FOR THE RECORD

Mutagenic exploration of the cross-seeding and fibrillation propensity of Alzheimer's β -amyloid peptide variants

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Abstract

Amyloid formation is a nucleation-dependent process that is accelerated dramatically in vivo and in vitro upon addition of appropriate fibril seeds. A potent species barrier can be effective in this reaction if donor and recipient come from different biological species. This species barrier is thought to reflect differences in the amino acid sequence between seed and target polypeptide. Here we present an in vitro mutagenic cross-seeding analysis of Alzheimer's A β (1-40) peptide in which we mapped out the effect of systematically varied amino acid replacements on the propensity of seed-dependent amyloid fibril formation. We find that the susceptibility of different peptides toward cross-seeding relates to the intrinsic aggregation propensity of the respective polypeptide chain and, therefore, to properties such as β -sheet propensity and hydrophobicity. These data imply that the seed-dependent formation of amyloid-like fibrils is affected by the intrinsic properties of the polypeptide chain in a manner that is similar to what has been described previously for aggregation reactions in general. Hence, the nucleus acts in this case as a catalyst that promotes the fibrillation of different polypeptide chains according to their intrinsic structural predilection.

Keywords: amyloid; conformational disease; neurodegeneration; protein folding; prion

The template-dependent outgrowth is a critical step in the formation of amyloid fibrils in vitro and in vivo (Varga et al. 1986; Dzwolak et al. 2004). Long-standing evidence shows that the development of murine splenic amyloid after an inflammatory stimulus can be accelerated substantially upon coinjection of amyloid-enhancing factor (AEF) (Varga et al. 1986). Recently, it was clarified that amyloid seeds are the predominant, if not the sole, biologically active component of AEF (Lundmark et al.

2002). Consistent with this, amyloid-like fibrils, formed from pure polypeptide chains in vitro, possess AEF activity when injected appropriately into mice (Johan et al. 1998). The underlying idea of conformational transmissibility associated with the nucleation-dependent outgrowth of aggregates has been expressed most explicitly in the prion concept of a conformation-based and protein-only infectivity (Prusiner 1998).

Interspecies cross-transmission studies have shown that the transmissibility of the abnormal phenotype can be affected strongly by a species barrier if donor and recipient are from different biological species (Prusiner 1998). This barrier is thought to reflect, at least in part, a molecular species barrier inherent in the sequential difference and potential incomplementarity between seed

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and target sequence (Chien et al. 2004). Indeed, previous *in vitro* studies have shown that the sequential similarity is a major factor contributing to the seeding efficiency *in vitro* (Krebs et al. 2004).

Here, we have mapped out systematically the effect of different side chains on the cross-seeding susceptibility of Alzheimer's A β (1-40) peptide *in vitro*. Our experimental setup is such that the sequence of the seed is kept constant [wild-type A β (1-40) peptide], while the target sequence is varied systematically. All target sequences are single-site mutants of the A β (1-40) peptide, differing only in residue 18 (which is a valine in the wild-type sequence). The potential of A β (1-40) to form fibrillar aggregates depends strongly on the origin of the peptide batch and the presence or formation of a fibrillation seed. For example, the material obtained from a recently established recombinant expression system results in aggregates that are predominantly, if not entirely, nonfibrillar in morphology (Fig. 1A) (Christopeit et al. 2005; Hortschansky et al. 2005a). In contrast to this, chemically synthetic and commercially available peptide batches form fibrils very readily (Fig. 1B).

Cross-seeding trials between these two batches show that the fibrillogenic peptide acts in a dominant manner, i.e., addition of 1% to 5% from this batch to the freshly dissolved recombinant peptide leads to very large quantities of fibrils (Fig. 1C). In contrast, addition of similar amounts from the recombinant peptide to freshly dissolved commercial A β (1-40) has no apparent effect (Fig.

1D); at least fibril formation is not obviously impaired. The ability of the chemically-synthetic peptide to form fibrils spontaneously is eliminated by disaggregation, and tryptic digestion shows protease resistance in the fibrillogenic batch under conditions where the recombinant peptide is fully digested (Fig. 2). These observations suggest the presence of preaggregated species of peptide that act as fibrillation seeds and that are absent in our peptide purifications.

Based on these observations, we wondered whether the nucleation ability of these seeds is affected by the sequence of the target peptide. To that end, we mixed and incubated freshly dissolved recombinant peptides containing different sequences, with a 2% aliquot from the seed-containing batch. A total of 16 variants of A β (1-40) peptide were used in this analysis, which differed only in residue 18. In the wild-type peptide, residue 18 is a valine and occurs after fibril formation within the center of a β -strand (Petkova et al. 2002; Williams et al. 2004). Analysis with electron microscopy shows that this treatment leads to discernible amounts of fibrils in most samples, except from the Lys or Pro variants (Table 1), where only nonfibrillar aggregates can be seen. Without fibril seeds, all samples contained no fibrils or only very small amounts of fibrils (Table 1). Fibril formation was quantified in seeded samples by critical concentration measurements. The critical concentration represents the maximum concentration of full peptide solubility and allows calculation of the Gibbs free energy of fibrillation

Figure 1. Seed-dependent formation of fibrillar aggregates. (A,B) Electron micrographs from recombinant A β (1-40) (A) and chemically synthetic peptide (B) after incubation for 6 d at 37°C. (C,D) Seeding of the recombinant peptide with 2% chemically synthetic peptide (C) and seeding of the chemically synthetic peptide with 2% recombinant A β (1-40) (D).

Figure 2. Tryptic digestion of A β (1-40) peptide. Ultraviolet absorption traces (215 nm) from reversed phase chromatography of different batches of A β (1-40) peptide before and after tryptic digestion for 2 h. (Trace A) Freshly dissolved recombinant peptide without addition of trypsin; (trace B) freshly dissolved chemically synthetic peptide after incubation with trypsin; (trace C) freshly dissolved recombinant peptide after incubation with trypsin. Full-length A β (1-40) runs at ~42% acetonitrile. Arrow shows residual undigested peptide in the chemically synthetic peptide batch. The peak profile is closely similar to a tryptic digest of A β (1-40) published by Kheterpal et al. (2001).

(ΔG_{fib}) (Oosawa and Asakura 1975). Within the precision of such measurements, fibril formation estimated by electron microscopy is consistent with the ΔG_{fib} -measurements (Table 1), i.e., the largest quantities of fibrils can be seen for peptide variants that are associated with very low ΔG_{fib} -values.

Next, we compared the ΔG_{fib} -values with intrinsic properties of residue 18. Previous studies could establish that the intrinsic properties of a polypeptide chain contribute substantially to its experimentally observed aggregation propensity (Chiti et al. 2003; DuBay et al. 2004; Fernandez-Escamilla et al. 2004; Tartaglia et al. 2004; Christopeit et al. 2005; Hortschansky et al. 2005b); however, these studies compared the intrinsic properties only with the case of aggregation (and without assuming specifically the formation of fibrils). We observe here similar correlations between ΔG_{fib} -values for fibril formation and the β -sheet propensity ($R = 0.72$) (Street and Mayo 1999) or hydrophobicity of residue 18 ($R = 0.63$) (Creighton 1993). Furthermore, the present ΔG_{fib} -values relate to the ΔG_{agg} -values associated with the formation of nonfibrillar aggregates determined previously (Hortschansky et al. 2005b). The correlation coefficient of the two data sets is 0.73. Hence, many of the principles revealed previously for the thermodynamics and kinetics of nonfibrillar aggregate formation apply also to the seed-dependent formation of amyloid fibrils studied here.

The similarity by which these macroscopically rather dissimilar types of aggregates respond to mutagenic changes implies that both contain structural microdomains that are sterically very similar, such as an aggregated β -strand conformation. Indeed, the fibrillar aggregates studied

here show green birefringence after Congo red binding (data not presented) which was seen also in the nonfibrillar aggregates studied previously (Hortschansky et al. 2005b). Infrared spectroscopy shows that amyloid fibrils and the nonfibrillar aggregates formed from denatured or intrinsically unstructured polypeptide chains produce the same type of amide I' spectrum (Jackson and Mantsch 1991; Fink 1998; Zandomenighi et al. 2004). In addition, fibrillar and nonfibrillar aggregates can give rise to very similar X-ray diffraction patterns (Fändrich et al. 2003).

The presently observed ability of fibrillation nuclei to induce and promote fibril formation in several different peptide variants testifies further to a concept according to which fibrillation nuclei can act as catalysts that promote the conversion of fibril precursors to an extent that is defined by the intrinsic structural predilection. Indeed, several studies show that amyloid fibrils and aggregates can be nucleated in vivo or in vitro from seeds that are chemically very different and without the need of any sequential relationship. These nuclei consist of silks (Kisilevsky et al. 1999), lipids (Gellermann et al. 2005), Teflon surfaces (Sluzky et al. 1991), glycosaminoglycans (Kisilevsky 2000), or polypeptides of rather unrelated sequence (Ganowiak et al. 1994). On the other hand, there is evidence that a given polypeptide chain can form and stably propagate nuclei of different conformation (Dzwolak et al. 2004). Moreover, several examples show that the sequence is, in some cases, clearly involved in determining the cross-seeding efficiency in aggregation reactions. For example, an artificial species barrier

Table 1. Structural properties of the aggregates and peptides

Variant	Seeded reactions			No seed
	c_c (μM)	ΔG_{fib} (kcal/mol)	EM	EM
Wild type	75.0	-5.85	+++	-
V18F	54.2	-6.05	+++	+
V18Y	65.9	-5.93	+++	+
V18I	86.2	-5.76	+++	+
V18T	107.8	-5.63	+++	+
V18W	124.6	-5.54	+++	-
V18H	127.8	-5.52	+	-
V18Q	137.2	-5.48	+++	-
V18S	139.4	-5.47	++	-
V18K	141.6	-5.46	+	-
V18G	144.2	-5.45	++	-
V18N	151.0	-5.42	++	-
V18P	179.8	-5.31	-	-
V18D	186.6	-5.29	+	-
V18M	187.8	-5.28	+	-
V18E	202.8	-5.24	-	-

(c_c) Critical concentration; (EM) presence of fibrils by electron microscopy; (+++) large amounts of fibrils; (++) small amounts of fibrils; (+) very small amounts of fibrils; (-) no fibrils.

is created for the transmissibility of mammalian prions when the prion protein is truncated to a 106-residue species (Supattapone et al. 1999). Mutation in the yeast protein sup35 affects the transmissibility of the [PSI⁺] phenotype and the structure of the resulting aggregate (Chien et al. 2004). Finally, susceptibility of lysozyme to cross-seeding by various sequences depends largely on the sequential similarity between nucleus and target sequence (Krebs et al. 2004).

Although the present data imply that those A β (1-40) variants that are most similar to the wild-type sequence, i.e., which comprise a large hydrophobic or an aromatic residue in position 18, are most susceptible to seed-dependent fibrillation (Table 1), it cannot be excluded that the structural context, and therefore the position of mutation, contributes significantly to determining some molecular species barriers in some cases. The presently studied mutations occur within an aggregated β -sheet conformation (Petkova et al. 2002; Williams et al. 2004), but other positions may be much more critical in defining specific tertiary or quaternary structural contacts. Mutation of such residues could have an even stronger effect when trying to establish a potential complementarity between seed and target sequence. Whether or not the latter is prone to aggregate, however, depends on its intrinsic properties in a given physicochemical environment. These data could provide further experimental information for rationalizing or predicting fibril formation based on intrinsic and environmental parameters.

Materials and methods

Source of the peptides

Chemically synthetic wild-type A β (1-40) peptide was purchased at >96% purity from Bachem. One batch of wild type and all A β (1-40) sequence variants were generated with a recombinant expression system (Christopeit et al. 2005; Hortschansky et al. 2005a).

Critical concentration measurements

Recombinant peptides were dissolved at 1.0 mg/mL concentration in 50 mM sodium borate (pH 9.0). In seeded reactions, these solutions were supplemented with 2% from a 1.0 mg/mL solution of commercial peptide in the same buffer. These reactions were incubated at 37°C for 14 d. Centrifugation was performed at 120,000 rpm (513,000g) at 4°C for 30 min using a Beckman TLA-100 tabletop centrifuge and a TLA-120.2 fixed angle rotor. After centrifugation, the supernatant was taken off and the soluble peptide fraction was determined by using the Micro-BC-Assay (Uptima). The Gibbs free energy of fibrillation (ΔG_{fib}) relates to the critical concentration as described by the simple equation $\Delta G_{\text{fib}} = -RT \ln(1/c_c)$, in which R represents the gas constant and T the absolute temperature.

Electron microscopy

Sample preparation and analysis with electron microscopy are described elsewhere (Fändrich et al. 2003; Christopeit et al. 2005) using an EM 900 electron microscope (Zeiss) that was operated at 80 kV.

Proteolytic digestion of the peptide

A total of 0.3 mg of recombinant or chemically synthetic A β (1-40) peptide was freshly dissolved in 1 mL of 50 mM sodium borate buffer (pH 9.0) and analyzed by reversed phase chromatography or incubated for 2 h at room temperature after addition of 0.025 mL of a trypsin solution (0.03 mg/mL in 50 mM sodium borate at pH 9.0). After incubation, proteolysis was stopped by addition of 2 μ L of trifluoroacetic acid (TFA). The samples were snap frozen in liquid nitrogen and stored until further analysis. Reversed phase chromatography was carried out on 480 μ L of these aliquots using a 3 mL Source 15RPC column (GE Healthcare) and a linear gradient between solvents A (0.1% v/v TFA in water) and B (0.1% v/v TFA in acetonitrile). Peak fractions were collected and analyzed by mass spectrometry.

Competing interests

The authors claim a conflict of interest arising from a commercial partner that precludes free distribution of any DNA constructs described here. Basis vectors are available from commercial vendors.

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