

Amyloidogenicity of rodent and human β A4 sequences

Thomas Dyrks^a, Elke Dyrks^b, Colin L. Masters^c and Konrad Beyreuther^b

^aResearch Laboratories of Schering AG, Berlin 65, Germany, ^bCenter for Molecular Biology Heidelberg, University Heidelberg, Heidelberg, Germany and ^cDepartment of Pathology, University of Melbourne, Parkville, Victoria 3052, Australia

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Previously we have shown that aggregation of the C-terminal 100 residues (A4CT) of the β A4 amyloid protein precursor (APP) and also of β A4 itself depends on the presence of metal-catalyzed oxidation systems [T. Dyrks et al. (1988) EMBO J. 7, 949–957]. We showed that aggregation of the amyloidogenic peptides induced by radical generation systems requires amino acid oxidation and protein cross-linking. Here we report that aggregation of A4CT and β A4 induced by radical generation systems involves oxidation of histidine, tyrosine and methionine residues. The rodent β A4 sequence lacking the single tyrosine and one of the three histidine residues of human β A4 and a β A4 variant in which the tyrosine and the three histidine residues were replaced showed a reduced tendency for aggregation. Thus our results may explain why β A4 amyloid deposits could so far not been detected in the rodent brain.

A4CT; β A4; Radical; Rodent sequence; Aggregation; Alzheimer's disease

1. INTRODUCTION

Alzheimer's disease (AD) is an irreversible neurodegenerative disorder of the human central nervous system. The neuronal dysfunction is correlated with massive deposition of fibrillar aggregates in the brain in the form of amyloid plaques [2–4]. These extracellular plaques consist mostly of a 42–43 amino acid polypeptide called the β A4 peptide, which is derived from one or several larger transmembrane proteins, i.e. the (APPs) [5]. Similar deposits have been detected in aged monkeys, dogs, and polar bears [6–10], but rarely have they been found in rats and mice [11]. The sequence of APP of mice [12] and rats [13], deduced from cDNA clones, are about 96% similar to the human APP protein [14]. Only three amino acid substitutions are found in the β A4 region of rodents: Arg→Gly, Tyr→Phe and His→Arg at β A4 positions 5, 10 and 13, respectively.

Proteolytic processing of amyloid protein precursors, leading to secretion of the extracellular part, occurs within the amyloid β A4 sequence [15–17]. This suggests that the N-terminus of the amyloid β A4 protein is not produced by the normal cleavage, which leads to secretory APP's. How β A4 is generated, and why its deposition as amyloid is largely restricted to the brain, may involve a combination of altered APP processing and the unusual amyloidogenic properties of the human β A4 sequence [18,19].

Recent reports have shown that β -A4-related peptides, corresponding to β A4 residues 1–40, are normally secreted by cultured cells and can be detected in human

cerebrospinal fluid [20–23]. These β A4 related peptides are soluble and show no tendency for plaque formation or neurotoxicity.

Here we show that β A4 and A4CT, an APP fragment which is likely to be an intermediate in the pathway leading to amyloid deposition [24], depend on amino acid oxidation and protein cross-linking to be amyloidogenic. Furthermore, we demonstrate that within aggregated A4CT, histidine, tyrosine and methionine residues are oxidized by the metal-catalyzed oxidation systems. In addition we give evidence for a different aggregational behavior of the rodent β A4 sequence.

2. EXPERIMENTAL

2.1. Cloning procedures

Preparation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis of DNA, DNA ligation and bacterial transformation were carried out as described by Sambrook et al. [25].

2.2. Plasmid construction

Construct SP65/A4CT and the prokaryotic expression vector NCO/A4CT were constructed as described previously [26,27].

The constructions of SP65/ β A4, SP65/ β A4rodent and SP65/ β A4H/Y were achieved by polymerase chain reaction (PCR) of the β A4 sequence using APP695 cDNA as template. Oligonucleotides 5' β A4 and 3' β A4 were used to amplify the human β A4 sequence (codons 597–638 of APP695 numbering is according to Kang et al. [5]) starting with the methionine codon 596 as initiation codon. Oligonucleotides β A4rodent and 3' β A4 were used to amplify the rodent β A4 sequence with the following substitutions Arg-601→Gly, Tyr-606→Phe and His-609→Arg. Oligonucleotides β A4H/Y and 3' β A4 were used to amplify the H/Y β A4 sequence with substitutions of all three histidine residues and the tyrosine residue (His-602→Arg, Tyr-606→Phe, His-609→Arg and His-610→Arg). The oligonucleotide sequences are shown below. The amplified cDNAs were digested with *EcoRI*/*HindIII* and subcloned into SP65/A4CTII. Plasmid SP65/A4CTII was obtained by cloning an oligonucleotide-linker (CATGCGGATC-CATGGATGCAGAATT) into SP65/A4CT. The resulting plasmids

Correspondence address. T. Dyrks, Schering AG, Neuropsychopharmakologie, Müllerstr. 170–178, 1000 Berlin 65, Germany. Fax: (49) (30) 469 16738.

were digested with *Bam*HI/*Hind*III and the β A4-sequences were cloned into SP65 digested with the same enzymes. The resulting in vitro expression vectors SP65/ β A4, SP65/ β A4rodent and SP65/ β A4H/Y included methionine codon 596 of APP695 as initiation codon and the human, rodent or H/Y amyloid β A4 sequence (codons 597–638 of APP695) followed by a stop codon.

The oligonucleotides used were:

5'/ β A4. GCAGAATTCCGACATGACTCAGG:

3'/ β A4. CGATGAAGCTTACGCTATGACAACACCGCCACC:

β A4rodent. GCAGAATTCGGACATGACTCAGGATTGAAGT-TCGTCATCAAAAATTGG.

H/Y β A4. GCAGAATTCGACGTGACTCAGGATTGAAGTT-CGTCGTCAAAATTGG.

2.3. In vitro transcription [28]

DNA templates (100 μ g/ml) were transcribed in 40 mM Tris, pH 7.5, 6 mM MgCl₂, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, RNasin (1 unit/ μ l), 100 μ g/ml BSA, 500 μ M each ATP, CTP and UTP, 50 μ M GTP, and 500 μ M m⁷G(5')ppp(5')G (Pharmacia, Freiburg, Germany). Typically, 1 unit of SP6 RNA polymerase (Boehringer, Mannheim, Germany) was added per μ g DNA template for a 1-hour synthesis at 40°C. DTT and rNTPs stocks were prepared with diethylpyrocarbonate treated water. The components of the transcription reaction were mixed at room temperature to prevent precipitation of DNA.

Following RNA synthesis, the DNA template was removed by the addition of RNase-free DNase and after a 10 min incubation at 37°C the reaction mixtures were phenol:chloroform extracted after addition of NaOAc (pH 5.2) to 0.3 M. The RNA was precipitated with ethanol and washed with 70% ethanol.

2.4. In vitro translation

Translation of mRNA in a cell-free rabbit reticulocyte lysate or a wheat germ extract followed the procedures as described in the supplier's manuals (Promega, Germany). Typically, the reactions were carried out for 60 min at 30°C in the presence of 50 μ Ci of [³⁵S]methionine and 0.5–1.0 μ g mRNA. The translation mixture was diluted with 2 $\times$ Laemmli sample buffer. After heating for 5 min at 100°C, labelled proteins were analyzed by SDS-PAGE and fluorography with EN³HANCE (DuPont).

2.5. Hemin and hemoglobin promoted peroxidation [29]

The hemin stock solution was always freshly dissolved in 5 mM NaOH and centrifuged for 10 min at 12,000 $\times$ g. The oxidation reaction was performed in the presence of 20 μ M hemin and 1 mM H₂O₂, or 0.5 mg/ml hemoglobin and 1 mM H₂O₂ in PBS at 37°C for 1 h. The reaction was terminated by the addition of 2 $\times$ Laemmli sample buffer (10% β -mercaptoethanol; see SDS-PAGE) and heating for 5 min at 100°C.

2.6. SDS-PAGE

Analysis of in vitro translated A4CT was by SDS-PAGE and of β A4 peptides was by 16.5% Tris-tricine gel separation [30]. The gels were analyzed by fluorography with EN³HANCE (DuPont, Boston).

2.7. Prokaryotic expression and purification of bacterially expressed A4CT

Construction and expression of prokaryotic expression vector NCO/A4CT were performed as described [26].

2.8. Amino acid analysis

A4CT was hydrolyzed with 6 N HCl/0.1% phenol for 20 h by 110°C under N₂ prior to analysis. Quantitative amino acids analysis was performed on an Applied Biosystems 130A/920A amino acid analyzer. For each analysis calibration was done with a standard mixture of all amino acids (200 pmol).

3. RESULTS

3.1. Inhibition of metal-catalyzed aggregation of A4CT by specific amino acids

Previously we showed that translation of A4CT in wheat germ extract results in a soluble non-aggregated protein which could be transformed to an aggregated molecule by incubation with hemoglobin/H₂O₂ or other metal-catalyzed oxidation systems [26]. By the addition of amino acids, vitamin C, and the vitamin E derivative trolox we were able to inhibit the aggregation of A4CT. We suggested that the aggregation of A4CT induced by radical initiation systems may be due to the oxidation of amino acids and subsequent covalent cross-linking. To verify the latter assumption we tested the inhibitory potential of different amino acids on the aggregation process.

For this purpose, A4CT was translated in the WG extract and incubated with hemoglobin/H₂O₂ in the absence or presence of different amino acids. Incubation of monomeric A4CT, translated in the WG extract, with hemoglobin/H₂O₂ resulted in the previously-reported aggregation pattern of A4CT (Fig. 1, lane 2) [26]. Fig. 1, lanes 3 to 13, shows that of the amino acids tested, only histidine and tyrosine showed a significant and reproducible inhibition of the aggregation. The same results were obtained if hemin/H₂O₂ were used as the radical generation system (data not shown).

3.2. Identification of amino acids modified by metal-catalyzed oxidation systems

To analyze the amino acid composition of the aggregated A4CT obtained in the presence of metal-catalyzed

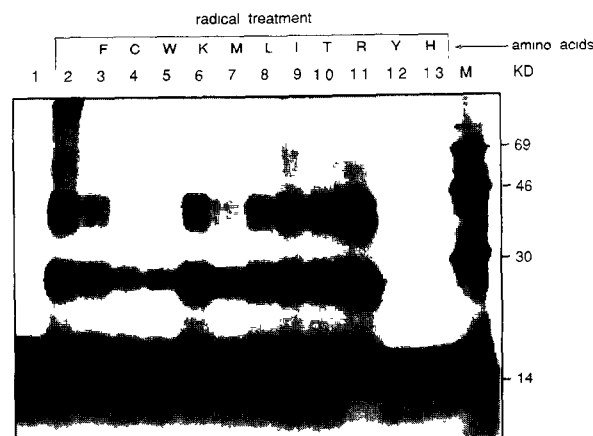


Fig. 1. Inhibition of metal-catalyzed aggregation of A4CT by specific amino acids. A4CT mRNA was transcribed from the corresponding SP6 vector, translated in the WG extract in the presence of [³⁵S]methionine, precipitated with TCA and after incubation with or without 0.5 mg/ml Hb/1 mM H₂O₂ and different amino acids (30 mmol), proteins were analyzed by SDS-PAGE (12.5%). Lane 1, A4CT translated in the WG, precipitated with TCA and incubated with PBS (0.2% SDS) for 1 h at 37°C; lane 2, same as lane 1, but in the presence of 0.5 mg/ml Hb/1 mM H₂O₂; lanes 3 to 13 same as lane 2, but in the presence of the indicated amino acids (30 mmol). M, molecular mass marker; KD, kilo dalton.

Table I

Amino acid compositions of A4CT before and after radical treatment

hem/H ₂ O ₂	-	+	A4CT/ A4CT + hem
Amino acid			
D	6,47 ± 1,75	5,73 ± 1,87	1,13
E	15,71 ± 4,16	13,37 ± 2,80	1,18
S	5,81 ± 0,54	7,26 ± 1,08	0,80
G	11,96 ± 3,89	17,13 ± 4,42	0,70
H	3,61 ± 0,91	1,46 ± 1,17	2,47
R	4,07 ± 0,24	4,67 ± 0,63	0,87
T	5,25 ± 0,38	5,53 ± 0,34	0,95
A	9,06 ± 0,74	9,23 ± 0,54	0,98
P	3,07 ± 0,47	3,50 ± 0,62	0,88
Y	3,68 ± 0,45	1,52 ± 0,94	2,43
V	8,33 ± 2,10	8,35 ± 1,12	1,00
M	0,21 ± 0,10	0,07 ± 0,10	2,99
I	4,01 ± 0,93	3,27 ± 1,66	1,23
L	7,65 ± 1,07	8,62 ± 0,97	0,89
F	4,79 ± 0,48	4,60 ± 0,51	1,04
K	6,11 ± 0,60	5,41 ± 0,79	1,13

Data are the means ± S.D. of six experiments in mol% amino acid.

oxidation systems, purified bacterial-expressed A4CT was incubated with and without hemin/H₂O₂ and the amino acid composition was analyzed by amino acid analysis. Table I shows the means ± SD of six experiments in mol% amino acid. After the radical treatment a significant decrease in mol% amino acid is only seen for histidine, tyrosine and methionine. Fig. 2 shows the quotient of mol% amino acid of untreated A4CT and A4CT after radical treatment. It is obvious that only histidine, tyrosine and methionine are oxidized by the radical treatment.

3.3. Aggregation behavior of different β A4 sequences

Because the inhibition assay and the amino acid analysis of oxidized A4CT strongly suggest that aggregation of A4CT is induced by oxidation of histidine, tyrosine and methionine, we analyzed whether substitution of some or all of these amino acids in the β A4 sequence had any effect on the aggregation properties of β A4. Therefore we constructed SP65/ β A4rodent and SP65/H/Y, in addition to SP65/ β A4, which codes for the human β A4 sequence. SP65/ β A4rodent codes for the rodent β A4 sequence, which shows three amino acid substitutions, two of which change one of the three histidines

and the single tyrosine residue of human β A4 respectively. SP65/ β A4H/Y codes for a β A4 sequence in which all three histidine residues are substituted with arginine and the single tyrosine with phenylalanine (Scheme 1).

To determine whether the different amino acid substitutions have any effect on the aggregation properties of β A4 induced by metal-catalyzed oxidation systems, we incubated the corresponding peptides, β A4human, β A4rodent, and β A4H/Y translated in the WG extract with the same radical generating systems as described previously [26].

Fig. 3, lanes 1 to 3, shows translations of the different β A4 sequences in the WG extract and analysis by Tris-tricine SDS-PAGE. Incubation of monomeric β A4human, translated in the WG extract, with hemoglobin/H₂O₂ resulted in the previously-reported aggregation pattern including three prominent bands at 4 kDa, 19 kDa and 46 kDa (Fig. 4, lane 4). In contrast, the β A4rodent peptide and the β A4H/Y peptide show a clear and reproducible decrease of the aggregational bands at around 19 kDa and 46 kDa. The same results are obtained if hemin/H₂O₂ are used as radical generation system (data not shown).

Densitometric measuring of the major aggregates in Fig. 3, lanes 4, 5 and 6, shows a 4- to 5-fold decrease for β A4H/Y and β A4rodent aggregates in relation to the human sequence (Fig. 4). This provides good evidence that the histidine and/or tyrosine residues of the β A4 sequence are responsible for the aggregation behavior of this amyloidogenic peptide.

4. DISCUSSION

Previously, we have presented experimental evidence that A4CT and β A4 are soluble and non amyloidogenic APP fragments. Both peptides, if expressed in the wheat germ translation system and analyzed by SDS-PAGE, resulted in only monomeric forms. Furthermore, we were able to transform monomeric A4CT and β A4 into aggregating amyloidogenic molecules by the addition of metal-catalyzed oxidation systems. This transformation could be inhibited by the addition of radical scavengers such as vitamin C, vitamin E analogue trolox and amino acids. We suggested that the aggregation of A4CT and β A4 requires amino acid oxidation and protein cross-linking induced by radical generation systems.

Rodent	GGA								TTT			CGA				
Human	GAT	GCA	GAA	TTC	CGA	CAT	GAC	TCA	GCA	TAT	GAA	GTT	CAT	CAT	CAA	AAA
Human	D	A	E	F	R	H	D	S	G	Y	E	V	H	H	Q	K
Rodent	-	-	-	-	G	-	-	-	-	F	-	-	R	-	-	-
H/Y	-	-	-	-	-	R	-	-	-	F	-	-	R	R	-	-

Scheme 1. Schematic diagram of the different β A4 sequences used in this study. Line 1 shows the rodent β A4-DNA sequence at codons where substitutions occur. Line 2 shows the human β A4 DNA sequence from Asp-597 to Lys-612 (numbering is according to [5]). Lines 3 to 5 represent the amino acid sequences in one letter code of β A4human, the β A4rodent and β A4H/Y from Asp-597 to Lys-612.

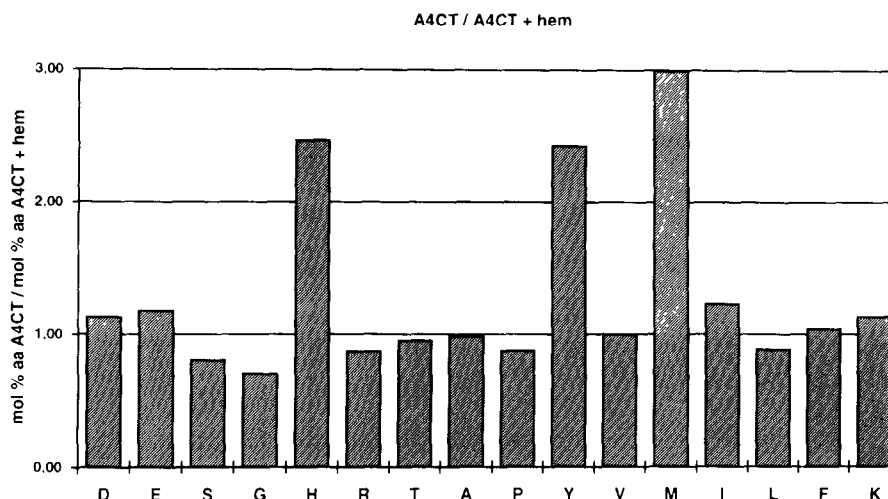


Fig. 2. Modification of the amino acid composition of A4CT by oxygen radicals. 500 pmol of purified bacterial A4CT was incubated with or without 20 μ M hemin/1 mM H_2O_2 for 90 min, precipitated with methanol/chloroform and hydrolyzed with 6 N HCl/0.1% phenol for 20 h at 110°C under N_2 . Amino acid analysis was performed on an Applied Biosystems amino acid analyzer. The ordinate presents the quotient of mol% amino acid of untreated A4CT and mol% amino acid of A4CT incubated with hem/ H_2O_2 .

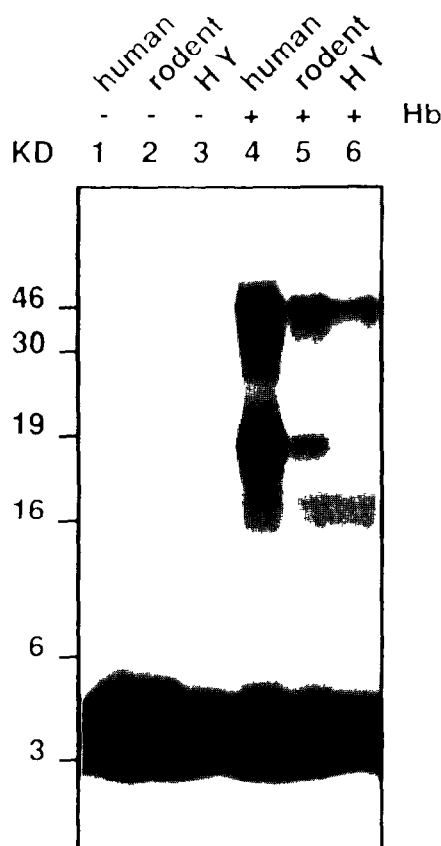


Fig. 3. Aggregation behavior of different $\beta A4$ -sequences. Translation was as described in Fig. 1. The different $\beta A4$ peptides were expressed in the WG extract, precipitated with TCA, and after incubation with 0.5 mg/ml Hb/1 mM H_2O_2 , peptides were analyzed by Tris-tricine SDS-PAGE (16.5%). Lanes 1, 2 and 3, $\beta A4$ human, $\beta A4$ rodent and $\beta A4$ H/Y incubated with PBS (0.2% SDS) for 1 h at 37°C; lanes 4, 5 and 6, same as lanes 1, 2 and 3, but in the presence of 0.5 mg/ml Hb/1 mM H_2O_2 ; KD, kilo dalton

To verify this, in the work reported here we have tested the inhibitory potential of different amino acids on the aggregation process. The specific inhibition of the aggregation by histidine and tyrosine strongly supports the idea that amino acid oxidation is involved in the $\beta A4$ aggregation process. Both amino acids are known to be particularly sensitive to modification by hydroxyl radicals [31].

Furthermore, direct evidence for the oxidative damage of these amino acids has been obtained by amino acid analysis of modified bacterial A4CT. Only methionine, histidine and tyrosine showed a 2- to 3-fold decrease after radical treatment.

From these results we suggest that those amino acids which are modified by the radical attack should also be necessary for the aggregation process. Substitution of these residues should lead to a peptide which is resistant to free radical-induced transformation into an aggregated molecule.

In the context of the on going discussion of whether the rodent $\beta A4$ sequence has an altered conformation and aggregational behavior, we analyzed the rodent $\beta A4$ sequence ($\beta A4$ rodent) which contains three substitutions, two of which change histidine and tyrosine residues, and also a human $\beta A4$ sequence ($\beta A4$ H/Y) without any histidine and tyrosine in our aggregation assay. We show that in the presence of radical generating systems there is a significant difference between the aggregational properties of the wild type human and the rodent $\beta A4$ sequences. Under the conditions used, only the human $\beta A4$ sequence shows a strong tendency to form higher aggregates. Thus in the presence of biological active radicals, only the human $\beta A4$ sequence was highly amyloidogenic. Because in addition the human $\beta A4$ sequence without histidine and tyrosine residues

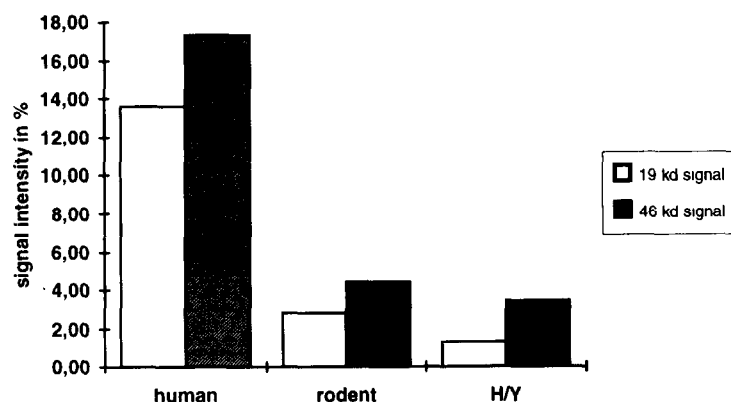


Fig. 4. Densitometric measuring of the major aggregates presented in Fig. 3. The autoradiography was analyzed by scanning densitometry (Elscscript 400, Hirschmann) and the area of the signals are calculated (HID-Calculationprogram, Hirschmann). The data represent the signal-intensity in percent of the 4 kDa signal (100%). The values are normalized to the 4 kDa band.

shows a further decrease in aggregation tendency in the presence of radicals, we suggest that the difference between aggregational behavior of the rodent and human sequence is based on the different content of histidine and tyrosine residues.

Our results are not in conflict with previously published data giving evidence that the human β A4 peptide, but not the rodent homologue, is capable of forming a β -pleated sheet structure at a low peptide concentration. Otvos et al. have already suggested that subtle interspecies amino-acid differences may account for the inability of the rodent peptide to form amyloid fibrils in situ [32]. In contrast, other results showed no difference in solubility and secondary structure of the full- and shorter sized A4 peptides derived from humans and rodents [14]. The latter studies however, did not take into account the effects of varying environmental factors such as radical attack which may contribute to the deposition process of the soluble β A4 peptide into insoluble fibrils. We propose that radical attack cross-links and stabilizes already-preformed β A4 tetramers, which then may be able to aggregate further. We show here that the substitutions in the rodent sequence are responsible for the reduction in protein cross-linking by amino acid oxidation but they also may be responsible for differences in the β -pleated sheet-forming ability of the β A4 peptide at low concentrations [32].

The results presented here provide further evidence for our previous suggestion that amino acid oxidation by metal-catalyzed oxidation systems is required to initiate β A4 aggregation in vitro and in Alzheimer's disease. The aggregation of β A4 or APP-fragments induced by radical attack may therefore be the primary event that leads to amyloid formation. After generation of a core of cross-linked amyloidogenic fragments, further APP-fragments may extend the deposition without involvement of metal-catalyzed oxidation systems. Furthermore we provide important clues as to why humans

and animal species expressing the human β A4 sequence, but not rodents, are capable of forming amyloid fibrils in the brain.

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