Biochimica et Biophysica Acta 1854 (2015) 218–228

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Role of monomer arrangement in the amyloid self-assembly

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ARTICLE INFO

Article history:
Received 19 June 2014
Received in revised form 24 November 2014
Accepted 10 December 2014
Available online 24 December 2014

Keywords:
Protein aggregation
Amyloids
AFM
Nanoimaging
Force spectroscopy
Neurodegenerative diseases

ABSTRACT

Assembly of amyloid proteins into aggregates requires the ordering of the monomers in oligomers and especially in such highly organized structures as fibrils. This ordering is accompanied by structural transitions leading to the formation of ordered β-structural motifs in proteins and peptides lacking secondary structures. To characterize the effect of the monomer arrangements on the aggregation process at various stages, we performed comparative studies of the yeast prion protein Sup35 heptapeptide (GNNQQNY) along with its dimeric form CGNNQQNY-(d-Pro)-G-GNNQQNY. The (d-Pro)-G linker in this construct is capable of adopting a β-turn, facilitating the assembly of the dimer into the dimeric antiparallel hairpin structure (AP-hairpin). We applied Atomic Force Microscopy (AFM) techniques to follow peptide–peptide interactions at the single molecule level, to visualize the morphology of aggregates formed by both constructs, thioflavin T (ThT) fluorescence to follow the aggregation kinetics, and circular dichroism (CD) spectroscopy to characterize the secondary structure of the constructs. The ThT fluorescence data showed that the AP-hairpin aggregation kinetics is insensitive to the external environment such as ionic strength and pH contrary to the monomers the kinetics of which depends dramatically on the ionic strength and pH. The AFM topographic imaging revealed that AP-hairpins primarily assemble into globular aggregates, whereas linear fibrils are primary assemblies of the monomers suggesting that both constructs follow different aggregation pathways during the self-assembly. These morphological differences are in line with the AFM force spectroscopy experiments and CD spectroscopy measurements, suggesting that the AP-hairpin is structurally rigid regardless of changes of environmental factors.

1. Introduction

Yeast Prion Protein, Sup35p, has been used as a model for the prion disease phenomenon, particularly for understanding of the structural aspects of such diseases [1,2]. Sup35p misfolding and aggregation mimic those associated with mammalian prion diseases [1,3]. Therefore, the understanding of the transient states of the misfolded Sup35p will pave the way for development of efficient diagnostics and remedies for the disease [4,5]. The N-terminal domain of the protein plays an important role in the aggregation of the entire Sup35 prion. More specifically, a seven amino acid sequence that spans from residues 7 to 13, GNNQQNY, has a significant involvement in aggregation of the whole protein [1,2,6–12]. In fact, the addition of this peptide seeded aggregation of the whole protein, dramatically accelerating the aggregation process.

The solid state NMR structural studies emerged to suggest strongly the origin of polymorphic variation in amyloid fibrils [11]. Differences in the packing of these β-sheets, originating from differences in the side-chain packing, register or topology of β-sheets, may explain morphological variants of fibrillar structure. Solid-state NMR studies clearly showed the coexistence of three distinct conformations of the GNNQQNY peptide within a single fibril. Different packing arrangements of peptides within a fibril were proposed to be responsible for the observed differences in NMR chemical shifts. These coexisting conformations differ by the degree of the local secondary structure

Abbreviations: AFM, Atomic Force Microscopy; AP-hairpin, CGNNQQNY-(d-pro)G-GNNQQNY; APS, aminopropyl silatrane; CD, circular dichroism; DFS, dynamic force spectroscopy; DMSO, dimethylsulfoxide; HFIP, 1,1,1,3,3,3-hexafluoro-propan-2-ol; k_{off}, off-rate constant; MD, molecular dynamics; NHS-PEG-MAL, N-hydroxysuccinimide-polyethylene glycol-maleimide; TCEP, tris(2-carboxyethyl)phosphine; ThT, thioflavin T; TMAO, trimethylamine-*N*-oxide; WLC, wormlike chain approximation; x_β, distance of the ground state to the transition state.

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http://dx.doi.org/10.1016/j.bbapap.2014.12.009
1570-9639/

(α-helical versus β-sheet) [11]. This structural variability is in line with the crystallographic studies where eight classes of so-called steric zippers have been identified [13]. Such variation involves mainly orientation of peptides and β-sheets with respect to each other.

The structure of the peptides and their arrangements within assemblies can depend on the aggregate size. In crystals, the Sup35 heptapeptide forms a steric zipper arrangement between two β-sheets with the parallel orientation of monomers within the sheets [3]. However, the monomer arrangement can be different if the aggregates are small and interact with the environment. This assumption is supported by a recent publication [14], in which molecular dynamics (MD) simulation of the Sup35 octapeptide was performed. The authors showed that the single β-sheet structure taken from the crystallographic structure depends on a number of factors including the aggregate size. The experiment revealed that an aggregate can disintegrate into smaller-sized oligomers or the edge peptides can dissociate sequentially. The authors also assumed that a heterogeneous mixture of oligomers of different sizes exist prior to the formation of the critical nucleus. The pH and ionic strength of the surrounding solution can also play a role which was confirmed by our recent AFM imaging and force spectroscopy studies [15].

These data suggest that assembly of monomeric unit in the aggregates and their secondary structure can define the aggregate morphology. To test this hypothesis, we performed comparative studies of heptapeptide Sup35 peptide (monomer) and its covalent dimeric form in which two monomers are covalently attached to each other in the tail-to-head orientation via the (d-Pro)G dipeptide. The latter according to NMR and circular dichroism (CD) spectroscopy studies [16] has the β-turn structure forcing the entire dimer to adopt antiparallel geometry (AP-hairpin). We show here that although both constructs are capable of self-assembly into amyloid aggregates, the pre-arrangement of the monomers into dimers changes dramatically the AP-hairpin conformational properties limiting the aggregation propensities of the AP-hairpin compared to the monomer.

2. Materials and methods

2.1. Materials

The peptides $NH3^+$-Cys-Gly-Asn-Asn-Gln-Gln-Asn-Tyr-$COOH^-$ (CGNNQQNY, Monomer) and the hairpin peptide $NH3^+$-Cys-Gly-Asn-Asn-Gln-Gln-Asn-Tyr-DPro-Gly-Gly-Asn-Asn-Gln-Gln-Asn-Tyr-$COOH^-$ (CGNNQQNY(d-Pro)G-GNNQQNY, AP dimer) were synthesized by Peptide 2.0, Inc. (Chantilly, VA). Synthesized peptides were purified by VYDAC-C18 reverse-phase HPLC, and their molecular weight was confirmed by MALDI-TOF mass spectrometry.

The aminopropyl silatrane (APS) was used at a concentration of 167 μM for 30 min for mica surface functionalization as described in ref. [17]. The 1.67 mM stock solution of NHS-PEG-MAL (N-hydroxysuccinimide-polyethylene glycol-maleimide, MW = 3400 g/mol), purchased from Laysan Bio, Inc. (Arab, AL), was prepared in DMSO (Sigma-Aldrich Inc., St. Louis, MO) and stored at −20 °C. The 10 mM Tris(2-carboxyethyl)phosphine (TCEP) hydrochloride (Hampton Research Inc.) was prepared in water, and was added to peptide solution and incubated for 10 min prior to deposition on the PEGylated substrates. TCEP was useful for reducing any disulfide bonds that may have formed between two peptides to make the thiol group available to the substrate.

2.2. Methods

2.2.1. Aggregation studies using ThT fluorescence

The extent of peptide aggregation was followed by characteristic changes in thioflavin T (ThT) fluorescence. First, the lyophilized peptide powder was dissolved in either water or buffer in an initial volume of 100 μL. The initial concentration was measured using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE), by detecting the absorbance of the tyrosine residue at 274 nm, and using an extinction coefficient of 1405 $L \cdot mol^{-1} \cdot cm^{-1}$ for tyrosine. Then, the peptide solution was diluted down to 500 μM, using either water or a buffer as a solvent [1,2]. For the ThT fluorescence assay, 2 μL aliquots from the samples were withdrawn periodically and added to 590 μL of 5 μM ThT solution. Fluorescence intensity was measured on a Cary Eclipse spectrofluorimeter (Varian Inc. Palo Alto, CA) at 485 nm while exciting at 450 nm. Each reported value is an average of 15 values of fluorescence intensity after subtracting out the fluorescence contribution from free ThT.

The initial fluorescence readings were taken immediately after the solution was prepared. Stirring the solution at intervals was done in order to accelerate the aggregation process. Magnetic stir bars were placed into the peptide solutions, and the samples were stirred on a magnetic stirrer (Hanna Instruments, Smithfield, RI) for 20 min once every 2 h. The ThT data were fitted with the following sigmoidal equation:

$$y = T_0 + \frac{T_f - T_0}{1 + 10^{(\log_{10}(T_{1/2}) - T) * S_{max}}}, \quad (1)$$

with T_0 being a time of 0 h, T_f being the final time, $T_{1/2}$ the half-time of aggregation, T the time point being measured, and S_{max} being the maximum fluorescence attained in the data set.

The working buffer solutions were prepared at four different pHs: pH 2.0 (HCl/KCl), pH 3.7 (sodium acetate/acetic acid), pH 5.6 (sodium acetate/acetic acid), and pH 7 (HEPES). A fifth buffer was used occasionally at pH 9.8 (sodium carbonate/sodium bicarbonate). The ionic strength was adjusted by adding NaCl.

2.2.2. AFM topographic imaging

At the plateau levels of ThT fluorescence, 2 μL of the 500 μM aggregation mixture was deposited on the mica and allowed to sit for 2 min, followed by the addition of 8 μL of distilled water, which was then allowed to sit for 2 min. The samples were then dried by 2 min of spin coating at 2000 rpm using a Model WS-400BZ-6NPP/LITE spin coater (Laurell Technologies Corporation, North Wales, PA).

Images were acquired in air using a MultiMode SPM NanoScope V Multimode 8 system (Bruker Nano, Santa Barbara, CA) operating in peak force mode. Silicon nitride (Si_3N_4) AFM probe tips (MSNL — Bruker, Santa Barbara, CA, USA) with nominal spring constants of 0.6 N/m were used for peak force imaging. Image analysis was done using Femtoscan Online (Advanced Technologies Center, Moscow, Russia). The aggregates in were analyzed manually by counting elongated fibrils and globular oligomers after subtracting anything in the background that was less than 1 nm in height.

2.2.3. AFM force spectroscopy

Freshly cleaved mica (Asheville-Schoonmaker Mica Co., Newport News, VA, USA) surfaces were treated with amino-propyl-silatrane (APS) for 30 min according to previously reported protocol [17] followed by rinsing with water and drying with argon gas flow. The APS modified mica surfaces were treated with 167 μM NHS-PEG-MAL in DMSO for 3 h followed by rinsing with DMSO to remove non-bound NHS-PEG-MAL, rinsing with water, and drying with argon gas flow. Maleimide-functionalized mica was incubated for 1 h in 190 nM of peptide solution (HEPES buffer, pH 7.0). Prior to immobilization of peptide, the peptide solution was treated with 0.25 mM TCEP hydrochloride for 10 min in pH 7.0 buffer to reduce any disulfide bonds. After washing with HEPES (pH 7.0, 10 mM HEPES, 50 mM NaCl) buffer, unreacted maleimide was quenched with 10 mM β-mercaptoethanol for 10 min followed by rinsing with HEPES pH 7.0 buffer.

Silicon nitride (Si_3N_4) AFM probe tips (MLCT — Bruker, Santa Barbara, CA) were washed in ethanol by immersion for 30 min and then activated by UV treatment for 30 min. The activated probe tips were treated with APS for 30 min. The APS modified probe tips were then treated with 167 μM NHS-PEG-MAL for 3 h followed by rinsing with DMSO, and thorough rinsing with double distilled water.