The potential stem-forming sequence consists of the polymerization in Pmel17

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Polymerization accelerated by Pmel17, advanced within the mildly acidic conditions of melanosomes, plays a vital role during pigment deposition. The polymers closely resemble amyloid fibrils, associated with amyloidosis. Concerning the formation of the amyloidogenic cross-β structure, the initial mechanism in the conversion to a β-structure is critically important. To explore the core regions forming a stem of the amyloid, we prepared a series of fragment peptides of the C-terminal part of the repeat domain (RPT, residues 315–444) and examined their ability to produce amyloids. Sequence alignment of the peptides bearing the ability to form amyloid structures revealed that β-consisting of 405VSIVVLSGTTAAQVTT420 are the core regions responsible for initiating the formation of cross-β structures and for further ordered aggregation.

Keywords Pmel17, amyloid fibrils, melanosome, pigment deposition

Introduction

Amyloid fibrils, which are caused by abnormal conformation and misassembly of proteins, are responsible for several conformational diseases, such as prion disease, Alzheimer’s disease, and dialysis amyloidosis. Although the lengths of the amino acid sequences vary in amyloid-forming proteins, amyloid fibrils are typically long, unbranched, and 10 nm in diameter. Amyloid fibrils can be identified by their unique ability to bind to Congo red dye and thioflavin T. To date, several structural models of amyloid fibrils have been proposed for different amyloid-forming proteins.

Most experimental data have supported that amyloid fibrils are structured according to the cross-β structure model, which involves the orientation of β-strands containing disease-associated mutations perpendicular to the fibril axes in regions forming cross-β structures [1, 2].

Pmel17, which is a transmembrane protein, has also been proteolytically processed to generate polymers, similar to amyloid-like fibrils, in melanosomes [3–5]. Pmel17, which is the main component of these fibrils, comprises 668 residues and is divided into nine individually folded domains.

Melanosomes mature through four morphological stages, assigned based on fibril formation, and melanin synthesis. Stages I and II lack pigment, but the formation of intraluminal proteinaceous fibrils is initiated in stage I and completed in stage II. Mature fibrils, which are 6–10 nm in diameter, form parallel arrays that span the length of the organelle. When the fibrous striations are fully formed in ellipsoidal melanosomes in stage II, melanin synthesis begins and melanins deposit on the fibrils, resulting in their thickening and blackening with maturation in stage III [6]. In stage IV, the organelle is completely enriched with melanin. In epidermal melanocytes, stage IV melanosomes are translocated along microtubules from the cell center to actin-rich dendritic tips, and then transferred to neighbouring keratinocytes [7]. In these stages of the process, it is known that the pH of the solution changes with melanosome maturation. Stages I and II are the most acidic, whereas in stages III and IV near neutral pH is reached [8, 9]. Interestingly, tyrosinase activity is also pH dependent, accompanying melanosome maturation under acidic conditions [10, 11]. Notably, Candace M et al. demonstrated that RPT fibril morphology can be transformed directly by weak acidic conditions [12]. Consequently, the formation of melanosome fibrils is essential for pigment deposition and synthesis.

Previously, Nikolaos N. Louros et al. had demonstrated that the 405VSIVVLSGTT413 peptide fragment possibly has an essential role in RPT domain fibrillogenesis [13]. Moreover, our previous method [14], which predicted the propensity for amyloidogenicity, strongly indicated that residues
including 405–413 of Pmel17 are related to the stem-forming region. Namely, it appears that one region constituted of one β-strand is relevant to the formation of amyloid-like fibrils. However, it seems difficult to form aggregates by the interaction with only one β-strand in each peptide because of their high structural flexibility. On the other hand, a 2-stranded β-structure is presumed to be more stable than a single strand as a fibril forming intermediate (Fig. 1). Therefore, in the present work, we focused on the stem-forming region of aggregates corresponding to the length of a 2-stranded β-structure. To identify the stem-forming region essential for amyloid formation of Pmel17, a series of peptides covering the sequence of RPT domain, including 405–413, were prepared.

Materials and Methods

**Peptide synthesis**

The peptides were prepared by solid-phase peptide synthesis using the Fmoc strategy, as previously described [15]. In each synthetic cycle, the terminal Fmoc group was removed by a DMF solution containing 1,8-diazabicyclo[5.4.0]-7-undecene, piperidine, and 1-hydroxybenzotriazole (1.1%, 7.7%, and 2.3%, respectively). Both the protecting groups and the resin were removed by shaking the peptidyl resin in trifluoroacetic acid in the presence of 5% triisoprolylsilane and 3% water for 1.5 h at room temperature. RP-HPLC purified the synthetic peptides.

**Fibril formation**

As mentioned in the introduction, RPT fibril morphology can be directly transformed in weak acidic conditions. Therefore, in a plastic microtube the lyophilized peptides were dissolved in 50 mM sodium acetate buffer (pH 6.0) to become 0.2 mM. The solutions were incubated for 7 d under static conditions at 4°C, and spectroscopic measurements were carried out with an aliquot of the solution.

**Thioflavin T assay**

The thioflavin T (ThT) assay was used for the detection of fibril formation by measuring the ThT fluorescence enhancement that occurs in the presence of fibrils. Synthetic peptides were prepared by adding 20 μL of incubated peptide solution to 2 mL of aqueous ThT. The formation of amyloid fibrils was monitored through fluorescence enhancement of fibril-bound ThT in 50 mM sodium acetate buffer (pH 6.0) with excitation at 450 nm, as previously described [15]. Fluorescence enhancement of ThT in the amyloid-bound state, ΔF, was defined as ΔF = (F_S – F_o)/F_o × 100, where F_s and F_o denote the fluorescence intensity of a sample and that of a control solution without peptides, respectively.

**Circular dichroism spectroscopy**

The circular dichroism (CD) spectrum was recorded in the far-UV region (200–260 nm) at 20°C with a JASCO J-725 spectropolarimeter using a quartz cuvette with a 1.0 mm path length. The spectral data were recorded in terms of mean residue ellipticity, [θ], in degrees centimeter per decimole.

**Scanning electron microscopy**

Scanning electron microscopy (SEM) imaging was performed using JSM-700F Field Emission Scanning Electron Microscope (JEOL). The peptide solution was deposited on the surface of freshly cleaved mica. The surface of the sample was observed using an acceleration voltage of 5.0 kV.

**Results and Discussion**

The stem-forming regions with cross-β-structures in amyloids were explored using synthetic peptides containing the parts of the native sequence that semiexhaustively covers the region of Pmel17 (396-430), including 405-413. The sequence regions and the compositions of the peptides are indicated in Fig. 2.

The results of the ThT binding assay to examine amyloid forming properties are shown in Fig. 3. Based on a previous report [16], systems showing a fluorescence intensity, F, of >100% were determined to be participating in a significant amount of amyloid formation.

In the peptides including 405-413, the peptides pm396-420, pm396-425, pm401-425, pm401-430, pm402-422, pm403-420, pm404-420 and pm405-420 exhibited significant amyloid-forming properties. On the other hand, although pm401-415 and pm403-419 included 405-413, they exhibited rather low amyloidogenicities. This may be attributed to the fact that these peptides were formed amyloid by...
However, a peripheral sequence outside of 405-413, Thr414-Thr420, influences amyloid formation, since pm405-420 exhibits significant amyloid-forming properties. These results support the structural stability of amyloid fibrils, which depends on the existence of the peripheral sequence near the core cross-β region as previously reported [16–17].

Furthermore, Val405-truncated pm406-420, has low amyloidogenicities, probably because Val405 plays a vital role in amyloidogenicity (Fig. 3). These data unambiguously

![Amino acid sequences of the synthetic peptides. All peptides were coded as “Pm” with a sequential number.](image)

![The results of a ThT binding assay to detect amyloidogenicity. The values of ΔF as a measure of amyloidogenicity are shown with bar graphs, of which the color is given in a two-step manner switched at 100% in order to zoom in lower values.](image)
demonstrate that the essential part for fibril formation is the region 405-420. To obtain structural information related to amyloid formation, CD measurement of pm405-420 was carried out as shown in Fig. 4. The amyloid forming peptide, pm405-420, resulted in a CD spectral pattern with negative bands at 220-230 nm, which are specific to the cross-β structure [16]. On the other hand, the peptides pm401-415, pm403-419 and pm406-420, with no amyloidogenicity as revealed by ThT binding gave weak signals with the intensity almost within -500 deg cm$^2$ dmol$^{-1}$ in the wide range 200–260 nm, which are amorphous aggregates (data not shown).

Amyloid fibrils were observed by electron microscopy to assess peptides with pm405-420 showing enhanced fluorescence. The observed fibrils resembled typical amyloid fibrils, as shown in Fig. 5.

The results are summarized as follows. The sequence of VSIVLSGTTAAQVTT (residues 405-420) is most likely to form an intramolecular β-sheet structure. This sequence would be the stem-forming region for full-length Pmel17 proteins as a framework of amyloids. Applying the β-sheet model established for some fibril-forming peptides to pm405-420 fibrils, it would be difficult to construct a cross-β structure with 16-residue β-strands if each strand is straight. Since the naturally abundant β-strands are generally less than ten residues long, the most probable model for pm405-420 is likely to include a turn structure. Based on the statistical data for frequency of β-turns, the region around Gly412 is principal candidate to contain a turn structure [18]. Thus, our results demonstrate the region 405-420 has the potential to form the stem formation. Moreover, Val405 and Thr420 could contribute definitively to the joining up of potential stem-forming regions.

Thus, the hydrophobic core region 405-420 plays a major role in forming amyloids. On the other hand, it had been reported that amyloid fiber formation occurs under acidic condition in Pmel17 [10–12]. This suggests that a native Pmel17, not polymer, may be prevented from forming amyloids by a charge balance unfavorable to amyloids. Since the stem forming region is composed mainly of non-hydrophilic residues, hydrophilic residues are likely to exist in peripheral sequences, outside of the stem forming regions.

**Conclusions**

Based on the experimental identification of the core of amyloids being stem-forming regions, it is proposed that amyloids formed from Pmel17 involve an intramolecular β-sheet derived from potential stem-forming regions by local transformation. The identification of stem-forming regions should help to protect the organism from amyloid toxicity. Furthermore, our results provide insights into the folding mechanism associated with amyloid formation for various amyloidogenic proteins. The identification of the stem-forming region should also help significantly in the elucidation of relevance to other isoforms.

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