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Biochemical and Biophysical Research Communications 342 (2006) 808–816
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Peripheral region for core cross-β plays important role in amyloidogenicity ☆

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Received 31 January 2006
Available online 14 February 2006

Abstract

The role of the peripheral sequence neighboring the core cross-β region was investigated using a peptide library constructed with all possible combinations of Lys, Glu, Ser, and Leu at three residue positions (X1–X3) forming the N-terminal region linked to the amyloid core sequence of the barnase-derived segment (A4–K22). By means of CD spectra and thioflavin T binding assay for 64 peptides, not only the composition but also the sequence in the peripheral region were found to be responsible for amyloid formation. The preferences of amino acid residues in the peripheral region of the amyloid-forming peptides were in the order of Leu ≈ Ser ⩾ Glu ≫ Lys. A balance of positive and negative charges was found to be essential for amyloid formation, suggesting that the electrostatic interaction at the surface of the amyloid fibrils is relevant to their stability. On the basis of the maximum fluorescence wavelength of fibril-bound thioflavin T, the highly amyloidogenic peptides were classified into two classes, which exhibited the sequence preferences of (Leu, Ser/Glu, and Leu) and (Glu, Leu, and Ser) for the peripheral sequence (X1, X2, and X3). The former class can be rationally assigned to the structural model with deep grooves along the fibril axis. Thus, the peripheral sequence regulates the manner of molecular packing in the fibrils as well as the amyloidogenicity. In addition, the chains of the peripheral sequence are most likely to form thioflavin T binding sites.

Keywords: Amyloid; Prion; Peripheral; Core cross-β; Thioflavin T; Binding site; Peptide library

Amyloid fibrils of various proteins are constructed through the consecutive formation of hydrogen bonds along the fibril axes. Most experimental data have suggested a 'cross-β structure' model as a common structural backbone [1,2]. In this model, β-strands run perpendicular to the fibril axes. Since the amyloids are recognized as relevant to or responsible for so-called conformational diseases, such as Alzheimer's and/or Creutzfeldt–Jakob diseases [3], structural studies of them are very important in terms of therapy and clinical analysis [4,5]. Undoubtedly, amyloid fibrils have highly regular structures organized across all the stages of the structural hierarchy. This can be recognized with the extreme sensitivity of amyloid formation for even point mutations, as well as the well-ordered physical appearance of amyloids. Recently, we have succeeded in identifying the essential interactions for amyloid formation. Among them, the most essential one is the hydrophobic interaction between amino acid residues effectively operating in inter-strand, inter-molecular, and inter-sheet manners [6]. On the basis of these interactions, and considering the symmetry of amyloids, the most rational total-structure model of amyloids was proposed there [6]. Our model well describes not only molecular packing but also the hierarchical formation of amyloid fibrils.

Thus, the hydrophobic core cross-β region plays a major role in forming the amyloids. On the other hand, it is reported that the charged residues are also relevant to the amyloidogenicity [7,8]. In fact, the prion protein shows

☆ *Abbreviations:* ThT, thioflavin T; CD, circular dichroism.

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doi:10.1016/j.bbrc.2006.02.012

more than a few disease-associated mutations at hydrophilic residues. As for the prion protein, although several structural models have been proposed using experimental data and computer simulation [9–12], the high-resolution structure of aberrantly folded isoforms, such as that of the scrapie prion, has not been clarified yet. It is notable that a metastable conformer of hamster prion involves a locally disordered region derived from helices B and C ranging (173–228) [13]. This region is in good accord with the core of amyloid structure revealed for *Podospora anserina* HET-s prion [14]. In the corresponding region of human prion, the mutations, D178N, V180I, T183A, H187R, T188R, E196K, F198S, E200K, D202N, R208H, V210I, E211Q, Q212P, and Q217R, are known to be disease-associated [15]. The frequency of mutations for hydrophilic residues is found to be 9/17 (53%) in the region (178–217). In contrast, that of hydrophobic ones is only 5/23 (22%). It is obvious that most of these causative mutations occurred for hydrophilic residues. Since the core cross-β region would be composed mainly of non-hydrophilic residues, as revealed by our original model of amyloid structure, most hydrophilic residues are likely to exist in peripheral regions near the core cross-β sequences. This implies that the hydrophilic residues in peripheral regions are relevant to the regulation of amyloid formation.

Therefore, in the present work we tried to shed light on the role of peripheral sequences in amyloid formation. Instead of using the prion protein, we employed a well-characterized sequence of barnase module I for this purpose [6,16]. Using a peptide library technique, we elucidated position-specific roles of some kinds of amino acid residues locating at the peripheral regions in detail.

Materials and methods

Peptide synthesis. The peptide XXXAQ VINTF DGVAD YLQTY HK, which is named here 'Plib', was derived from barnase module I [16] and designed to be coupled with N-terminal library region (X1–X3). The peptides were synthesized in a manner similar to that described previously [6,17]. The C-termini of the peptides were free carboxyl groups prepared with 2-chlorotrityl chloride resin (Nova Biochem). The peptide chain (A4–H21) was automatically synthesized stepwise with a PSSM8 peptide synthesizer (Shimadzu), and the three subsequent residues (X1–X3) were elongated manually in each of 64 different reaction vessels, where four kinds of amino acids—Lys, Glu, Ser, and Leu—were systematically incorporated at every position of the library sequence to provide $4 \times 4 \times 4$ kinds of peptide sequences. These are named Plib-XXX using a sequence of three residues at N-terminal region. The peptides were purified by reversed-phase HPLC as usual, and then identified by electro-spray ionization mass spectroscopy by using an LCMS-QP8000α (Shimadzu).

Incubation for fibril formation. The peptide solutions, each at a concentration of 0.2 mM (calculated with the extinction coefficient 1450 M^{-1} cm^{-1} at 274.5 nm for tyrosine), were incubated for 14 days at 4 °C. The buffer systems were 50 mM sodium phosphate-citrate (pH 4.5 and pH 5.5), 50 mM sodium phosphate (pH 6.5), 50 mM Tris chloride (pH 7.5), and 50 mM sodium borate-chloride (pH 8.5).

ThT binding assay. The formation of amyloid fibrils was examined by fluorescence enhancement of fibril-bound ThT in 10 mM sodium phosphate buffer (pH 7.0) using the excitation light at 450 nm [18]. The protocols are the same as described previously [6]. The obtained fluorescence intensities were expressed as relative values in which a 'unit' was based on control data without peptides.

Electron microscopy and circular dichroism. Electron microscopic observation and CD spectroscopic measurements were carried out in manners similar to those used previously [6].

Results and discussion

Fluorescent thioflavin T assay for peptide library

The fluorescence intensities of ThT in the presence of the Plib peptides bearing a common core cross-β region (A4–T19) and a library sequence (X1–X3) are shown in Fig. 1. The formation of amyloids, which was reflected by the enhancement of ThT fluorescence, depended remarkably on the three residues of the N-terminal region, each arranged with the combination of Lys, Glu, Ser, and Leu. This suggests that even the peripheral region, i.e., (X1–X3), neighboring the intrinsic core cross-β region, is responsible for the regulation of amyloid formation. Though a group of Plib peptides with a common amino acid composition gave dissimilar fluorescence intensities of ThT, the amyloidogenic properties are consistent to some extent in all members of the group. This means that the composition of the three residues from the N-terminus is a primary factor destining a peptide to either form amyloids or not.

Several peptides, such as Plib-SLS and Plib-KLS, gave marked fluorescence intensities of ThT irrespective of the different pH conditions. Thus, the correlation between the fluorescence intensities obtained after incubation at two different pH implies some sequence-specific contribution not only to amyloid formation but also to ThT fluorescence.

Classification and characterization of amyloids

The CD spectra of the Plib peptides on the 14th day of incubation at pH 6.5 are shown in Fig. 2. The spectral patterns of the samples could be classified mainly into two categories. One exhibited a spectrum with a deep trough at 197 nm, of which the negative molar ellipticity is more intensive than $-10{,}000$ deg cm^2 $dmol^{-1}$. The other gave a spectrum with a trough around 220 nm and a peak around 200 nm. The former indicates that the sample peptides have random coil structures. On the other hand, the latter is likely to be assigned to the cross-β structure of amyloids, since the negative band at 220 nm is close to the 215 nm observed for the β-sheet structure of general globular proteins. Most of the CD spectra in the second category gave very similar spectral shapes, suggesting a unique higher-order structure, probably due to amyloid formation with a common core cross-β region (A4–T19). These amyloid-forming peptides mostly showed the fluorescence intensities of ThT larger than 8 units. Therefore, we categorize these peptides with high-amyloidogenicity as Class I.

In addition, to discuss the peptides with weak but not negligible amyloidogenicity, we have defined the second