The core Alzheimer's peptide NAC forms amyloid fibrils which seed and are seeded by β-amyloid: is NAC a common trigger or target in neurodegenerative disease?

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Background: NAC is a 35-amino-acid peptide which has been isolated from the insoluble core of Alzheimer's disease (AD) amyloid plaque. It is a fragment of α-synuclein (or NACP), a neuronal protein of unknown function. We noted a striking sequence similarity between NAC, the carboxyl terminus of the β-amyloid protein, and a region of the scrapie prion protein (PrP) which has been implicated in amyloid formation.

Results: NAC was prepared by chemical synthesis and was found to form amyloid fibrils via a nucleation-dependent polymerization mechanism. NAC amyloid fibrils effectively seed β1–40 amyloid formation. Amyloid fibrils comprising peptide models of the homologous β and PrP sequences were also found to seed amyloid formation by NAC.

Conclusions: The *in vitro* model studies presented here suggest that seeding of NAC amyloid formation by the β-amyloid protein, or seeding of amyloid fibrils of the β-amyloid protein by NAC, may occur *in vivo*. Accumulation of ordered NAC aggregates in the synapse may be responsible for the neurodegeneration observed in AD and the prion disorders. Alternatively, neurodegeneration may be caused by the loss of α-synuclein (NACP) function.

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Introduction

Amyloid plaque is characteristic of Alzheimer's disease (AD) [1–3] . The major protein components of the insoluble core of AD amyloid plaque are short peptide fragments (39–43 amino acids in length) of the β protein (also called β-amyloid, β/A4, βAP, or Aβ) (Fig. 1) [4–8]. Recently, the peptide NAC (non-Aβ component of AD amyloid) was identified as an intrinsic component of the amyloid plaque core based on its copurification with the β protein and immunological localization on amyloid fibrils by electron microscopy (EM) [9]. NAC is a peptide of at least 35 amino acids (Fig 2). It is a fragment of a 140-amino-acid precursor, a protein known as NACP or α-synuclein, which is localized to the presynaptic nerve terminal [9–13] (Fig. 1). Interestingly, NACP is found primarily in the regions of the brain that degenerate in AD [13].

The prion diseases (for example, scrapie, kuru and Creutzfeldt–Jakob disease) are also characterized by abnormal protein deposition in brain tissue, in this case, of a species known as the prion protein (PrP) [14]. We have noted a sequence similarity between PrP and the β protein which may direct amyloidogenesis [15, 16]. Both the β protein [17] and peptides based on the PrP sequence [18] exhibit neurotoxicity in cell culture. Overexpression of the β protein in transgenic mice induces amyloid plaque formation [19] and neurodegeneration [20]. Aggregation of these proteins is a requirement for neurotoxicity *in vitro* [21]. Since amyloid deposits may be causative in neurodegenerative diseases [1,22], it is critical to understand the mechanism by which amyloid deposition occurs, and the role of endogenous factors such as NAC.

The formation of β and PrP amyloid fibrils, like a crystallization, appears to occur by a nucleation-dependent mechanism (Fig. 3) [16,23,24]. Nucleus formation involves a series of thermodynamically-unfavorable protein association equilibria ($K_n \ll 1$). Once a nucleus has formed, further addition of monomer is thermodynamically favorable ($K_g \gg 1$). Consequently, at a protein concentration slightly exceeding the thermodynamic solubility (critical concentration), amyloid formation exhibits a lag time before rapid fibril growth. Addition of preformed fibrils (or 'seeds') can decrease the length of the lag time by eliminating the need for a slow nucleation step. Seeds can be homogeneous (derived from an identical molecule of peptide or protein) or heterogeneous (derived from a different molecular species). Alternatively, endogenous factors could inhibit nucleus formation and/or fibril growth [25].

Here we show that a synthetic peptide based on the minimal 35-amino-acid NAC sequence can form amyloid fibrils via a nucleation-dependent kinetic mechanism. We examined the effect of NAC on the solubility and the aggregation kinetics of amyloidogenic β- and PrP-derived

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                      1                           26                  43
β1-43                 DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLM  VGGVVIAT
                                                    61                                 95
α-synuclein/NACP      GKTKEGVLYVGSKTKEGVVHGVATVAEKTKEQVTN  VGGAVVTG  VTAVAQKTVEGAGSIAAATGFVKKDQ
  (31-99)
PrP (82-150)A117V     GQPHGGGWGQGGGTHSQWNKPSKPKTNMKHMAGAA  VAGAVVGG  LGGYMLGSAMSRPIIHFGSDYEDRYY
                                            106            117       126
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Fig. 1. A comparison of sequences from β1-43, α-synuclein, and PrP. Sequence identities are highlighted. The box indicates the eight-residue hydrophobic region with local sequence homology. The A117V mutation (asterisk) segregates with the human prion disease Gerstmann–Straussler–Scheinker syndrome (GSS) [31,32].

peptides (Fig. 2), and *vice versa*. It has been proposed that NAC could promote the formation of β protein amyloid *in vivo* [9, 26]. Our *in vitro* results indicate that NAC amyloid effectively seeds β-protein fibril formation, and, in addition, that NAC amyloid formation can be seeded by either β or PrP peptide seeds. These results may shed light on the role of NAC in the pathogenesis of AD and related neurodegenerative disorders.

Results

NAC peptide aggregates from supersaturated solution to form amyloid fibrils

The precise length of NAC associated with β protein amyloid fibrils has not been determined [9]. However, a minimal sequence of 35 amino acids was determined by enzymatic digestion of amyloid plaque core and confirmed by cDNA sequencing of NACP. We therefore synthesized a 35-amino-acid NAC peptide (Fig. 2). A supersaturated solution of this NAC peptide was stirred and aggregation was monitored by turbidity measurements [15,23]. The precipitate was isolated by centrifugation and visualized by electron microscopy (EM) (Fig. 4a), showing that it formed ordered amyloid fibrils. The peptide film stained with Congo Red dye, like true amyloid fibrils, and, under a polarizing light microscope, exhibited the birefringence characteristic of amyloid [27,28]. The Fourier transform infrared (FTIR) spectrum of fibrillar NAC was dominated by two low-frequency absorption amide I bands (1633 and 1627 cm^{-1}), indicative of antiparallel β-sheet structure (Fig. 4b) [28].

The solubility of β and PrP peptides does not significantly decrease in the presence of NAC

We compared the solubility of the β and PrP peptides alone with their solubility in the presence of another amyloidogenic peptide. Fibrils were prepared by stirring supersaturated mixtures, containing one peptide (40 μM) or equimolar in two peptides (40 μM each). After aggregation, the concentration of each peptide in the soluble phase was compared using quantitative amino acid analysis. As well as the β and PrP peptides themselves, we also tested several shortened forms of the the β peptide and a mutant form of the PrP peptide (Ala117→Val, A117V). The solubility of isolated peptides, in μM was: β1–40, 0.9±0.8; β26–40, 6.2±1.2; β26–42, 3.4±0.3; β26–43, 3.1±0.2; PrP106–126, 15.9±2.5; PrP106–126A117V, 8.7±1.0. The individual measured solubilities in mixtures of β and PrP-derived peptides were indistinguishable from the solubilities of the isolated peptides (small effects are not measurable by this method, however, and these values may not represent the true thermodynamic solubility [29,30]). In contrast, mixtures of NAC with β or PrP peptides produced anomalous effects; although the solubility of NAC was not significantly changed by the presence of other peptides (10.9±0.8 μM), some of the β and PrP peptides had increased solubility in the presence of NAC (solubility in the presence of 40 μM NAC, in μM: β1–40, 0.5±0.1; β26–40, 11.8±1.2; β26–42, 7.2±0.3; β26–43, 6.5±0.5; PrP106–126, 24.1±3.0; PrP106–126A117V, 16.4±2.5).

Amyloid formation by NAC is nucleation-dependent and can be seeded by NAC fibrils

Supersaturated solutions of NAC were metastable. Amyloid formation by NAC at 80 μM did not proceed immediately but exhibited a lag time (666 ± 88 min) characteristic of nucleation-dependent polymerization (Fig. 5a). The lag time preceding NAC fibril formation could be eliminated by addition of NAC seed fibrils (20 μM) (Fig 5a).

β1–40 Amyloid fibril formation can be seeded by NAC fibrils

The rates of amyloid formation from a supersaturated solution of β1–40 (80 μM) in the presence and the absence of NAC seed fibrils (20 μM) were compared by monitoring the increase in turbidity (Fig. 5b); the turbidity due to the presence of the seed peptide was subtracted from all data points. In the presence of NAC, the length of the nucleation phase preceding amyloid formation

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                 1                         26                 43
1-43         H2N-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIAT-CO2H
26-43                                  AcHN-SNKGAIIGLMVGGVVIAT-CO2H
26-42                                  AcHN-SNKGAIIGLMVGGVVIA-CO2H
26-40                                  AcHN-SNKGAIIGLMVGGVV-CO2H

                61                                 95
NAC          H2N-EQVTNVGGAVVTGVTAVAQKTVEGAGSIAAATGFV-CO2H
                       106        117      126
PrP106-126         AcHN-KTNMKHMAGAAAAGAVVGGLG-CONH2
PrP106-126A117V    AcHN-KTNMKHMAGAAVAGAVVGGLG-CONH2
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Fig. 2. The sequences of NAC, PrP, the β protein and corresponding truncated model peptides. NAC comprises residues 61–95 of human α-synuclein/NACP. The sequence difference between PrP106–126 and PrP106–126A117V is marked with an asterisk.

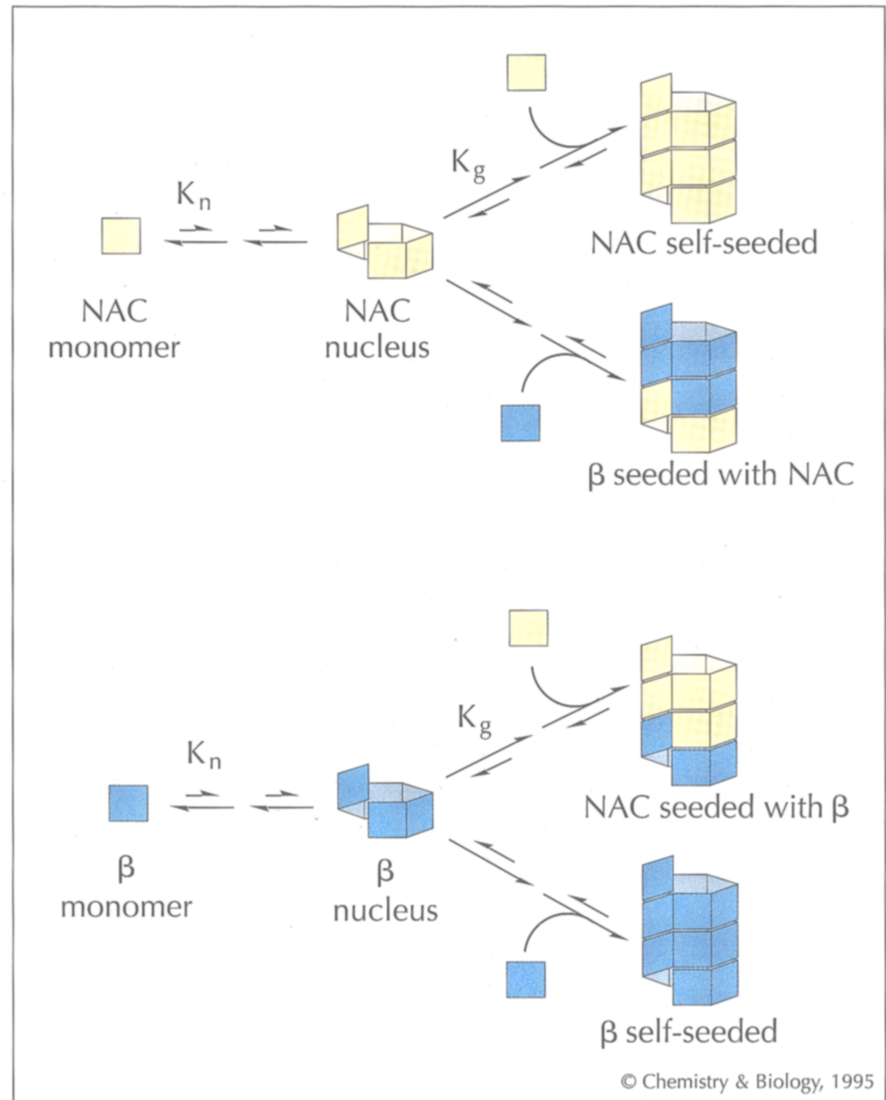

Fig. 3. A general mechanism for nucleation-dependent amyloid fibril formation, including a working hypothesis for NAC. Nucleation (association equilibria $K_n \ll 1$) and growth steps (association equilibria $K_g \gg 1$) are indicated. A similar mechanism is applicable for PrP. Heterogeneous seeding which is demonstrated herein to be effective, albeit less effective than homogeneous seeding, may be critical *in vivo*.

decreased by more than 50 % (Table 1, g). A similar effect was observed for model peptide β26–40 seeded with NAC (Table 1, h). In contrast, PrP106–126 and PrP106–126A117V were not seeded by NAC (data not shown). The prion peptides showed no significant ability to seed β26–40 (Table 1, i,j) or β1–40 (data not shown).

NAC fibril formation can be seeded by β and PrP peptide fibrils

The β protein analogs shown in Table 1 seeded NAC amyloid formation, albeit less efficiently than did NAC itself (Fig. 5a). Short β (β1–40 and the model peptide β26–40), which does not contain the entire homologous sequence, was a less effective seed than models of long β (β26–42, β26–43) (Table 1, a–d). Addition of fibrils comprising PrP106–126 and PrP106–126A117V (a mutation which segregates with Gerstmann–Straussler-Scheinker syndrome [31,32]) also led to a decrease in the observed nucleation time (Fig. 5a; Table 1, e–f). No significant difference between the abilities of the Ala- and Val- containing variants to seed NAC was observed. Neither NAC nor β was an effective seed for either PrP analog (data not shown).

Discussion

Amyloid plaque is the pathological hallmark of AD [1,2]. The recent discovery of NAC as an intrinsic component of the insoluble amyloid plaque core suggests that this peptide may be important in AD pathogenesis. While a number of components (for example, apolipoprotein E [33,34], α_1-antichymotrypsin [35] and heparan sulfate proteoglycan [36], among others) are loosely associated with plaques, NAC copurifies with the β protein even in the presence of SDS, and can be localized to amyloid fibrils using electron microscopy [9]. The primary sequence of an amino-terminal portion of NAC (residues 66–73) resembles the amyloidogenic carboxyl terminus of the β protein (residues 36–43) (Fig. 1). This is a critical region of the β protein: truncated variants of β, which form amyloid more slowly *in vitro* [23], are the primary circulating proteins while the longer, more rapidly aggregating variants are the primary plaque proteins [37]. A similar sequence is also found in a region of the prion protein (PrP, residues 117–124, Fig. 1). The substitution of Val for Ala at codon 117 of PrP is found in Gerstmann–Straussler–Scheinker syndrome (GSS) [31,32]. A peptide containing this region (PrP106–126)

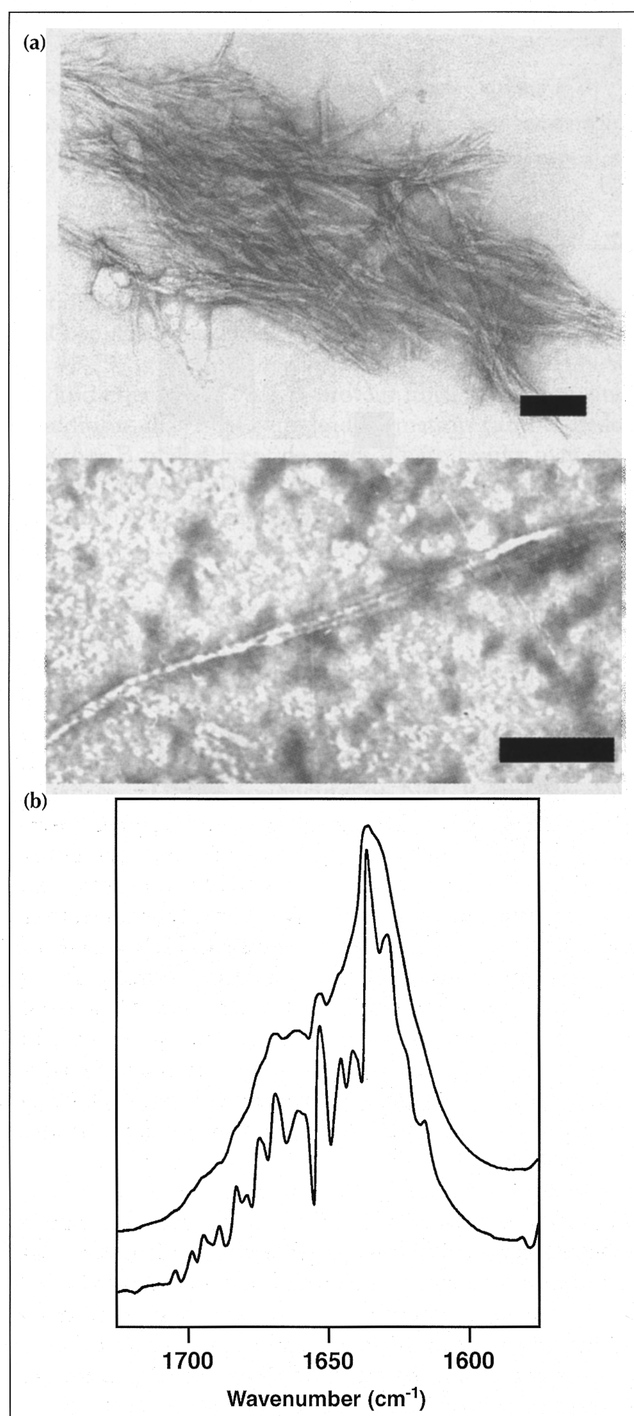

Fig. 4. The NAC peptide forms amyloid fibrils. **(a)** Electron micrograph of NAC fibrils (Bar = 1000 Å). **(b)** Upper trace: FTIR spectrum of NAC fibrils; lower trace: Fourier-deconvoluted spectrum.

is known to form amyloid fibrils and is neurotoxic [18]. The sequence similarities suggest that specific hydrophobic interactions between NAC (or NACP), PrP, and the β protein could occur *in vivo*.

A 35-amino-acid peptide with the minimal NAC sequence was chemically synthesized on the solid phase. When a supersaturated solution of this peptide in phosphate buffered saline (PBS) was prepared, NAC formed ordered amyloid fibrils which fit the characteristic definition of amyloid: unbranched, twisted fibrils as viewed by EM (Fig. 4a), and staining and birefringence under polarized light with the dye Congo Red [22]. Fourier transform IR (FTIR) indicates predominantly antiparallel β-sheet conformation (Fig. 4b) [28].

The colocalization of NAC and β in amyloid plaque could be due to the formation of highly insoluble coaggregates of these two species [29,30]. To test this hypothesis, we compared the solubility of each isolated peptide to the solubility of that peptide in the presence of one other peptide. No evidence was found for the formation of a stable coaggregate. In fact, several β protein- and PrP-derived peptides actually showed an increase in solubility in the presence of NAC. This phenomenon is not easily interpreted, and an effect on solubility does not necessarily correlate with the ability to serve as a seed.

The kinetics of amyloid formation from a supersaturated solution of NAC indicate that this process is a nucleation-dependent polymerization (Fig. 3). An identical mechanism governs amyloid formation by a number of amyloidogenic species [15,16,23,24,38]. One feature of this mechanism is that addition of a preformed fibril (seed) eliminates the need for nucleation. Thus, addition of NAC fibrils to a supersaturated NAC solution eliminated the lag time completely, and immediate seeded growth was observed ('homogeneous seeding') (Fig. 5a).

Heterogeneous seeding was also observed. Fig. 5b shows the result of seeding β1–40 with synthetic NAC. Addition of NAC seed fibrils led to a significant decrease in the lag time observed before β1–40 fibril growth. In this case, seeded growth does not occur immediately, as it does in self-seeding experiments; seeding manifests itself as a reduction, rather than an elimination, of the observed lag time. This observation is consistent with the idea that a greater degree of primary sequence homology leads to more efficient seeding. Seeding is a chemically discriminating event; small changes in primary sequence can eliminate the ability of a peptide to serve as a seed [15,16,24]. This property has been demonstrated using a series of peptides derived from a similar amyloidogenic sequence [15]. Our experimental data thus support the hypothesis [9,26] that NAC, which comprises 10 % of the total protein concentration in the plaque, could promote β protein amyloid formation *in vivo* (Fig. 3).

A second possibility is that the promotion of NAC amyloid formation by β or prion proteins could be pathogenic. In Fig 5a, we show the results of heterogeneous seeding of NAC amyloid formation. Peptides derived from both β protein (β1–40, β26–40, β26–42, β26–43) and prion protein (PrP106–126, PrP106–126A117V) sequences exhibited the ability to seed NAC amyloid formation in our *in vitro* assay (Table 1). The 'long β' models, which have a greater sequence homology to NAC, were more efficient seeds, once again supporting the importance of the sequence homology. Thus,

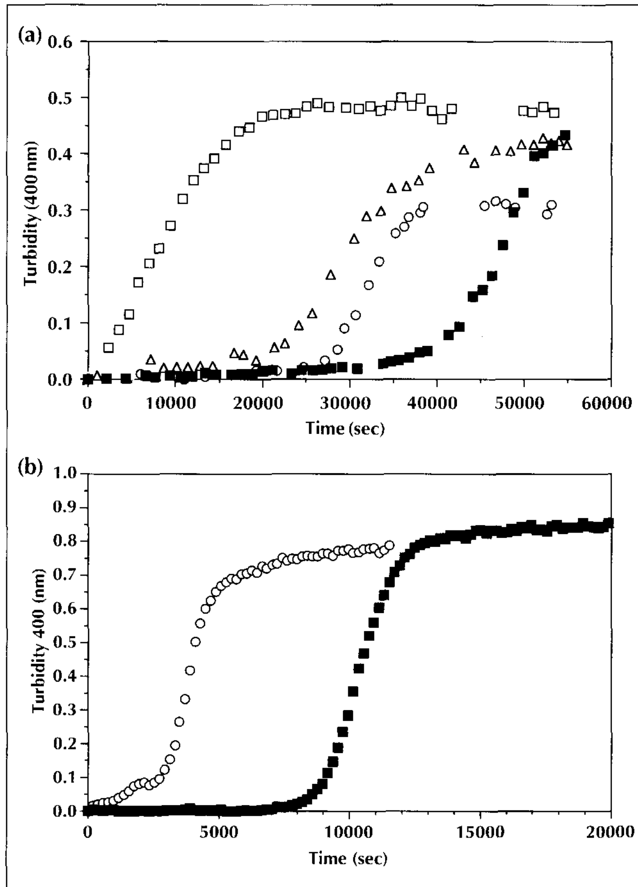

Fig. 5. Representative kinetics for amyloid fibril formation measured by turbidity (400 nm). The vertical axis indicates the increase in turbidity relative to an initial measurement. The turbidity due to added seeds was subtracted. **(a)** Unseeded NAC (■); NAC self-seeded (□); NAC seeded with β1–40 (O); NAC seeded with PrP106-126A117V (Δ). **(b)** β1–40 unseeded (■); β1–40 seeded with NAC (O). Calculated lag times and standard deviations are given in Table 1.

Table 1. Nucleation (lag) times measured by turbidity.

	Solution (80 μM)	Seed (20 μM)	Lag time relative to unseeded*
a	NAC	β1–40	0.70 (0.02)
b	NAC	β26–40	0.79 (0.19)
c	NAC	β26–42	0.47 (0.09)
d	NAC	β26–43	0.44 (0.14)
e	NAC	PrP106–126	0.71 (0.22)
f	NAC	PrP106–126A117V	0.60 (0.12)
g	β1–40	NAC	0.4 (0.1)
h	β26–40	NAC	0.60 (0.04)
i	β26–40	PrP106–126	0.9 (0.2)
j	β26–40	PrP106–126A117V	1.1 (0.3)

*Lag time of aggregating peptide solutions (80 μM) in the presence of fibrils (20 μM) as a seed. Data shown are an average over at least 3 trials. Each value was determined by solving the best fit line to the growth phase of the aggregation curve for y = 0 and is presented as a fraction relative to unseeded lag times (in min (±S.D.)): NAC, 666 (88); β1–40, 119 (25); β26–40, 115 (3).

aggregation of NAC itself may be promoted by other amyloid proteins *in vivo* (Fig. 3).

This possibility leads back to the role of NACP/α-synuclein, the synaptic protein from which NAC is derived. Although the function of NACP is unknown, it is found primarily in brain tissue, and is localized within neurons at the presynaptic nerve terminal [10,11,13,39]. Synaptic amyloid deposition may be a common theme among amyloid-characterized neurodegenerative disorders [13]. Production of NAC in high local concentration at the synapse could seed amyloid formation and have a neurotoxic effect [9,26]. Alternatively, β amyloid (or PrP^{Sc}) could seed the aggregation of NAC or NACP. Further information on the structure and function of NACP/α-synuclein will be required to elucidate the possible role of this protein in neurodegenerative disorders.

Significance

Brain amyloid is the major pathological diagnostic feature of AD, although there is no consensus as to whether these plaques cause disease [1]. Evidence is accumulating that suggests a possible causative role for amyloid plaque and its primary protein component, the β protein. In addition to AD, which is characterized by β protein amyloid formation, other neurodegenerative diseases are characterized by abnormal ordered-protein aggregates, for example, the prion diseases [14]. It is therefore important to study the mechanism of amyloid deposition, as well as to determine the role of factors which might affect this process. One such factor is the non-Aβ component of AD amyloid (NAC). Although a number of proteins are associated with the plaques *in vivo*, only NAC has been identified as a second intrinsic component of the insoluble plaque core.

We have shown that NAC forms ordered fibrils which meet the characteristic definition of amyloid. Amyloid formation by NAC proceeds via a nucleation-dependent mechanism similar to a crystallization. Addition of a preformed NAC fibril seeds amyloid formation by eliminating the need for a nucleation step.

Seeding is a sequence-dependent event; a region of high local sequence homology between β, PrP, and NAC suggests to us that specific local interactions may lead to heterogeneous seeding between these species. In fact, we find that NAC seeds β1–40 amyloid formation, and both β1–40 and PrP106–126 seed NAC amyloidogenesis. However, neither NAC nor β seeds PrP.

These results suggest that NAC may be a common target or trigger for amyloid plaque formation in AD. The identification of the precursor to NAC as NACP or α-synuclein, a synaptic protein, suggests

a connection between these proteins and the neurodegeneration observed in AD [13,26]. Aggregation of NAC, or the loss of NACP/ α-synuclein function, could be pathogenic.

Materials and methods

Peptide synthesis, purification, and characterization

NAC was synthesized by standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry on the Wang resin (Fmoc-Val-resin, Novabiochem, La Jolla, CA) and purified to homogeneity by reverse-phase HPLC (water/acetonitrile/0.1 % trifluoroacetic acid). Purity of the peptide was determined to be >90 % by reverse-phase HPLC on a C4 analytical column (5-μm TP, 0.46x25 cm,Vydac, Hesperia, CA) under isocratic conditions (70 % H_2O/30 % acetonitrile, 2 ml min^{-1} flow rate, R_v=17–20 ml). Amino acid analysis (Applied Biosystems Model 420 PTH amino acid analyzer), amino acid sequencing (Applied Biosystems Model 477A protein sequencer), and plasma desorption mass spectrometry (PDMS) were consistent with the desired product: $(M+H)^+$, calc'd. 3262.7, found 3258.4. β26–40, β26–42, β26–43, PrP106–126, and PrP106–126A117V were prepared by methods reported previously [16,23]. β1–40 was purchased from Bachem (Torrance, CA).

Electron microscopy (EM) and Congo Red (CR) staining

Samples for EM were prepared by stirring supersaturated solutions of peptide (80 μM) in 100 mM NaCl/8.2 mM Na_2HPO_4/1.8 mM NaH_2PO_4, pH 7.4/10 % dimethyl sulfoxide (DMSO) (hereafter referred to as the standard buffer). EM samples were placed on carbon-coated copper grids, negatively stained with 2 % uranyl acetate, and viewed at 60K–120K on a JEOL 1200 CX EM at 80 kV. CR staining of aggregated peptides dried on a glass microscope slide was performed as described [15].

FTIR spectroscopy

Aggregated peptides were centrifuged at 4000 rpm for 10 min and spread on a CaF_2 plate. After air drying, the infrared spectra were recorded on a Perkin-Elmer 1600 series FTIR spectrometer.

Solubility

A supersaturated solution of peptide (40 μM) or mixtures of two peptides (40 μM each) in standard buffer was stirred at 1800 rpm for 72 h. The suspension was filtered through a Millex-GV 0.22-μm aqueous filter (Millipore).Total peptide concentrations were determined by quantitative amino acid analysis, and the relative ratios were determined based on unshared amino acids (data shown represent an average of three trials).

Aggregation kinetics and seeding experiments

A supersaturated solution of peptide (80 μM) in standard buffer was prepared by addition of peptide dissolved in DMSO to phosphate buffer and stirred continuously at 1800 rpm (1550 rpm for β1–40 and β26–40). Samples were vortexed briefly (except for β1–40 and β26–40) and aggregation was measured by turbidity at 400 nm versus a buffer blank. Seeding experiments were initiated by addition of a suspension of fibrils (20 μM final) from a previous aggregation trial. The turbidity due to the seed peptide was accounted for by blanking on the suspension of seeds in buffer immediately before addition of the DMSO stock solution.

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