Colloids and Surfaces B: Biointerfaces 149 (2017) 72–79

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Amyloid formation characteristics of GNNQQNY from yeast prion protein Sup35 and its seeding with heterogeneous polypeptides

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ARTICLE INFO

Article history:
Received 7 July 2016
Received in revised form 3 September 2016
Accepted 6 October 2016
Available online 7 October 2016

Keywords:
Prion
Yeast
Amyloid
Aggregation
β-Sheet
Thioflavin T
Seeding

ABSTRACT

Sup35 is a prion-like protein from yeast and shares the ability to transmit its aberrant fold and to aggregate into amyloid fibrils. ^{7}GNNQQNY^{13} from the prion-determining domain of Sup35 was reported to form an amyloid. We first investigated the self-aggregation transition behavior of GNNQQNY to the β-sheet amyloid state under various conditions. Mechanical stirring using a magnetic bar resulted in accelerated aggregation of the GNNQQNY. The aggregation rate of GNNQQNY was also dependent on its concentration; the higher the GNNQQNY concentration, the faster the aggregation. Circular dichroism and Fourier transform-infrared spectral data indicated the formation of the β-sheet structure in the GNNQQNY aggregates. The fluorescence experiments using an amyloid-specific thioflavin T also demonstrated that the GNNQQNY aggregates formed the amyloid structures. The amyloid structure of the GNNQQNY aggregates served as seeds for the elongation of the monomeric GNNQQNY in the solution state. We further studied the ability of the GNNQQNY amyloid fibrils to act as seeds for the elongation of the amyloid-forming monomeric proteins (albumin, lysozyme and insulin). The cross-seeding experiments suggested that the GNNQQNY aggregate could possibly promote the amyloid fibril formation of heterogeneous insulin. The inverse monomeric GNNQQNY would have a binding capacity for the heterogeneous already-formed amyloid-β fibrils on a mice brain section. These basic data could be informative for elucidating the pathogenic and/or propagation mechanisms of prion agents and developing effective therapeutics and/or diagnosis for prion diseases.

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1. Introduction

Amyloid-related diseases are characterized by the misfolding of proteins into amyloid fibrils and subsequent deposition of amyloid aggregates in the body. More than 30 amyloid-related diseases have been described, among which are Alzheimer's disease, Parkinson's disease, prion diseases and type 2 diabetes mellitus [1–3]. Each disease is associated with a particular protein, and aggregates of the proteins are thought to be the direct or indirect origin of the pathological conditions associated with the disease. In some cases, the quantity of material involved is enormous with several kilograms of protein being deposited in certain manifestations of systemic amyloidosis [4–7]. The aggregated forms of the proteins have many characteristics in common, although all of which have a unique native folding [8]. A protein-based fibril is identified as an amyloid by its structural and tinctorial properties; amyloid fibrils are unbranched, and bind the dye Congo red. The fibrils also produce a characteristic cross-β X-ray diffraction pattern, consistent with a model in which stacked β-sheets form parallel to the fiber axis having their individual β-strands perpendicular to the fiber axis [9]. However, the process of amyloid formation and its structural details are still unknown [10,11].

Prion diseases are lethal neurodegenerative disorders that occur in humans as well as in animals [4], which involve Creutzfeldt-Jakob disease (CJD) in humans, scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, etc. [12]. A prion is a proteinaceous particle that resists inactivation by procedures that modify nucleic acids [13]. Thus, the protein structure is distinctively passed at the protein, and not at the nucleic acid level. Fundamental to the diseases is the conversion of a normally folded, globular, mainly

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http://dx.doi.org/10.1016/j.colsurfb.2016.10.011
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α-helical protein (PrP^C, 'C' for 'cellular') into a misfolded, insoluble, largely β-strand rich, pathological form (PrP^{Sc}, 'Sc' for 'scrapie') that can aggregate and accumulate in the brain [14]. Another distinguishing feature of the prion diseases is only infectious in the amyloid-related diseases. In the 1980s, an epidemic of BSE in England that killed over 140,000 cows seems to have been caused by feed containing spinal cords and brains of cattle and/or sheep that may have died of a related disease such as scrapie. In 1996, the variant form of CJD was finally spread to humans through the consumption of meat products from BSE-infected cattle [15,16].

PrP^C is a membrane-associated protein occurring in a wide range of eukaryotic cells. The physiological functions of PrP^C are still unknown, although the broad distribution among mammalian species and the high conservation of PrP^C implies a role of general importance. During the propagation and onset of prion diseases, irreversible structural conversion of endogenous PrP^C in the native conformation into PrP^{Sc} in an aberrant 'killer' conformation is thought to occur and then aggregate into an infectious form with the amyloid structure [4,17]. PrP^C and PrP^{Sc} are conformationally isomeric forms of each other; the PrP^C to PrP^{Sc} transition involves conversion of the α-helix to β-sheet in large parts of the protein. The killer conformer and its aggregates are thought to be the disease-causing agents in the prion diseases. The decisive process i.e., the irreversible conversion of PrP^C into PrP^{Sc}, initiates an 'autocatalytic' reaction which leads to the amyloid accumulation in the central nervous system [18]. Actually, inhibitors of pathological amyloid fibril formation could be useful in the development of therapeutics against prions. Currently, the molecular details of the structure transmission from PrP^{Sc} to PrP^C are still hardly known.

Sup35 is a prion-like protein from yeast and shares the ability to transmit its aberrant folding and to aggregate into amyloid fibrils. Its normal cellular function is to terminate translation [19–22]. ^{7}Gly-Asn-Asn-Gln-Gln-Asn-Tyr^{13} (GNNQQNY) from the prion-determining domain (residues 1–123) of Sup35 was reported to form an amyloid [1]. This heptapeptide segment can form closely related microcrystals, from which Nelson et al. have determined the atomic structure of the cross-β spine [23,24]. GNNQQNY aggregates could be used as a model of amyloid fibrils in order to obtain basic information for elucidation of the propagation mechanism and the development of therapeutic and preventive agents. In this study, we investigated the self-aggregation transition behavior of GNNQQNY to the β-sheet amyloid state under various conditions, and the ability of GNNQQNY fibrils to serve as seeds for the elongation of several amyloid-forming monomeric proteins.

2. Materials and methods

2.1. Materials

Heptapeptide (H-Gly-Asn-Asn-Gln-Gln-Asn-Tyr-OH, GNNQQNY) was obtained from Takara Bio, Inc. (Tokyo, Japan) (Fig. S1). Fluorescamine (FS), human serum albumin (HSA), lysozyme from chicken egg white (LYS) and insulin from bovine pancreas (INS) were purchased from Sigma Co., Ltd. (St. Louis, MO). Thioflavin T (ThT) was from Nacalai Tesque, Inc. (Kyoto, Japan). Water used throughout this study was generated using a Milli-Q Biocel system (Millipore Corp., Billerica, MA). All other chemicals were of commercial reagent grade and used as received. Brain sections from *Tg2576* transgenic mice were prepared according to a previously reported procedure [25]. Fluorescently labeled GNNQQNY was prepared as briefly described; FS (0.67 mg/mL) and GNNQQNY (1 mg/mL) were mixed in acetonitrile and then stirred with a magnetic bar at room temperature for 15 min. The resulting mixture was subjected to reverse-phase liquid chromatography to isolate the FS-labeled GNNQQNY (Fig. S2).

2.2. Measurements of turbidity and free peptide concentration

After gentle pipetting several times, an aliquot of the sample solution was transferred into a 1-cm path quartz cuvette. The turbidity produced by the aggregation was monitored at 400 nm by a V-660 UV–vis spectrophotometer (Jasco Corp., Tokyo, Japan). The peptide solution was treated using an L-80 centrifuge with a SW-40Ti rotor (Beckman Coulter, Inc., Brea, CA) at 10,000 min^{-1} and 25 °C for 10 min, and then the absorbance of the supernatant was monitored at 276 nm. The free peptide concentration was expressed in% with the initial GNNQQNY concentration (0.33 mg mL^{-1}) being defined as 100%.

2.3. Circular dichroism

After the sample solutions were appropriately diluted with the buffer solution, circular dichroism spectra were measured by a J725 (Jasco Corp., Tokyo, Japan) using a 1-cm path quartz cuvette from 190 to 400 nm at ambient temperature.

2.4. Fourier transform-infrared spectroscopy

The aggregate suspensions were ultrafiltered through an Ultrafree-MC (molecular weight cutoff: 10,000, Millipore Corp.) at 5000 *g* for desalting. The filtrate (≈10 μL) was placed on a Zn-Se prism of PRO410-S attenuated total reflectance (ATR, Jasco Corp.), and allowed to dry at ambient temperature. The Fourier transform infrared spectra were measured by the ATR method using an FT/IR 4200 spectrometer (Jasco Corp.).

2.5. Measurement of ThT fluorescence spectra

After the addition of 1 mM ThT dissolved in Milli-Q water, and gentle pipetting several times, an aliquot of the sample solution was transferred into a 1-cm path quartz cuvette, then the fluorescence spectra were measured by a FP-6600 spectrofluorometer (Jasco Corp.) using an excitation wavelength of 450 nm.

2.6. Microscopic observations

The peptide aggregates were collected by centrifugation of the samples at 4000 min^{-1} for 15 min. The obtained precipitates (2.5 μL) were mixed with an equal volume of ThT solution (40 μM), and washed with water by the centrifugation. Fluorescence images were acquired by a BZ-8100 fluorescence microscope (Keyence Corp., Tokyo, Japan). For the electron microscopy, the specimens were put on a polycarbonate membrane in a SEM Pore (pore size 0.6 μm, Jeol Ltd., Tokyo, Japan), then washed with Milli-Q water to remove the inorganic salts. After drying at ambient temperature for 1 day, the specimens were subjected to a gold sputter coating for 1 min. Electron micrograms were acquired using a JSM-7500F scanning microscope equipped with a field emission gun (Jeol Ltd., Tokyo, Japan).

2.7. GNNQQNY aggregation experiments

GNNQQNY was dispersed in 0.01 M phosphate buffer (pH 7.4). To completely solubilize the peptide, the resulting solutions were sonicated for ≈10 min and subsequently left in a water-bath at 65 °C (final GNNQQNY concentration: 0.082–1.0 mg mL^{-1}). The GNNQQNY solutions were pass through a DISMIC-25CS cellulose acetate membrane filter (pore size: 0.2 μm). The obtained clear fresh solution was transferred to a 10-mL Erlenmeyer flask and vigorously stirred by a 1.5-cm length magnetic stirrer bar (≈300 rpm) on a SR-506 (Advantec, Tokyo, Japan) or gently in a water bath PERSONAL-11 (Taitec, Tokyo, Japan) at 37 °C and 60 stroke min^{-1}.

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