degradation by NEP (Tsubuki et al., 2003). This apparent contradiction likely results from differences in the preparation of peptides prior to, or aggregation during, the incubation with NEP. We were very careful to use monomeric peptide preparations completely devoid of any detectable A β aggregates (SFig. 1). In addition to characterising the peptides before use, we also employed the sedimentation assay to investigate the aggregation state of peptides after incubation at 37°C for two hours, confirming that no significant aggregation occurred during the incubation with proteases (data not shown). Furthermore, to exclude any differences arising due to chemical impurities, we used at least two different batches of each peptide and observed similar degradation profiles with each batch. We conclude that in their monomeric state all 6 peptides are readily degraded by the 3 most well-studied A β degrading proteases. However, in all experiments, whether assessed using the SDS-PAGE/silver stain or HPLC paradigms, the A21G peptide was less efficiently degraded by NEP than wt A β (1–40) (Fig. 4). Consequently, a more detailed study comparing the degradation of A β (1–40)A21G and wt peptide was undertaken.

NEP degrades A β (1–40)G21 less well than A β (1–40)

The observed resistance of the A21G (Flemish) peptide to degradation by NEP suggests that impaired degradation could underlie the enhanced parenchymal and vascular accumulation

of A β that characterizes the brains of individuals carrying this mutation. In order to investigate this further, kinetic studies were undertaken. NEP was incubated with wt A β (1–40) or A β (1–40)A21G for the indicated times, samples were electrophoresed and silver stained, and densitometry was used to estimate the amount of intact peptide remaining at each time point. Under the conditions used, wt A β (1–40) peptide had a half-life ($t_{1/2}$) of ~20 minutes, whereas the $t_{1/2}$ for the A β (1–40)A21G peptide was almost 3 times as long, taking ~60 min for 50% of the peptide to be degraded (Fig. 5B). In a separate set of experiments, HPLC was used to measure NEP-mediated degradation of wt A β (1–40) and A β (1–40)A21G. This analysis produced results qualitatively similar to those obtained using the SDS-PAGE/silver stain assay: the half lives for wt A β (1–40) and A β (1–40)A21G were ~20 min and ~70 min, respectively (Fig. 5C). HPLC also revealed that the NEP degradation profile of A β (1–40)A21G differed from that of the other peptides studied. Specifically, two product peaks, present in the NEP-digested wt A β (1–40), as well as the A β (1–40)E22G, A β (1–40)E22K, A β (1–40)E22Q and A β (1–40)D23N samples, were not found in the NEP-digested A β (1–40)A21G reactions (Fig. 5D and E). This latter observation suggests that substitution of alanine by glycine at position 21 changes the accessibility of certain peptide bonds such that NEP does not cleave A β (1–40)A21G at all the positions at which it cleaves wt A β (1–40).

To address this question, we analyzed the proteolytic fragments of wt A β (1–40) and A β (1–40)A21G generated by NEP treatment using MALDI-TOF-MS. This revealed that NEP degradation of wt A β (1–40) produced a number of proteolytic fragments, including 4–16, 4–17, 1–16, 1–17, 1–19, 1–30 and 1–33 (Fig. 5F). A similar profile was also obtained for A β (1–40)A21G (Fig. 5G), but the 1–19 fragment was not detected in 4 separate experiments. However, as in our HPLC analysis (Fig. 5D and E), the levels of proteolytic fragments were much reduced in the A β (1–40)A21G samples. Indeed even in samples that were incubated with NEP such that approximately 50% of the parent peptide was degraded the intensity of the A21G products was substantially lower than that observed for the wt peptide (Fig. 5F and G). Thus this makes it difficult to discern whether 1–19 was not produced by NEP proteolysis of A β (1–40)A21G or simply that it was produced at levels too low to detect.

DISCUSSION

This is the first study to simultaneously compare the relative aggregation rates and the susceptibility to degradation of A β peptides containing amino acid substitutions corresponding to all the known intra-A β mutations linked to AD-like disorders. Here we report that A β (1–40)E22G shows the highest propensity for aggregation (i.e., both rapid nucleation and fibril extension) and that A β (1–40)E22Q and A β (1–40)D23N show slightly slower, but similar aggregation kinetics. Aggregation of A β (1–40)E22K is intermediate between that of A β (1–40)E22Q and wt A β , with a longer lag phase than wt, but a more rapid extension phase. A β (1–40)A21G is similar to wt, but with a less rapid extension phase. Increased aggregation has long been assumed to underlie the diseases linked to the E22G, E22K, E22Q, and D23N mutations, however, the relative rates of *in vitro* aggregation do not show a simple correlation with either the age of disease onset or the type of pathology observed. For instance, the E22G mutation causes a disease indistinguishable from idiopathic AD with an average age of onset of 57+/- 2.7 years (Nilsberth et al., 2001) whereas, the Iowa disease is characterized by severe congophilic amyloid angiopathy (CAA) and a disease onset of 68.9 +/- 4.7 years (Grabowski et al., 2001). Indeed even within the same kindred the disease phenotype can vary dramatically. For instance the A21G (Flemish) mutation can manifest as presenile dementia or cerebral haemorrhage or a mixture of the two, with mean dementia onset at 46.2 +/- 6.1 years (Brooks et al., 2004; Roks et al., 2000). The diseases associated with the E22K and E22Q mutations are characterized by severe CAA but display a highly heterogeneous age of onset (Wattendorff et al., 1995). Thus, while

an increased propensity for aggregation is likely to contribute to the aetiology of these diseases; other factors must also influence the disease outcome. Indeed mouse models expressing human APP bearing the A22G mutation form amyloid plaques much more rapidly than mice expressing comparable levels of wild type APP (Cheng et al., 2004), whereas mice expressing E22Q APP form few parenchymal plaques, but exhibit prominent CAA (Herzig et al., 2004).

Because intra-A β mutations have the potential to impact upon A β degradation as well as aggregation, we asked if peptides bearing intra-A β mutations were degraded differently by 3 proteases known to be important for cerebral A β catabolism *in vivo*. Using wt A β , we found that proteolysis was highly dependent upon aggregation state and that IDE, NEP and plasmin could not readily degrade fibrillar A β . Thus, to distinguish between altered proteolysis resulting from a change in the primary amino acid sequence *per se* as opposed to a change in aggregation propensity, we were careful to use peptides devoid of aggregates and which exhibited no detectable secondary structure. Owing to the dynamic nature and difficulty obtaining soluble oligomers we did not investigate if oligomers produced from mutant A β peptides are catabolized differently, rather we focused on studying degradation of monomeric A β peptides. Employing these monomeric, aggregate-free preparations, we found that peptides bearing disease-associated mutations were in general readily degraded by IDE, NEP and plasmin.

Our findings are in conflict with reports that peptides bearing E22Q, A21G, E22K and E22G mutations are almost totally resistant to proteolysis by NEP (Tsubuki et al., 2003) and that the E22G and E22Q peptides are degraded poorly by IDE (Morelli et al., 2003). The most likely explanation for this divergence in results lies in differences in the aggregation state of the peptides used. In this regard, it is particularly noteworthy that in the case of the study by Morelli *et al.* the authors reported that their A β (1–40)E22Q (and to a lesser extent A β (1–40)E22G) contained substantial β -structure (Morelli et al., 2003). Both A β fibrils and protofibrils have a high β -sheet content, whereas A β monomer is natively unfolded (Walsh et al., 1999). Since we found that IDE degrades fibrillar A β much less readily than A β monomer (Fig. 3), it seems likely that the reported resistance of A β (1–40)E22G and A β (1–40)E22Q to degradation by IDE (Morelli et al., 2003) was due to the presence of A β fibrils and/or protofibrils and not a simple consequence of the amino substitutions rendering these peptides less good substrates. Similarly, the report that NEP did not readily degrade A β (1–40)A21G, A β (1–40)E22G, A β (1–40)E22K and A β (1–40)E22Q may also have resulted from aggregation of the peptides used. Although Tsubuki *et al.* reported that the peptide solutions had not formed fibrils or protofibrils, the extraordinarily high concentration (40 g/L i.e. ~9.2 mM) of peptide used would suggest otherwise (Tsubuki et al., 2003). Another possible explanation for the divergence between our results and those of Tsubuki *et al.* (Tsubuki et al., 2003) may be the species and form of the NEP used. In the current study, soluble human NEP was used, whereas Tsubuki *et al.* used mouse NEP of an undefined nature (Tsubuki et al., 2003). It is conceivable that mouse NEP, although highly homologous with human NEP (Li et al., 1995), may act on mutant A β differently, or that if Tsubuki *et al.* used a membrane-bound form of NEP that the mutant peptides readily associated with the membrane and were less accessible for proteolysis.

Whatever the reason for these discordant findings, in numerous experiments using different synthetic peptide batches and different methods to assess degradation we have consistently found that all disease-associated intra-A β mutant peptides are degraded similarly by IDE and plasmin and that all except the A21G (Flemish) peptide are degraded equally well by NEP. Given that A β (1–40)A21G was readily degraded by IDE and plasmin and that this peptide showed similar aggregation kinetics to wt A β (1–40), the observed resistance to NEP is unlikely to be due to altered aggregation but rather is a direct result of the change in

primary sequence. NEP preferentially hydrolyses oligopeptides on the amide side of hydrophobic amino acids (Turner et al., 2001) and is thought to cleave A β at multiple sites including at phenylalanine 20 which results in liberation of the 1–19 fragment. Glycine is highly disruptive of secondary structure, and it is possible that the A21G substitution distorts the A β monomer rendering it a less good substrate for NEP. This notion is supported by a recent structural study that indicates A β 21–30 containing the A21G substitution is more resistant to digestion by trypsin than wild type A β 21–30 or A β 21–30 containing other disease relevant substitutions (Grant et al., 2007). In turn this resistance to NEP-mediated proteolysis would facilitate a build up of A β monomer and may explain why the A21G mutation causes increased intracerebral accumulation of A β .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Drs. Zuhair Nasrallah (UCD) Francesca Paradisi (UCD) and Kevin O'Connor (UCD) for their assistance using the HPLC system and Ann Molloy (UCD) for use of the micro-balance. We also thank Dr. Mark Findeis (Satori Pharmaceuticals Inc, Boston, MA, USA) for advice on the Thioflavin T binding assay and Drs. Celine Adessi and Fiona Grueninger (F. Hoffman-La Roche Ltd, Basel, Switzerland) for the gift of soluble NEP.

This work was supported by Wellcome Trust grant 067660 (DMW), NIH grant AG027443 (DMW & DJS) and the Alzheimer's Association (RW). The mass spectrometry study was in part supported by an NIH/NCRR Shared Instrumentation Grant.

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Fig. 1. Primary sequences of wt and disease-associated mutant A β peptides

The sequence of wt A β (1–40) is shown in black letters, with residues 21, 22 and 23 denoted in bold and larger type. Single amino acid substitutions corresponding to intra-A β mutations found in familial AD-related disorders are shown in grey: the A21G, Flemish; E22Q, Dutch; E22K, Italian; E22G, Arctic; D23N, Iowa. The α -cleavage site which facilitates APP ectodomain shedding and which precludes A β formation is indicated with an arrow.

(A).

(B).

Fig. 2. Most disease-associated intra-Aβ mutations increase the aggregation kinetics of Aβ peptides

(A) Duplicate samples of Aβ peptides (10 μM) were incubated at 37°C and aggregation measured by loss of peptides from solution following centrifugation at 100,000 g for 1 h. Results shown are typical of three separate experiments. Gels have been cropped to show the region between 3 and 7 kDa, because no higher migrating species were detected. (B) For continuous assessment of aggregation, Aβ peptides were incubated with Thioflavin T in the wells of a 96 well micro-titre plate, shaken at 650 rpm, and fluorescence measurements made at regular intervals. The results shown are representative of two such experiments, in which 6 replicates were used per peptide and are presented as the mean $\pm$ standard error.

Fig. 3. Aggregated A β is degraded less well than monomeric A β

Incubation of monomeric wt A β (1–40) (20 μ M) with IDE, NEP or Plasmin caused a time-dependent loss of peptide, whereas wt A β (1–40) levels remained constant when the protease had first been specifically inhibited (I). Aggregated wt A β (1–40) was not significantly degraded by IDE, NEP or plasmin. In experiments using active proteases, proteolysis was halted by addition of inhibitors (2 mM 1,10 Phenanthroline, 1 mM PEFA, 5 mM EDTA, 200 μ M leupeptin, 1.5 μ M aprotinin, 15 μ M pepstatin, final concentrations); samples were resolved on 10–20% tris-tricine gels, and the proteins detected by silver stain. As in Fig. 2b gels have been cropped to show the region between 3 and 7 kDa, because no higher migrating species were detected.

Fig. 4. IDE, NEP and Plasmin readily degrade A β peptides bearing disease-associated amino acid substitutions

Monomeric peptide solutions were incubated +/- IDE (A), NEP (B) or Plasmin (C) for 45, 60 and 30 min respectively, protease inhibitors were added and samples electrophoresed on 10–20% tricine gels and peptides detected by silver stain. Upper panels show results from a representative experiment and lower panels show average results +/- SD of three independent experiments (A–C). In a separate set of experiments monomeric peptide solutions were incubated +/- IDE (D), NEP (E) or Plasmin (F) for 45, 60 and 30 min respectively, protease inhibitors added and then chromatographed on a Discovery[®] C18 HPLC column (Supelco, Bellefonte, PA) column and the change in the parent A β peak area measured. The results shown are averages +/- SD of triplicate samples run in a single experiment. The difference in the extent of degradation between the experiments described in panels A–C and D–F is due to variances in enzyme activity, as experiments were carried out on different days, and the amount of enzyme used was determined by protein content and not activity.

Fig. 5. NEP degrades A β (1-40)A21G less well than wt A β (1-40)

wt A β (1-40) and Flemish A21G peptides (20 μ M) were incubated with NEP and degradation monitored over a 105 min interval by either densitometric quantitation of silver stained A β bands (A and B) or by HPLC (C). The results shown in (A) are representative of three experiments. The graph in (B) represents the mean $\pm$ SD of densitometric analysis of one such experiment in which triplicate samples were used. The data shown in (C) represents the mean $\pm$ SD of a typical experiment analysed in duplicate. In (D and E) HPLC chromatograms are shown, and the product peaks labelled a-d have the following elution times a: 11.30; b: 12.25; c: 12.52; d: 13.10 (min). Note the absence of two peaks (a and d) from the NEP digestion of the A21G peptide. Data shown in (D and E) are representative of

3 such experiments. The peaks that elute ~20 and 22 min were also present in the buffer controls. (F and G) Mass spectrometry analyses. Peptides were prepared as described above and adjusted to a concentration of 20 μM . NEP (1/10th volume) was added to the peptide solution and digestion allowed to proceed at 37°C until approximately 50% degradation was achieved (as assessed by SDS-PAGE/silver staining). Reactions were stopped by boiling and samples stored at -80°C until analysed by MALDI-TOF mass spectrometry.

Table 1

MALDI-TOF Mass spectrometry of synthetic wt and disease-associated A β peptides.

A β peptide	Predicted Mass	Observed Mass
A β (1–40)	4330.9	4330.0
A β (1–40)A21G	4316	4315.6
A β (1–40)E22G	4258.0	4258.0
A β (1–40)E22K	4329.9	4328.8
A β (1–40)E22Q	4329.9	4329.9
A β (1–40)D23N	4329.9	4330.1

Table 2

All disease-associated A β peptides, except G21 show increased aggregation kinetics.

Peptide	Time to reach $\frac{1}{2}$ maximal ThT binding (min)
G22	18
N23	51
Q22	59
K22	84
WT	107.5
G21	138