

Elucidation of Primary Structure Elements Controlling Early Amyloid β -Protein Oligomerization*[§]

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Assembly of monomeric amyloid β -protein ($A\beta$) into oligomeric structures is an important pathogenetic feature of Alzheimer's disease. The oligomer size distributions of aggregate-free, low molecular weight $A\beta$ 40 and $A\beta$ 42 can be assessed quantitatively using the technique of photo-induced cross-linking of unmodified proteins. This approach revealed that low molecular weight $A\beta$ 40 is a mixture of monomer, dimer, trimer, and tetramer, in rapid equilibrium, whereas low molecular weight $A\beta$ 42 preferentially exists as pentamer/hexamer units (paranuclei), which self-associate to form larger oligomers. Here, photo-induced cross-linking of unmodified proteins was used to evaluate systematically the oligomerization of 34 physiologically relevant $A\beta$ alloforms, including those containing familial Alzheimer's disease-linked amino acid substitutions, naturally occurring N-terminal truncations, and modifications altering the charge, the hydrophobicity, or the conformation of the peptide. The most important structural feature controlling early oligomerization was the length of the C terminus. Specifically, the side-chain of residue 41 in $A\beta$ 42 was important both for effective formation of paranuclei and for self-association of paranuclei into larger oligomers. The side-chain of residue 42, and the C-terminal carboxyl group, affected paranucleus self-association. $A\beta$ 40 oligomerization was particularly sensitive to substitutions of Glu²² or Asp²³ and to truncation of the N terminus, but not to substitutions of Phe¹⁹ or Ala²¹. $A\beta$ 42 oligomerization, in contrast, was largely unaffected by substitutions at positions 22 or 23 or by N-terminal truncations, but was affected significantly by substitutions of Phe¹⁹ or Ala²¹. These results reveal how specific regions and residues control $A\beta$ oligomerization and show that these controlling elements differ between $A\beta$ 40 and $A\beta$ 42.

with its tendency to form amyloid fibrils (1–4). However, accumulating evidence suggests that soluble $A\beta$ oligomers and pre-fibrillar aggregates are the proximal effectors of neurotoxicity in early stages of AD (for reviews, see Refs. 5–7). Soluble oligomeric forms of $A\beta$, including dimers and trimers (8, 9), $A\beta$ -derived diffusible ligands (10–16), and protofibrils (17–20) are neurotoxic. Transgenic mice expressing the human amyloid β -protein precursor ($A\beta$ PP) exhibit neurologic deficits prior to the appearance of amyloid deposits (21–23). In humans, $A\beta$ oligomers have been detected in cerebrospinal fluid of Alzheimer's patients but not in age-matched controls (24). An $A\beta$ PP mutation causing reduction of total $A\beta$ levels accompanied by accelerated $A\beta$ assembly into protofibrils has been linked to early onset familial Alzheimer's disease (FAD) in a Swedish kindred (25). These findings support an important role for $A\beta$ oligomers in the etiology of AD and thus make $A\beta$ oligomers attractive therapeutic targets.

If effective drug design strategies targeting $A\beta$ oligomers are to be developed, detailed knowledge of the structures and assembly dynamics of these oligomers must be obtained. However, the biophysical and structural characterization of oligomeric $A\beta$ assemblies has been difficult due to their metastable nature. Previously, we demonstrated that the size distribution of $A\beta$ oligomers could be determined quantitatively (26, 27) using the technique photo-induced cross-linking of unmodified proteins (PICUP) (28, 29). This approach revealed that low molecular weight (LMW) $A\beta$ 40, rather than existing in a stable monomeric or dimeric state, as previously suggested (30, 31), comprised a mixture of monomer, dimer, trimer, and tetramer in rapid equilibrium. In contrast, LMW $A\beta$ 42 produced a distinct oligomer distribution in which the main components were pentamer/hexamer units (paranuclei), which then formed larger oligomers through self-association (27). Here, PICUP was used to determine systematically the effects of primary structure modifications on early $A\beta$ oligomerization. We studied $A\beta$ 42 analogues bearing modifications at the C-terminal dipeptide, $A\beta$ 40 and $A\beta$ 42 analogues containing clinically relevant mutations at or near the central hydrophobic cluster (CHC), N-terminally truncated alloforms of $A\beta$ 40 and $A\beta$ 42 found in amyloid plaques, and $A\beta$ 40 analogues containing substitutions that alter the net charge of the peptide. The results advance our understanding of early $A\beta$ assembly, provide deeper mechanistic insight into the distinct oligomerization behaviors of $A\beta$ 40 and $A\beta$ 42, and suggest new targets for AD therapy.

Past studies of the role of the amyloid β -protein ($A\beta$)¹ in Alzheimer's disease (AD) have linked the neurotoxicity of $A\beta$

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¹ The abbreviations used are: $A\beta$, amyloid β -protein; $A\beta$ PP, amyloid β -protein precursor; AD, Alzheimer's disease; CHC, central hydropho-

bic cluster; FAD, familial Alzheimer's disease; LMW, low molecular weight; NTT, N-terminally truncated; PICUP, photo-induced cross-linking of unmodified proteins; Ru(Bpy), Tris(2,2'-bipyridyl)dichlororuthenium(II); SEC, size exclusion chromatography; WT, wild type.

MATERIALS AND METHODS

Peptides and Reagents—A β 40, A β 42, and analogues thereof (Table I) were synthesized by Fmoc (*N*-9-fluorenyl)methoxycarbonyl chemistry, purified by reversed-phase high performance liquid chromatography, and characterized by mass spectroscopy and amino acid analysis, as described (32). Tris(2,2'-bipyridyl)dichlororuthenium(II) (Ru(Bpy)) and ammonium persulfate were purchased from Aldrich. Polyacrylamide gels, buffers, stains, standards, and equipment for SDS-PAGE were from Invitrogen.

Isolation of LMW A β —LMW fractions of A β alloforms were isolated by size exclusion chromatography (SEC), as described previously (26). Briefly, 170 μ l of a 2 mg/ml peptide solution prepared in Me₂SO was fractionated using a 10/30 Superdex 75 HR column eluted at 0.5 ml/min with 10 mM sodium phosphate, pH 7.4. Peaks were detected by UV absorbance at 254 nm. A 10- μ l aliquot of each fraction was taken for amino acid analysis *a posteriori* to determine the peptide concentration. Typical concentrations were 30 \pm 10 μ M. SEC reproducibly yielded comparable LMW fractions for all of the peptides used. Recent studies (27) have shown that LMW A β prepared using SEC readily forms paranuclei and higher order oligomers, facilitating study of these assembly processes. For this purpose, SEC was superior to base pre-treatment protocols (34).

Cross-linking and SDS-PAGE Analysis—Freshly isolated LMW peptides were immediately subjected to PICUP, as described (26). Briefly, 1 μ l of 1 mM Ru(Bpy) and 1 μ l of 20 mM ammonium persulfate in 10 mM sodium phosphate, pH 7.4, were added to 18 μ l of freshly isolated LMW peptide. The mixture was irradiated for 1 s with visible light, and the reaction was quenched immediately with 10 μ l tricine sample buffer (Invitrogen) containing 5% β -mercaptoethanol. Concentration differences caused some variability in the relative abundance of each oligomer but did not alter the overall profile of the oligomer size distribution of each peptide (26). To examine further the question of whether inter-peptide oligomerization differences reflected fundamental differences in peptide assembly and not simply the effects of differing peptide concentrations, a series of cross-linking experiments were performed using A β 40 and A β 42 at varying concentrations. At all concentrations, from 1–300 μ M, the oligomer size distributions of A β 40 were distinct from those of A β 42 (data not shown). This confirmed that the assembly differences we observed in the experiments reported here, and in prior work (26, 27), reflected fundamental differences between peptides and not concentration effects.

In principle, radical formation may occur at any site along the polypeptide chain. However, the radical would form at, and further react preferentially with, amino acid side-chains that offer stabilization through aromatic or neighboring-group effects (*e.g.* Tyr, Phe, or Met (35)). The primary factors determining how A β is cross-linked are the proximity of a susceptible group to the radical and the lifetime of the radical itself. If the lifetime of the radical is long enough to allow intermolecular cross-linking as opposed to quenching by solvent, then the actual chemical nature of the radical is relatively unimportant. Cross-linked A β samples were analyzed by SDS-PAGE and silver staining, as described previously (26). The nominal total amount of peptide in each lane of the gels was identical. Gels were dried, scanned, and the intensities of the resulting monomer and oligomer bands quantified by densitometry using the program One-Dscan (Scanalytics, Fairfax, VA). The densitometric data for each of the figures is available as supporting online material. The relative amount of each band, as a percentage of all bands, was determined by calculating the quotient of its intensity and the sum of all band intensities and then multiplying by 100.

RESULTS

Effects of Structural Modifications at the C Terminus of A β 42 on Oligomerization—Our previous studies (26, 27) revealed that LMW A β 40 and A β 42 had distinct oligomer size distributions. LMW A β 40 existed as an equilibrium mixture of monomer, dimer, trimer, and tetramer (26). In contrast, LMW A β 42 comprised three groups of oligomers: 1) monomer through trimer, displaying decreasing intensity with increasing oligomer order; 2) a Gaussian-like distribution between tetramer and octamer, with a maximum at pentamer and hexamer; and 3) oligomers of M_r ~30–60 kDa, among which two intensity maxima, at dodecamer and octadecamer, were observed (27). These higher order oligomers appeared to form through self-association of pentamer/hexamer units (paranuclei). The data demonstrated that Ile⁴¹ was essential for formation of paranuclei,

whereas Ala⁴² was required for rapid self-association of paranuclei into larger oligomers (27). To better understand how amino acid side-chain structure in the C-terminal dipeptide of A β 42 controls oligomerization, seven peptide alloforms (C terminus, Table I) were prepared and their oligomer size distributions were determined by PICUP/SDS-PAGE (Fig. 1).

Substitution of Ile⁴¹ by Gly, eliminating both the side-chain and the stereocenter of the C α group, yielded a distribution that was qualitatively similar to that of A β 40 (26) (*cf.* Fig. 1, lane 2 and Fig. 2A, lane 1). Monomer through trimer formed in similar amounts, and a rapid decrease in oligomer abundance was observed above trimer, whereas A β 40 showed abundant tetramer, above which abundances decreased. [Gly⁴¹]A β 42 thus existed in a dynamic equilibrium involving monomer, dimer, and trimer, but not tetramer (the mathematical foundation for this analysis has been published (26)). When Ile⁴¹ was substituted by Ala, dimer and trimer were the predominant cross-linking products, a characteristic of A β 40 distributions. However, in contrast to wild type (WT) A β 40 distributions, tetramer abundance was low, whereas pentamer abundance was relatively high. This polytonic distribution is characteristic of A β 42. The oligomer size distribution of [Ala⁴¹]A β 42 thus appeared to be a composite of A β 40- and A β 42-like distributions. The majority of the peptide existed as an equilibrium mixture of monomer, dimer, and trimer, as in the case of [Gly⁴¹]A β 42, but the methyl side-chain of Ala⁴¹ facilitated limited paranucleus formation. Substitution of Ile⁴¹ by Val or Leu led to formation of abundant paranuclei. However, in the oligomer size distribution of [Val⁴¹]A β 42, no oligomers at ~30–60 kDa were detected. In the distribution of [Leu⁴¹]A β 42 the amount of these high molecular weight oligomers was substantially reduced relative to that of WT A β 42. Consistent with the low amounts of high molecular weight oligomers, a higher abundance of dimer and trimer were observed for [Val⁴¹]A β 42 and [Leu⁴¹]A β 42 (~18% dimer and ~14% trimer for each) relative to WT A β 42 (~14% dimer and ~10% trimer), demonstrating that these oligomers were in equilibrium with tetramer through octamer. Thus, the side-chain in position 41 is involved in the formation and self-association of A β 42 paranuclei. Examination of the distributions produced by Gly⁴¹-, Ala⁴¹-, and Val⁴¹-substituted A β 42 reveals a correlation between paranucleus formation and side-chain size. [Val⁴¹]A β 42 and [Leu⁴¹]A β 42, containing iso-propyl and iso-butyl side-chains, respectively, do not facilitate self-association of paranuclei, whereas the *sec*-butyl side-chain of Ile does.

Substitution of Ala⁴² by Gly or Val had little effect on formation of paranuclei (Fig. 1). However, no high molecular mass oligomers (~30–60 kDa) were observed for [Gly⁴²]A β 42, demonstrating a role for the methyl side-chain of Ala⁴² in the self-association of paranuclei. When the C-terminal carboxyl group was replaced by a carboxamide, a large increase in the abundance of high molecular weight oligomers was seen (Fig. 1). Thus, hydrophobic interactions involving the side-chains in residues 41 and 42 appear to be a driving force in the association of A β 42 paranuclei into higher oligomers, whereas the C-terminal carboxylate anion moderates this assembly effect.

Effects of Structural Modifications in the Central Region of A β on Oligomerization—Five naturally occurring, autosomal dominant mutations in the A β PP gene region encoding the CHC of amino acids in A β have been reported (25, 36–40). All result in cerebrovascular or parenchymal amyloid deposition, leading to AD-like diseases often characterized by recurrent cerebral hemorrhage. The mutations are thought to cause disease through alteration of A β PP processing or A β aggregation kinetics (due to the resulting amino acid substitutions) (41, 42).

TABLE I
 A β peptides: primary structure and isoelectric point (pI)^a

Group	Position	Modification ^b	A β 40 ^c	A β 42 ^c	pI ^d
N terminus	1-2	Δ	+	+	5.77
	1-2	Δ , <Glu ³	+	-	6.03
	1-4	Δ	+	+	6.30
	1-9	Δ	+	+	6.03
	1-10	Δ	+	+	6.03
	1-10	Δ , <Glu ¹¹	+	-	6.03
Central region	Phe ¹⁹	Pro	+	+	5.22
	Ala ²¹	Gly (Flemish)	+	+	5.22
	Glu ²²	Gly (Arctic)	+	+	5.76
	Glu ²²	Gln (Dutch)	+	+	5.76
	Glu ²²	Lys (Italian)	+	+	6.31
	Asp ²³	Asn (Iowa)	+	+	5.77
C terminus	Ile ⁴¹	Gly	-	+	5.22
	Ile ⁴¹	Ala	-	+	5.22
	Ile ⁴¹	Val	-	+	5.22
	Ile ⁴¹	Leu	-	+	5.22
	Ala ⁴²	Gly	-	+	5.22
	Ala ⁴²	Val	-	+	5.22
Asp/His	-COOH	-CONH ₂	-	+	5.77
	Asp ¹	Asn	+	-	5.77
	Asp ⁷	Asn	+	-	5.77
	Asp ²³	Asn	+	-	5.77
	His ⁶	Gln	+	-	4.74
	His ¹³	Gln	+	-	4.74
	His ¹⁴	Gln	+	-	4.74
	Asp ^{1, 7, 23}	Asn	+	-	7.26
	His ^{6, 13, 14}	Gln	+	-	4.08
	Asp ^{1, 7, 23} His ^{6, 13, 14}	Asn ^{1, 7, 23} Gln ^{6, 13, 14}	+	-	6.36

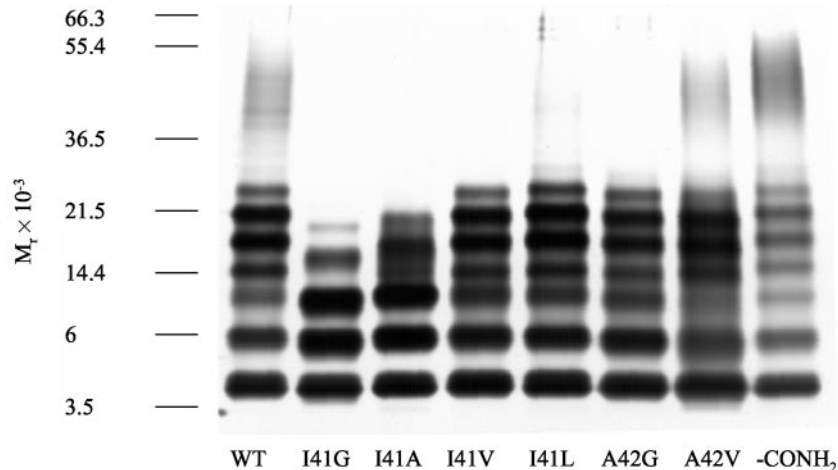
^a The WT A β 42 sequence is: (H)-DAEFRHDSGYEVHHQKLVFFAEDVSGNKGAIIGLMVGGVVIA-(OH).

^b The symbols Δ and < signify amino acid deletion and pyroglutamyl N terminus, respectively. The common name for each mutation is listed parenthetically.

^c The columns indicate on which backbone the primary structure changes were made. The symbols + and - signify whether the specific peptide was or was not studied.

^d Isoelectric point (pI) values were estimated using MacVector, v. 7.1 (Accelrys, San Diego, CA).

FIG. 1. Effects of C terminus structure on paranucleus formation and assembly. PICUP was applied to LMW A β 42 analogues containing the modifications Ile⁴¹ \rightarrow Gly, Ile⁴¹ \rightarrow Ala, Ile⁴¹ \rightarrow Val, Ile⁴¹ \rightarrow Leu, Ala⁴² \rightarrow Gly, Ala⁴² \rightarrow Val, or A β 42-carboxamide. WT A β 42 was used as control. Molecular weight markers are shown on the left. The gel is representative of each of 3 independent experiments. The densitometric analysis of these experiments is available as supporting online material.



The substitutions include Ala²¹ \rightarrow Gly, associated with a familial form of cerebral amyloid angiopathy in a Flemish kindred (36); Glu²² \rightarrow Gln, which causes hereditary cerebral hemorrhage with Amyloidosis-Dutch type (37); Glu²² \rightarrow Lys, which is associated with cerebral amyloid angiopathy and hemorrhagic stroke in an Italian kindred (38); Glu²² \rightarrow Gly, the "Arctic" mutation, which was linked to early onset AD in a Swedish kindred (25, 39); and Asp²³ \rightarrow Asn, recently discovered in an Iowa family and found to cause severe, early onset cerebral amyloid angiopathy (33, 40). A β congeners bearing these mutations display distinct aggregation kinetics. The rate of fibril formation by the Flemish and Italian mutants is decreased relative to WT A β (43, 44), whereas the Dutch and Iowa mutant peptides form fibrils substantially faster (33, 44). The Arctic peptide does not show an overall change in the rate of fibrillogenesis relative to WT A β 40, but rather accelerated protofibril

formation (25). In addition to these five naturally occurring A β alloforms, a Phe¹⁹ \rightarrow Pro substitution has been shown to prevent fibril formation *in vitro* (45, 46). Each of these A β alloforms was synthesized on both A β 40 and A β 42 backbones to determine how A β central region structure controls oligomerization (Table I, Central Region).

LMW A β preparations from each peptide were isolated, cross-linked, and analyzed by SDS-PAGE. A β 40 alloforms displayed distributions characterized by intense monomer through tetramer bands, followed by a sharp exponential decrease in the levels of higher order oligomers (Fig. 2A). The two substitutions lying within the CHC, Phe¹⁹ \rightarrow Pro and Ala²¹ \rightarrow Gly, had little effect on the oligomer distribution. This result was particularly unexpected for the non-fibrillogenic Phe¹⁹ \rightarrow Pro alloform. We have shown that monomeric peptides, when subjected to PICUP, yield oligomer distributions

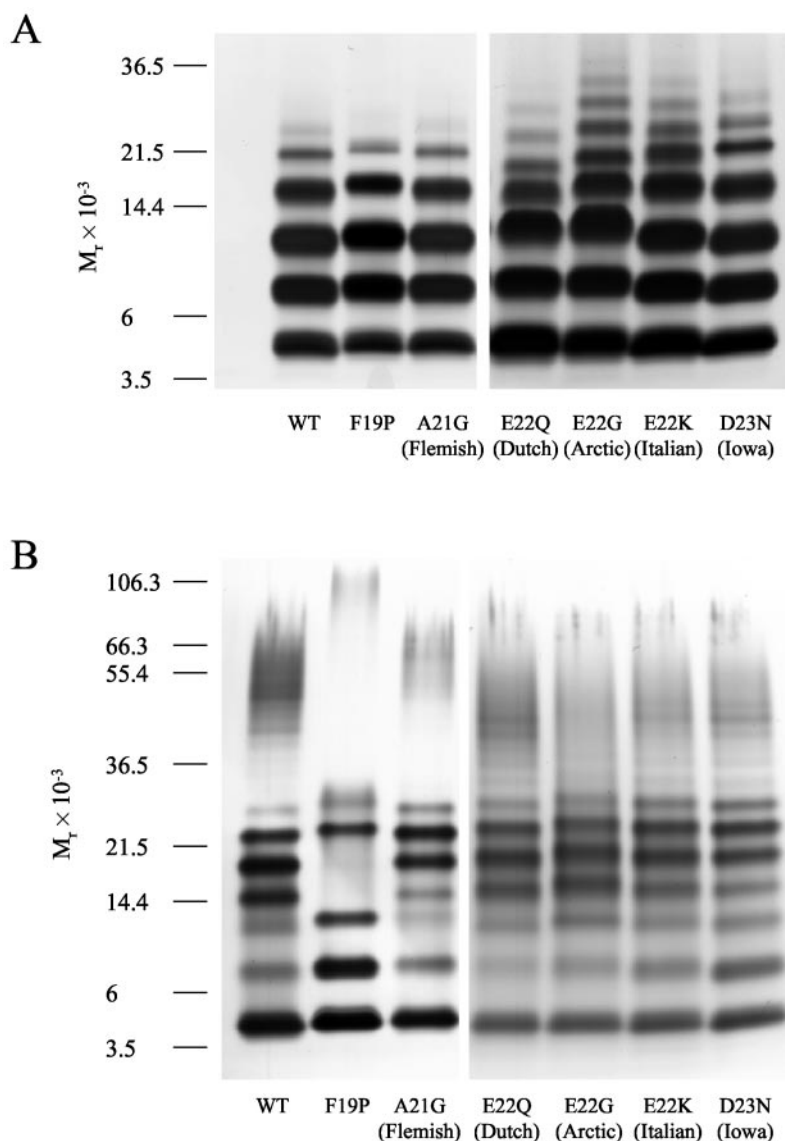


FIG. 2. Effects of central region structure on early A β assembly. LMW A β 40 (A) and A β 42 (B), each containing the substitutions Phe¹⁹ \rightarrow Pro, Ala²¹ \rightarrow Gly, Glu²² \rightarrow Gln, Glu²² \rightarrow Gly, Glu²² \rightarrow Lys, or Asp²³ \rightarrow Asn, were cross-linked, analyzed by SDS-PAGE, and visualized by silver staining. WT A β 40 and A β 42 were used as controls. Molecular weight markers are shown on the *left* of each *panel*. The gels are representative of each of 6 independent experiments. The densitometric analysis of these experiments is available as supporting online material.

characterized by a shallow exponential decrease in oligomer abundance starting at monomer or dimer and extending up to dodecamer or tridecamer (26). The distribution obtained for the [Pro¹⁹]A β 40 indicates that although this analogue does not form fibrils, it associates into small oligomers in a manner similar to WT A β 40. Despite the similarity in the oligomer size distribution between the WT and the Pro¹⁹-substituted analogue, the gel mobilities of [Pro¹⁹]A β 40 oligomers were lower than those of WT A β 40 oligomers. This result is consistent with disruption of a putative compact structure of the CHC of WT A β 40 by the Phe¹⁹ \rightarrow Pro substitution. All the peptides with substitutions at positions 22 or 23, particularly [Gly²²]A β 40, produced distributions extending to relatively high oligomer order. The distributions reflect the increased propensity of these analogues to form higher order oligomers and suggest an important role for Glu²² and Asp²³ in controlling A β 40 oligomerization. The gel mobilities of tetrameric and higher order oligomers of A β analogues containing substitutions at Glu²² were larger than those of the counterpart WT A β 40 oligomers, suggesting that the substitutions facilitated formation of a compact structure in the resulting oligomers.

The A β 42 (Fig. 2B) alloforms generally displayed polytonic distributions characterized by decreasing band intensities from monomer to trimer, a Gaussian-like distribution of intensities centered at pentamer/hexamer, and finally, a group of higher

molecular weight oligomers. The distribution obtained for Ala²¹ \rightarrow Gly was qualitatively similar to that of the WT. However, the distribution of paranuclei was narrower than that of WT A β 42, and densitometric analysis revealed that the relative abundance of paranuclei in the [Gly²¹]A β 42 distribution was 25%, compared with a relative abundance of 16% for WT paranuclei. Higher molecular mass oligomers of the Flemish mutant were centered at \sim 60 kDa, compared with those of WT A β 40, which were centered at \sim 50 kDa. The relative abundances of the high molecular weight oligomers were 25 and 48% for the Flemish and WT A β 42 peptides, respectively. The Flemish mutation thus appears to facilitate paranucleus formation. The higher oligomers formed by self-assembly of the Flemish paranuclei are larger, on average, than those formed by WT A β 42, but their rate of formation is lower. The data are consistent with the lower rate of fibril formation reported for the Ala²¹ \rightarrow Gly mutant relative to WT A β (30, 46, 47). The distribution of [Pro¹⁹]A β 42 comprised primarily monomer, dimer, trimer, hexamer, and a small amount of heptamer. Little or no tetramer and pentamer were observed. Higher order assemblies also formed, but unlike those of WT A β 42 and the other alloforms, which ranged in molecular mass from \sim 40–80 kDa, those of the [Pro¹⁹]A β 42 peptide had molecular masses of \sim 120–150 kDa (Fig. 2B). Substitutions at positions 22 or 23 produced distributions of monomer through octamer, which

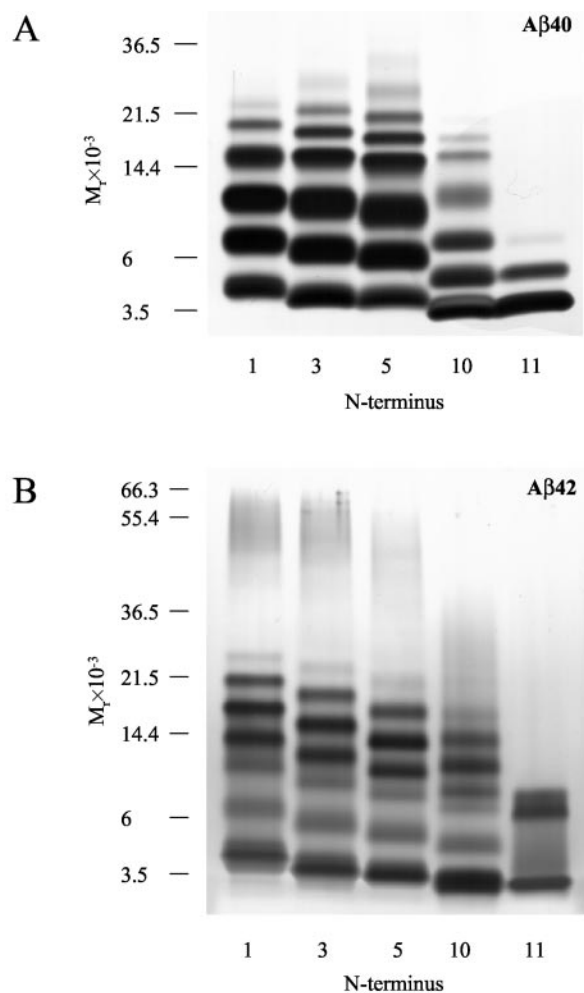


FIG. 3. Effects of N terminus structure on early A β assembly. LMW A β (X-40) (A) and A β (X-42) (B), in which X = 1, 3, 5, 10, or 11, were cross-linked, analyzed by SDS-PAGE, and the resulting bands visualized by silver staining. Molecular weight markers are shown on the left of each panel. The gels are representative of each of 4 independent experiments. The densitometric analysis of these experiments is available as supporting online material.

were similar to that produced by WT A β 42. However, the intense bands of high order oligomers observed at $M_r \approx 45$ –70 kDa with the WT peptide were substantially less intense in the Glu²²- and Asp²³-substituted alloforms.

Effect of N-terminal Truncations on A β Oligomerization—N-terminally truncated (NTT) A β is found in significant amounts in amyloid plaques (48, 49). In addition, cell culture studies have shown that NTT-A β peptides are secreted as a part of normal cellular metabolism (50, 51). Thus, NTT-A β has been hypothesized to have a role in the etiology of AD (49, 50, 52–54). The most abundant NTT-A β analogues found in AD plaques are those beginning at Glu³, Arg⁵, or Glu¹¹ (41, 53). Therefore, the oligomer size distributions of analogues starting at these positions and ending either at Val⁴⁰ or Ala⁴² were analyzed (N terminus, Table I). In A β (11–40) and A β (11–42), Tyr, one of the most reactive residues in radical cross-linking reactions (35), is missing. Therefore, to evaluate the relative contribution of Tyr¹⁰ to the observed profile of PICUP-generated A β oligomers, A β (10–40) and A β (10–42) also were included.

Deletion of Asp¹ and Ala² of A β 40 (Fig. 3A) led to extension of the observed oligomer distribution up to an octamer. Deletion of two additional residues, Glu³ and Phe⁴, extended the size distribution to nonamer. An irregular distribution was

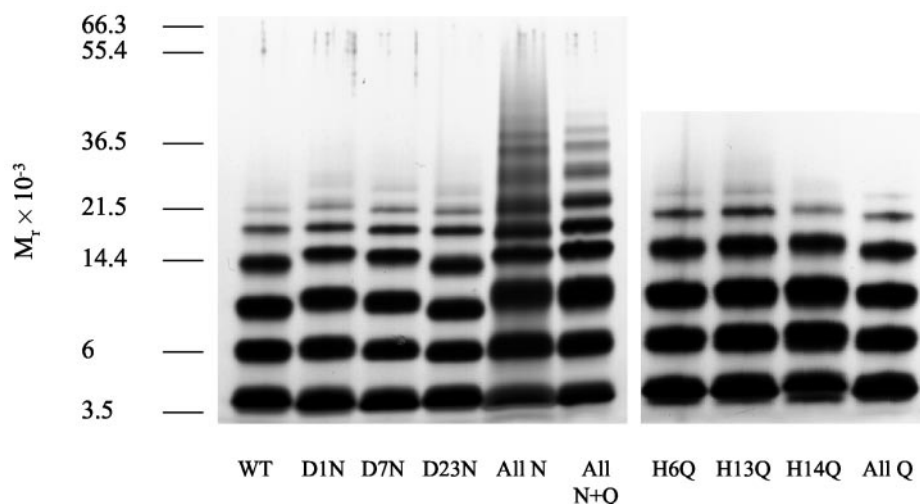
obtained for A β (10–40). The distribution extended to heptamer and contained a two-color monomer band and a broad, nebulous tetramer band. The cross-linking yield ($(A\beta_{total} - A\beta_{monomer})/(A\beta_{total})$) was lower in the absence of Tyr¹⁰. Approximately 40% of A β (11–40) were cross-linked, compared with ~70% for A β (10–40), and the highest order oligomer observed was a trimer. In contrast to the effects of N-terminal deletions on the distribution of A β (X–40) peptides, the same deletions had little effect on the oligomer size distributions of A β (X–42) analogues, with the exception of A β (11–42) (Fig. 3B). The A β (X-42) peptides beginning at Glu³, Arg⁵, or Tyr¹⁰ showed oligomer distributions essentially identical to that of A β 42, albeit of lower M_r . Somewhat reduced amounts of high molecular weight oligomers were observed for A β (3–42) and A β (5–42). These results demonstrate the primacy of the C terminus of A β 42 in oligomerization. As observed for A β 40, deletion of Tyr¹⁰ decreased the cross-linking yield, and in addition produced a pronounced, nebulous, trimer band, which has been shown previously to be an SDS-induced artifact (27).

A significant portion of NTT-A β peptides found within amyloid deposits *in vivo* begins with pyroglutamylated (<Glu) Glu³ or Glu¹¹ (55, 56). Therefore, the effects of these post-translational modifications were examined. An A β 40 backbone was used because it was found to be sensitive to N-terminal structure modifications (Fig. 3A). The oligomer size distributions of cross-linked oligomers produced by [$<$ Glu³]A β (3–40) or [$<$ Glu¹¹]A β (11–40) were identical to those of their uncyclized homologues (data not shown).

Effects of Charge-altering Substitutions on A β Oligomerization—The results obtained for A β analogues containing substitutions of Glu²² or Asp²³ (Fig. 2A), or N-terminal deletions (Fig. 3A), revealed that Coulombic interactions may be particularly important in A β 40 oligomerization. Recently, based on solid state NMR data (57), a salt bridge between Asp²³ and Lys²⁸ has been implicated in stabilizing a turn structure in A β 40 fibrils. This salt bridge, and other ionic interactions, may occur during the initial oligomerization of A β , before extensive β -sheet structures form. Thus, amino acid substitutions altering charged residues may provide information about specific Coulombic interactions involved in early A β assembly. Charge alterations also change the isoelectric point (pI) of the protein. Substitutions raising the A β pI, estimated to be 5.2–5.5 (58–60), could facilitate peptide aggregation at physiologic pH. In addition, substitutions of Asp or His residues previously have been shown to retard the formation of an α -helix-rich, oligomeric assembly intermediate, possibly by stabilizing structures that precede the α -helix-rich intermediate (61).

To examine systematically the effects of charge neutralization on A β oligomerization, Asp \rightarrow Asn and His \rightarrow Gln substitutions were incorporated into A β 40. The substitutions included Asp¹ \rightarrow Asn, Asp⁷ \rightarrow Asn, Asp²³ \rightarrow Asn, His⁶ \rightarrow Gln, His¹³ \rightarrow Gln, or His¹⁴ \rightarrow Gln (Asp/His, Table I). The strategy for selecting the substitutions was based upon theoretical considerations of protein evolution and chemical biology, as well as results of published experimental work (61). Each peptide was cross-linked, and the products were analyzed by SDS-PAGE. In addition, analogues containing substitution of all three Asp residues, all three His residues, or all Asp and all His residues, were also examined. Substitution of all three Asp residues promoted formation of oligomers extending at least to undecamer (Fig. 4). Analogues containing single Asp \rightarrow Asn substitutions displayed oligomer size distributions similar to that of WT A β 40. The formation of higher order oligomers by the triply substituted analogue may be related to the change in its pI, to ~7.3, a value almost identical to the pH of the medium, 7.4. A similar distribution was obtained for the analogue in

FIG. 4. Effects of charged residue substitutions on early A β assembly. PICUP was applied to LMW A β 40 analogues containing the substitutions [Asn¹], [Asn⁷], [Asn²³], [Asn^{1,7,23}], [Gln⁶], [Gln¹³], [Gln¹⁴], [Gln^{6,13,14}], or [Asn^{1,7,13}, Gln^{6,13,14}]. WT A β 40 was used as control. Molecular weight markers are shown on the left. The gel is representative of each of 3 independent experiments. The densitometric analysis of these experiments is available as supporting online material.



which all the Asp residues were substituted by Asn and all His residues were substituted by Gln. It should be noted that His residues are largely uncharged at pH 7.4, thus substitution of the native imidazole side-chain of His by the propylamide side-chain of the Gln residue did not significantly affect A β oligomerization. The gel mobilities of [Asn¹]A β 40 and [Asn⁷]A β 40 oligomers were lower than those of WT A β 40, suggesting that the oligomers formed by these analogs had a more extended structure relative to the WT oligomers. This phenomenon was seen also for [Asn^{1,7,23}]A β 40 and [Asn^{1,7,23}, Gln^{6,13,14}]A β 40, but was not observed for [Asn²³]A β 40 or for the analogues containing His substitutions. Replacement of His residues, singly or *en masse*, by Gln did not have a significant effect on the oligomer size distribution.

DISCUSSION

We have shown recently that the A β 42 C-terminal dipeptide, Ile⁴¹-Ala⁴², mediates formation and self-association of paranuclei (27). Ile⁴¹ was essential for paranucleus formation, whereas Ala⁴² was required for paranucleus self-association. Here, a detailed investigation of the role of amino acid side-chain structure in the C-terminal dipeptide of A β 42 revealed how the configuration of the side-chain in position 41 controls paranucleus formation and self-assembly. An unsubstituted residue 41 C α , even in the presence of Ala⁴², oligomerized similarly to WT A β 40. Methylation of the C α , as in the Ala⁴¹-Ala⁴² analogue, resulted in an oligomer distribution with features of both A β 40 and A β 42 oligomerization, but no high order oligomers. More typical A β 42-like oligomerization occurred only if the C α was alkylated with propyl or butyl groups. The *sec*-butyl side-chain of Ile⁴¹ was particularly important for self-assembly of paranuclei into larger oligomers. The iso-propyl or iso-butyl side-chains of Val⁴¹ and Leu⁴¹, respectively, did not have this activity. This result suggests that it is not merely the hydrophobicity of the side-chain in position 41 that controls paranucleus association, because the iso-butyl side-chain of Leu is more hydrophobic than the *sec*-butyl side-chain of Ile (62). The requirement for the Ile residue indicates that the conformation and configuration of the side-chain in position 41 are critical for paranucleus self-association.

The C-terminal carboxylate group moderates paranucleus self-association. Thus, replacement of the charged carboxylate group by a polar, yet uncharged, carboxamide function substantially accelerated paranucleus association. Although Ala⁴² is required for paranucleus association (27), the size of the side-chain in position 42 was not critically important, suggesting that it is the length of the peptide (42 *versus* 41 amino

acids) rather than the nature of residue 42, which controls paranucleus self-association. The data emphasize the key role of the C-terminal dipeptide, Ile⁴¹-Ala⁴², in determining the number and abundance of early A β oligomers. Essentially all of the A β 42 analogues yielded polytonic distributions, with pentamers or hexamers being the predominant products, whereas the oligomer size distributions of A β 40 analogues were monotonic and consistent with a rapid equilibrium among smaller oligomers (monomer through trimer or tetramer). The distinct oligomerization products produced by A β 40 and A β 42 may be responsible for the distinct neurotoxic activities reported recently in comparative studies of A β 40 and A β 42 (16). The data obtained here suggest that drugs targeting the C-terminal dipeptide of A β 42, particularly Ile⁴¹, could cause the longer A β alloform to oligomerize in the same manner as A β 40, leading to reduced neurotoxicity and decelerated disease progression.

In addition to structural alteration in the C-terminal dipeptide, Phe¹⁹ \rightarrow Pro or Ala²¹ \rightarrow Gly substitutions within the A β CHC also affected A β 42 oligomerization. These data show that the CHC is an important determinant controlling the size distribution of A β 42 paranuclei, possibly through direct, intra- or intermolecular interactions with the C terminus. Substitution of Phe¹⁹ by Pro, a modification known to abolish fibril formation (45), resulted in formation of paranuclei comprising largely hexamers. The absence of tetramers and pentamers suggests that the Pro substitution acts by stabilizing the hexamer form of the peptide. These hexamers can associate to form high molecular mass oligomers (~120–150 kDa), but the conformational rearrangements required for maturation of these high order oligomers into fibrils is inhibited by the substitution.

Relative to WT A β 42, the Flemish variant, [Gly²¹]A β 42, also produced a narrower paranucleus size distribution and decreased amounts of high molecular weight oligomers. Nevertheless, on average, these high molecular weight oligomers were larger than those of WT A β 42. These results are in agreement with the reduced rate of fibrillogenesis observed for [Gly²¹]A β (47) and are consistent with the hypothesis that accumulation of [Gly²¹]A β 42 paranuclei has a role in the etiology of the Flemish form of FAD. In addition, the stabilization of paranuclei increases the relative amount of soluble *versus* insoluble [Gly²¹]A β 42. The increased solubility may facilitate the diffusion or transport of the peptide from the brain parenchyma into cerebral blood vessels, providing an explanation for the angiopathy and hemorrhagic components characteristic of the Flemish disease.

Interestingly, neither the Phe¹⁹ \rightarrow Pro nor Ala²¹ \rightarrow Gly sub-

stitution had a significant effect on A β 40 oligomerization, whereas substitutions at positions 22 and 23, or truncation of N-terminal residues, had clear effects. In contradistinction, these latter structural modifications had little effect on A β 42 oligomerization. Taken together, these data support the hypothesis that the early assembly of A β 40 and A β 42 occurs via distinct mechanisms (27).

The finding that substitutions of Glu²² or Asp²³ facilitated formation of higher order oligomers of A β 40 suggests that early assembly of this peptide involves, at least in part, Coulombic interactions. Recent solid-state NMR studies of A β fibrils provide evidence that salt bridges form between Asp²³ and Lys²⁸ and possibly between Lys¹⁶ and Glu²² (57). Our data suggest that salt bridges involving Asp²³ and Glu²² may already have formed during early oligomerization of A β . Amino acid substitutions that affect these interactions could destabilize the structure of the resulting peptides. For example, molecular dynamics analysis of peptide solvation have shown that a [Gln²²]A β (10–35)NH₂ (Dutch) peptide monomer has a larger solvent-exposed hydrophobic surface area than does the WT analogue (63). Not surprisingly, a more recent comparative *in silico* analysis of peptide/water interactions of A β (10–35)NH₂ and [Gln²²]A β (10–35)NH₂ has shown that the Dutch peptide had weaker interactions with water than did the WT peptide (64). The side-chain of Asp²³ was found to form hydrogen bonds, both with water and with atoms of the peptide, at an especially high frequency. This propensity is consistent with the effects of Asn substitution at this site, which we have observed causes more facile formation of higher order oligomers, both for the singly substituted Asp²³ A β 40 analogue and for the triply substituted A β 40 analogue. This effect could be due to the elimination of salt bridges involving the Asp²³ residue and because of the decrease in solvation free energy associated with substitution of the Asp²³ acetate side-chain with an acetamide function. The propylamide substitution found in the Dutch peptide would have a similar effect on solvation free energy and thus would be expected to cause increased oligomerization, which is what we observed here experimentally. The mechanisms of action of the Arctic (Glu²² \rightarrow Gly) and Italian (Glu²² \rightarrow Lys) A β 40 substitutions also are likely to involve destabilization of the CHC region and disruption of attractive Coulombic interactions. Our observation here that the Arctic peptide forms relatively large oligomers, and the report by Nilsberth *et al.* (25) that the Arctic substitution causes enhanced protofibril formation, are consistent with the mechanism proposed.

In addition to causing specific structural effects, amino acid substitutions can alter general properties of A β , especially its pI. For example, the pI of the triply substituted A β analogue [Asn^{1,7,23}]A β 40 is 7.3 compared with \sim 5.2–5.5 for WT A β . Thus, in experiments performed at pH 7.4, this peptide was more prone to aggregation than the analogues containing individual Asp \rightarrow Asn substitutions, each of which had a pI \approx 5.8. However, the [Asn^{1,7,23}, Gln^{6,13,14}]A β 40 peptide (pI \approx 6.3) behaved similarly to [Asn^{1,7,23}]A β 40, suggesting that in addition to the change in the pI, other effects contributed to the enhanced formation of higher order oligomers. For example, the conformation of [Asn^{1,7,23}]A β 40 oligomers appeared to be extended relative to WT A β 40 because their gel mobilities were lower than those of the oligomers formed by the WT peptide. This is in contrast to the gel mobilities of oligomers of analogues containing substitutions of Gly²², which are larger than those of WT A β 40. Thus, perturbation of oligomerization by amino acid substitutions may result, in part, from effects on the conformation of the specific alloform, producing a more extended or a more compact conformation. Histidine residues

have been shown to be important in metal chelation by A β , a process that can affect the rate of fibrillogenesis (65–67). Here, substitution of His residues individually or *en masse* did not affect A β oligomerization, suggesting that the effects of His-mediated metal chelation come into play at later assembly stages.

In summary, our results reveal how specific regions and residues control A β oligomerization and show that these controlling elements differ between A β 40 and A β 42. The results show how each functional group in the C-terminal Ile⁴¹-Ala⁴² dipeptide acts in determining the pathway through which A β oligomerizes. Additional evidence is provided for the existence of distinct oligomerization pathways controlling early A β assembly. Oligomerization of A β 40 involves a rapid equilibrium among monomer, dimer, trimer, and tetramer, and is controlled to a significant degree by the peptide N terminus and charged residues at positions 22 and 23. In paranucleus formation by A β 42, the predominant controlling structural elements are the C-terminal dipeptide and the CHC. Paranucleus formation depends upon the presence of a hydrophobic side-chain in amino acid 41 with a size at least as large as that of a methyl group. Paranucleus self-association is facilitated by the *sec*-butyl side-chain of Ile⁴¹ but not by the iso-propyl or iso-butyl side-chains of Val or Leu, respectively. The self-association step requires the presence of residue 42, and is substantially facilitated by substitution of the C-terminal carboxylate group by a carboxamide. Distinct drug design approaches for A β 40 and A β 42 oligomers thus may be required for efficacious treatment of specific types of FAD. The data suggest that the regions 19–21 and 41–42 are rational targets for development of assembly inhibitors for A β 42 and that regions 1–10 and 22–23 are targets for development of A β 40 assembly inhibitors.

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