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Unwinding fibril formation of medin, the peptide of the most common form of human amyloid

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Abstract

Medin amyloid affects the medial layer of the thoracic aorta of most people above 50 years of age. The consequences of this amyloid are not completely known but the deposits may contribute to diseases such as thoracic aortic aneurysm and dissection or to the general diminished elasticity of blood vessels seen in elderly people. We show that the 50-amino acid residue peptide medin forms amyloid-like fibrils *in vitro*. With the use of Congo red staining, Thioflavin T fluorescence, electron microscopy, and a solid-phase binding assay on different synthetic peptides, we identified the last 18–19 amino acid residues to constitute the amyloid-promoting region of medin. We also demonstrate that the two C-terminal phenylalanines, previously suggested to be of importance for amyloid formation, are not required for medin amyloid formation.

Keywords: Amyloid; Fibril formation; Lactadherin; Medin

Amyloid is composed of fibrillar peptide aggregates and some additional components such as serum amyloid P component, glycosaminoglycans, and apolipoprotein E. It is mainly found extracellularly and is involved in diseases like Alzheimer's disease and type II diabetes [1,2]. In the amyloid fibril the peptide monomers are hydrogen bonded to each other to form a very stable β-sheet structure, which accounts for the characteristics of amyloid including its fibrillar ultrastructure, X-ray diffraction pattern, and staining properties with Congo red and Thioflavin T. There is still no cure for amyloid diseases but among the emerging therapies one approach is focused on inhibiting fibril formation (for review see [3]). Studies on amyloid fibril formation are therefore of great importance for the development of new clinical treatments.

Medin amyloid is a localized amyloidosis mainly found in the media of the thoracic aortic wall [4,5]. It is the most prevalent amyloidosis known, being present in 97% of the population above 50 years of age [6]. The medin fragment is a 50 amino acid residues long internal cleavage product of the full-length protein lactadherin [4]. The effect of the deposition is not fully known. However, since the deposits usually are found in contact with the elastic laminae [6], medin amyloid may affect the elasticity of blood vessels. In a recent study we saw that aortas from patients suffering from thoracic aortic aneurysm and dissection contain higher levels of Congo red-negative medin than normal-dimensioned aortas. The results indicate that aneurysm and dissection specimens possibly contain more toxic medin oligomers (these are not stained by Congo red), which may be deleterious to the aortic wall and lead to

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these diseases (Peng *et al.*, unpublished results). Cell studies on medin and many other amyloid proteins show that oligomeric pre-amyloid aggregates, rather than mature amyloid, induce apoptosis of surrounding cells [7,8].

Hydrophobicity, charge attraction, β-sheet propensity, and aromatic residues are factors that have been shown to govern fibrillogenesis [9–13]. Hydrophobic residues with a high β-sheet propensity are frequently found in amyloid fibrils. Aggregation is inversely correlated to the net charge of a peptide [9] due to repellation. For instance, the 4-amino acid residue peptides KFFE and KVVE (both with a net charge of 0) form amyloid-like fibrils *in vitro*, however KFFK and EFFE, with a net charge of +2 and −2, respectively, do not [10]. Kallberg *et al.* showed that many of the amyloidogenic proteins contain discordant α-helix stretches, sequences that are α-helical in structure but should form β-strands according to secondary structure predictions [11]. Aromatic residues are found more frequently in amyloid-forming proteins and have been suggested to be involved in the self-assembly process of amyloid peptides into fibrils. The self-assembly is accomplished by attractive non-bonded interactions between the planar aromatic rings and is referred to as π-stacking [12]. However, recent data from Bemporad *et al.* indicate that the aromaticity *per se* is not a determinant for amyloid formation, rather the intrinsic hydrophobicity and β-sheet propensity of aromatic residues [13].

Until now no fibrillization study has been performed on the complete medin peptide *in vitro* and the aggregation prone region(s) has not been identified. We have earlier demonstrated that the octapeptide NFGSVQFV, in the C-terminus, is very amyloidogenic [4] and Gazit *et al.* have shown in a few studies that the phenylalanines are important for the fibrillization of that peptide [14,15]. In the present study, we show that the complete medin peptide forms amyloid-like fibrils *in vitro*, that the amyloidogenic part is located in the C-terminus, and that the C-terminal phenylalanines are not essential for fibrillization.

Materials and methods

Synthesized medin peptides. All peptides are shown in Table 1. $Medin_{1–50}$ was prepared by custom synthesis (Keck Biotechnology Resource Laboratory, Yale University, New Haven, CT). $Medin_{11–50}$, $medin_{31–50}$, and $medin_{1–12}$ were synthesized by Biotechnology Center of Oslo (University of Oslo, Norway). $Medin_{14–22}$, $medin_{16–24}$, $medin_{32–41}$, and $medin_{42–49}$ were synthesized at the Ludwig Institute for Cancer Research (Uppsala, Sweden). The purity of all these peptides was evaluated by reversed phase high performance liquid chromatography and the identity was confirmed with mass spectroscopy.

Recombinant medin peptides. The remaining peptides were recombinantly expressed in a bacterial system (Table 1). The gene encoding lactadherin was purchased from American Type Culture Collection (ATCC No. 84574, HIBAA24 construct) and PCR was carried out to amplify the medin sequence. The PCR product was subcloned into a pCR-Blunt II-TOPO vector followed by insertion into the pGEX-6P-3 expression vector (Amersham Biosciences, Uppsala, Sweden), containing an N-terminal glutathione S-transferase (GST) tag. $Medin_{1–25}$ was generated by inserting a stop codon after Asp in position 25 of medin.

Table 1
Peptides used for fibrillization experiments

Synthesized peptides	
$Medin_{1-50}$:	rldkqgnfnawvagsygndqwlqvdlgsskevtgiitqgarnfgsvqfva
$Medin_{1-12}$:	rldkqgnfnawv
$Medin_{11-50}$:	wvagsygndqwlqvdlgsskevtgiitqgarnfgsvqfva
$Medin_{14-22}$:	gsygndqwl
$Medin_{16-24}$:	ygndqwlqv
$Medin_{31-50}$:	evtgiitqgarnfgsvqfva
$Medin_{32-41}$:	vtgiitqgar
$Medin_{42-49}$:	nfgsvqfv
Medin-aa_{42-50}:	n**a**gsvq**a**va
Recombinant peptides	
$Medin_{1-50}$:	gprldkqgnfnawvagsygndqwlqvdlgsskevtgiitqgarnfgsvqfva
Medin-aa_{1-50}:	gprldkqgnfnawvagsygndqwlqvdlgsskevtgiitqgarn**a**gsvq**a**va
$Medin_{1-25}$:	gprldkqgnfnawvagsygndqwlqvd

In two of the peptides (medin-$aa_{42–50}$ and medin-$aa_{1–50}$) two phenylalanine residues (positions 43 and 48, in bold) were exchanged for alanines.

The GST–medin fusion proteins were expressed in the *E. coli* BL21-Gold strain (Stratagene, La Jolla, CA). Cells were harvested and cell pellet lysed in lysis buffer (50 μl/ml cell culture) (phosphate-buffered saline (PBS), pH 7.4, supplemented with lysozyme (1 mg/ml, Sigma, St. Louis, MO), DNAse I (10 μg/ml, Sigma), and Complete™ Protease Inhibitor Cocktail (1 tablet/50 ml, Roche Diagnostics GmbH, Mannheim, Germany)). Cell lysates were clarified by centrifugation at 48,000*g* for 30 min at 4 °C and passed through a 0.45 μm filter before being applied onto a GSTrap FF column (Amersham Biosciences), equilibrated in PBS. The column was washed with PBS and elution of GST–medin was carried out with 10 mM reduced glutathione.

To get non-GST tagged medin peptides, removal of the GST moiety was performed by PreScission protease (Amersham Biosciences) cleavage while the GST–medin still was bound to the column. Before elution of non-tagged medin peptide, a second GSTrap FF column was connected downstream to the first column, to allow binding of any free GST moiety and/or PreScission protease during the elution.

Peptide aggregation. In order to remove preformed aggregates the peptides were dissolved in a mixture of equal volumes of trifluoroacetic acid (TFA) and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP). The peptide solution was centrifuged at 16,000*g* and the supernatant was filtered through a 0.2 μm mesh filter to remove any residual aggregates and lyophilized. The dried peptides were dissolved in dimethyl sulfoxide (DMSO) and then diluted 1:10 with water to a concentration of 0.25–0.5 mM. The peptides were incubated for up to 3 months at room temperature. To avoid bacterial growth 0.02% sodium azide was added to the peptide solutions. The aggregation of the different peptides was studied with Congo red staining, transmission electron microscopy, and a Thioflavin T assay.

Congo red staining. Droplets (0.8 μl) of the peptide solutions were placed twice on the same spot on a glass slide. When dry, the slides were put in Congo B solution [16] for 20 min, twice in absolute ethanol for 10 s, twice in xylene for 2 min and then mounted.

Thioflavin T assay. The aggregation process was followed by removing aliquots of the 0.25–0.5 mM peptide solutions and mixing with 1% of 10 mM Thioflavin T solution (Sigma), which resulted in a final peptide concentration of 10 μM in a 96-well plate (100 μl/well). The fluorescence was determined by excitation at 440 nm and emission at 486 nm on a