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An Aβ concatemer with altered aggregation propensities

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ABSTRACT

We present an analysis of the conformational and aggregative properties of an Aβ concatemer (Con-Alz) of interest for vaccine development against Alzheimer's disease. Con-Alz consists of 3 copies of the 43 residues of the Aβ peptide separated by the P2 and P30 T-cell epitopes from the tetanus toxin. Even in the presence of high concentrations of denaturants or fluorinated alcohols, Con-Alz has a very high propensity to form aggregates which slowly coalesce over time with changes in secondary, tertiary and quaternary structure. Only micellar concentrations of SDS were able to inhibit aggregation. The increase in the ability to bind the fibril-binding dye ThT increases without lag time, which is characteristic of relatively amorphous aggregates. Confirming this, electron microscopy reveals that Con-Alz adopts a morphology resembling truncated protofibrils after prolonged incubation, but it is unable to assemble into classical amyloid fibrils. Despite its high propensity to aggregate, Con-Alz does not show any significant ability to permeabilize vesicles, which for fibrillating proteins is taken to be a key factor in aggregate cytotoxicity and is attributed to oligomers formed at an early stage in the fibrillation process. Physically linking multiple copies of the Aβ-peptide may thus sterically restrict Con-Alz against forming cytotoxic oligomers, forcing it instead to adopt a less well-organized assembly of intermeshed polypeptide chains.

1. Introduction

Alzheimer's disease (AD) is the most prevalent dementia disease affecting more than 5% of all people older than 65. The main hallmarks of AD are extracellular senile plaques (SP) and intracellular neurofibrillary tangles (NFT), both found in brain areas important for memory and cognition. The NFT are composed of the microtubule-associated protein Tau, a protein that stabilizes and promotes formation of axonal microtubules [1]. The major constituent of SP is the 39–43 residue amyloid β peptide (Aβ), an aberrant proteolytic fragment of the membrane protein amyloid precursor protein (APP). Aβ can form β-sheet rich structures called amyloid fibrils, which *in vitro* form via a complex multi-step nucleation polymerization mechanism involving intermediate species. These are called Aβ derived diffusible ligands (ADDLS) [2] or protofibrils [3] and disappear upon fibril formation. During the last few years, there has been a paradigm shift towards these pre-fibrillar species rather than the mature fibrils being the main cause of neurodegeneration [4,5]. Aβ's cytotoxicity is believed to be linked to their ability to form membrane permeable pores via an oligomeric state [6,7].

Current AD treatment only relieves some symptoms for a period of time for a subset of patients, and does not address the underlying pathologic process or substantially slow clinical progression [8]. Several curative strategies are being explored. One is to target the intermediate Aβ aggregates, since antibodies directed against these aggregates can rescue cells from the deleterious effects of protein aggregation [9]. However, while such an antibody reduced plaque load in AD-transgenic mice, it had no functional effect [10]. Another approach is to reduce the concentration of the Aβ in the AD patients using Aβ-specific antibodies (reviewed in [11]). This may be done by administering therapeutic antibodies to the patients (passive immunization) [12] or by vaccination with Aβ [13] or derivatives thereof (active immunization). The Aβ analog K6Aβ1–30[$E_{18}E_{19}$], designed to remove two T-cell epitopes which have been linked to the microencephalitis observed in a few cases when vaccinating with full-length pre-aggregated Aβ [14], was shown to improve cognition and reduces Aβ burden when used with an adjuvant suitable for humans, without increasing vascular Aβ deposits or microhemorrhages [15]. An 11-mer tandem repeat of Aβ1–6 also led

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to a predominantly IgG1 response, where the induced antibodies reacted strongly with Aβ plaques without inflammation-related pathology [16]. However, the structural properties of these constructs and their possible link to the extent of the immune response have not been reported.

As Aβ is an endogenous peptide, the immunogenicity of an Aβ containing vaccine may be increased by fusing Aβ molecules or fragments thereof to known T-cell epitopes from the tetanus toxin, (P2 and P30), which are known to enhance immune responses [17]. Such a promising vaccine candidate is the protein Con-Alz, which contains three copies of the Aβ(1–43) sequence connected by P2 and P30 (see Fig. 1). The addition of epitopes P2 and P30 in the vaccine serves a double purpose: 1) P2 and P30 are potentially more efficient at activating Helper T cells (CD4+ T cells) than Aβ itself, leading to a potentially higher titer of Aβ-specific antibodies; 2) since P2 and P30 are immunodominant, there should be no response towards the T cell epitopes contained within Aβ upon vaccination with the concatemer, and thus no activation of Aβ-specific Th1 T cells and, in turn, no adverse auto-immune response. Thus, the Con-Alz construct is a potentially safer and more efficient vaccine than Aβ alone. *In vivo* the precursor protein APP is cleaved in different positions by the γ-secretase, leading to Aβ peptides of 38–43 residues [18]. Our Aβ construct represents the maximum possible length of the Aβ peptide to ensure that in its eventual use as a vaccine, all Aβ residues which may occur *in vivo* are exposed to the immune system.

An obvious question is whether Con-Alz has inherited the fibrillogenic properties of Aβ. It could be expected that the juxtaposition of several highly fibrillogenic sequences would accelerate fibrillation since the rate-limiting nucleation step would become intra- rather than intermolecular. This has been shown to be the case for several other systems [19,20]. On the other hand, when two copies of one of the shortest fibrillating sequences known (KFFE) are linked together by a four-residue intervening sequence, the resulting dodecamer either remains as a monomeric random coil (if the linker is flexible) or forms a stable oligomeric β-hairpin (if the linker is a turn) [21]. An increased aggregation propensity would be a challenge to a reliable and reproducible formulation of the vaccine species due to both the variability in the relative amount of differentially aggregated species and the formation of potentially cytotoxic species. Here we show that even under strongly denaturing conditions Con-Alz has a very high propensity to form aggregates which slowly coalesce over time with changes in secondary, tertiary and quaternary structure. However, this strong aggregative behavior is not linked to pronounced vesicle permeabilizing abilities presumably because the linking together of Aβ concatemers sterically restrict Con-Alz against forming cytotoxic oligomers, forcing it instead to adopt a less well-organized assembly of intermeshed polypeptide chains.

2. Materials and methods

2.1. Materials

Con-Alz (batch: LEP0149 D, suspended in 10 mM TRIS pH 8, purity > 90%) was kindly provided by Lundbeck A/S (Valby, Denmark). Western blots run together with SDS-PAGE showed that the protein formed higher order structures as well as monomers (see Results) but also confirmed that all protein bands were Con-Alz. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and 1,2-dioleoyl-*sn*-glycero-3-phospho-*rac*-1-glycerol-sodium salt (DOPG) were from Avanti Polar Lipids. Aβ(42) was from American Peptide Company Inc. (Evelyn Ave, Sunny Vale, CA). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

2.2. Buffer compositions at different pH values

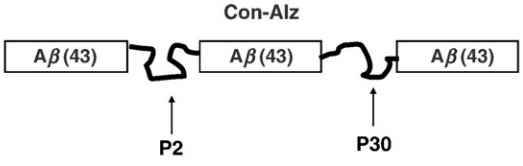
pH 1: 100 mM HCl, pH 2: 10 mM HCl, pH 3: 50 mM Glycine (adjusted with HCL), pHs 4 and 5: 50 mM sodium acetate, pH 6 and pH 7: 10 mM phosphate, pH 10: 50 mM Glycine (adjusted with NaOH), pH 10.8 50 mM Glycine (adjusted with NaOH). PBS: 10 mM sodium phosphate pH 7 150 mM NaCl. All buffers were filtered through a 0.22 μm filter.

2.3. Stock solutions of Con-Alz

Con-Alz was dialyzed extensively against water, lyophilized and resuspended to a clear solution in (a) 15 mM SDS 10 mM phosphate pH 7 150 mM NaCl for CD measurements or (b) 5 M GdmSCN pH 10 for all other experiments. These two solvents lead to the smallest aggregate sizes; in addition it is stable against further aggregation over a time period of several days (data not shown). The high far-UV absorption of GdmSCN precludes its use for CD spectroscopy. Con-Alz concentration (~500 μM) was determined using an estimated ε_{280} of 10.81 mM^{-1} cm^{-1}.

2.4. Fluorescence and absorption assays

The affinity of Con-Alz towards Thioflavin T (ThT) was followed using a SpectraMax Gemini XS fluorescence plate reader (Molecular Devices, Sunnyvale, CA) or a LS55 Luminescence Spectroflourometer (Perkin Elmer, Wellesley, MA). Unless otherwise stated all measurements were conducted in triplicate at 25 °C with a total sample volume of 100 μl and a final concentration of 40 μM ThT and 20 μM Con-Alz 10 mM phosphate 150 mM NaCl pH 7. Contributions from ThT were subtracted. ThT excitation was at 450 nm with emission at 485 nm, Trp excitation was at 295 nm with emission at 330 and 350 nm. ThT was dissolved in 10 mM phosphate buffer pH 7 and the



Aβ:
D_1AEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIAT$_{43}$

Con-Alz:
MD$_1$AEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIAT$_{43}$QYIKANSKF
GITELD$_1$AEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIAT$_{43}$GS*FNNF*
*TVSFWLRVPKVSASHLE*D$_1$AEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVG
GVVIAT$_{43}$

Fig. 1. Primary structure of Con-Alz. The three white boxes represent three Aβ(43) peptides connected by the P2 epitope and P30 epitope (bold black line). Amino acid sequence of Con-Alz is illustrated below, where bold black letters denote amino acids in the Aβ(43) black letters P2 epitope and italic black letters are the P30 epitope, underlined letters are amino acids incorporated for cloning purposes.