Biochimica et Biophysica Acta 1854 (2015) 249–257

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Structural characterization of amyloid fibrils from the human parathyroid hormone

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ARTICLE INFO

Article history:
Received 16 October 2014
Received in revised form 2 December 2014
Accepted 21 December 2014
Available online 30 December 2014

Keywords:
Human parathyroid hormone
Amyloid fibrils
EGCG
Electron microscopy
NMR spectroscopy
MALDI-TOF mass spectrometry

ABSTRACT

Amyloid deposits are common in various tissues as a consequence of misfolded proteins. However, secretory protein and peptides are often stored in membrane coated granules as functional amyloids. In this article, we present a detailed characterization of *in vitro* generated amyloid fibrils from human parathyroid hormone (hPTH(1–84)). Fully mature fibrils could be obtained after a short lag phase within less than one hour at 65 °C. These fibrils showed all characteristic of a cross-β structure. Protease cleavage combined with mass spectrometry identified the central region of the peptide hormone involved in the fibril core formation. EGCG, an inhibitor of amyloid fibril formation, showed binding to residues in the peptide monomers corresponding to the later fibril core and thus explaining the inhibition of the fibril growth. Conformational and dynamic studies by solid-state NMR further corroborated the cross-β core of the fibrils, but also identified highly mobile segments with a random coil structure not belonging to the rigid fibril core.

1. Introduction

Failure of proteins to reach a stable native structure may result in the population of misfolded or aggregated species [1–3]. Amyloid deposits are common in particular polypeptide producing tissues and typically represent a characteristic feature of diseases affecting these tissues including Alzheimer's or Parkinson's disease and type II diabetes [4]. On the other hand, because of their optimized packing properties, amyloids are found in nature for storage of proteins and peptides. Such amyloids have a non-pathological biological function and are called 'functional amyloids'. These are present in *Escherichia coli* [5], silkworms [6], fungi [7], and mammalian skin [8]. Some functional amyloids of fungal prions are involved in prion replication, and the amyloid protein Pmel17 is involved in mammalian skin pigmentation. Formation of functional amyloids is also common to the secretory proteins such as hormones [9]. Hormones and secretory proteins stored in membrane coated secretory granules over long time periods at very high concentration before release into the blood stream occurs. Often, this high concentration leads to self-association of the peptide chains. It has been reported that peptide and hormones are found in the pituitary secretory granules of the endocrine system in the form of functional amyloids [9]. Few hormones including insulin and glucagon tend to form amyloid fibrils under certain *in vitro* conditions [10]. The basic architecture of self-assembled amyloid fibrils is the cross-β structure [11]. The cross-β motif is composed of intermolecular β-sheets arranged along the fibril axis with the β-strands aligned perpendicularly to the axis of fibrils. The amyloid-like cross-β-rich conformation was also observed for functional amyloids in secretory granules of the endocrine system [9].

Human parathyroid hormone is secreted from the parathyroid glands if the Ca^{2+} level in the blood drops or the blood phosphate concentration increases [12]. Human parathyroid hormone is translated as a 115 residues comprising pre-pro protein. The 25 N-terminal residues (pre sequence) are required as signal for efficient transport to the endoplasmic reticulum [13,14]. This signal sequence is rapidly [15] cleaved off by a signal peptidase and the resulting pro-hPTH peptide subsequently transferred to the Golgi apparatus [16]. The 6 residues of the pro sequence at the N-terminus are proteolytically removed [17] and the mature hPTH(1–84) is packaged and stored in secretory granules until the release into the blood. It has been reported that peptide and hormones found in the pituitary secretory granules of the endocrine system are stored in the form of functional amyloids [9]. Few hormones including insulin [10], glucagon [10], and PTH [18] tend to form amyloid

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http://dx.doi.org/10.1016/j.bbapap.2014.12.020
1570-9639/

fibrils under certain *in vivo* and *in vitro* conditions [9]. Often, mutations result in the accumulation of a conformationally defective protein in the ER which contributes to diseases such as Alzheimer's, Parkinson's, Huntington's, or type 1 diabetes [19–21]. C18R mutation in the pre sequence of pre-pro-PTH disrupts the hydrophobic core of the signal sequence leading to intracellular accumulation and causes the familial isolated hypoparathyroidism (FIH) [22].

In the present study, we test the hypothesis whether hPTH(1–84) undergoes fibril formation under *in vitro* conditions. Indeed, we could explore in detail amyloid fibril formation of hPTH(1–84) and elucidate its structural properties. The fibrils are curvilinear, long structures and exhibited the characteristic of repeating cross-β motif. Primarily, the N-terminal residues 25R–37L form the cross-β core structure of the fibrils. Most of the diseases related to the secreted proteins are caused by accumulation of misfolded proteins and the progressive degeneration of the associated tissues. An amyloid-like structure of peptide and protein hormones could explain most of their properties.

2. Material and methods

2.1. Protein expression and purification

Human PTH(1–84) was purified by recombinant expression following the previously reported procedure [23,24]. In brief, pET SUMO adapt vector containing hPTH(1–84) with C-terminal His-tags was transformed in *E. coli* BL21codon + cells. Isotope labeling was achieved in M9 media with ^{15}N NH_4Cl as nitrogen source. Ni-NTA purified samples were mixed with SUMO protease (1:100 ratio) 50–150 μg/ml. Cleaved products were further purified by S-75 gel filtration chromatography. Aβ peptides were recombinantly expressed and purified according to reported procedure [25].

2.2. Thioflavin T (ThT) kinetic and hPTH(1–84) fibril preparation

Fibril formation of hPTH(1–84) was achieved by dissolving recombinant purified hPTH(1–84) in 50 mM borate buffer, pH 9.0 at a concentration 10 mg/ml and followed by incubation at 65 °C. ThT kinetics was performed in borate buffer and individual aliquots were prepared and incubated. The reaction was stopped by dilution with addition of pre-cooled buffer at 4 °C. The kinetics was followed at 0–3 h of incubation time. These samples were finally diluted to 35 μM of hPTH(1–84) and ThT was added in equal ratio. ThT fluorescence was monitored by excitation wavelength of 450 nm. For the EM analysis the hPTH(1–84) samples were incubated at least for 1 h.

2.3. Aβ fibril formation

Aβ fibrils were grown in 50 mM HEPES buffer at pH 7 by dissolving 50 μM of the peptide followed by incubation at 37 °C [26].

2.4. Attenuated total reflection–Fourier-transform infrared (ATR-FTIR) spectrometry

ATR-FTIR spectra for hPTH(1–84), hPTH(1–84) fibrils and Aβ(1–40) fibrils were recorded with a Tensor 27 FTIR spectrometer (Bruker, Germany) equipped with a BIOATR II cell and a MCT detector that was cooled with liquid nitrogen. 15 μl of the samples (protein concentration: 5 mg/ml) was placed onto the crystal of the ATR cell and measured at room temperature. Collected spectra represent averages of 64 scans at 4 cm^{-1} resolution.

2.5. Monomer release assay

Amyloid fibrils were grown as mentioned above at 918 μM of hPTH(1–84) monomers, pH 9.0. Amyloid fibrils were separated by centrifugation at 30,000 rpm, 4 °C for 30 min. These fibrils were mixed with the buffer at pH 7.0 in 20 mM Na_2HPO_4 and 25 mM NaCl. Subsequently, this sample was incubated at 37 °C for 24 h and supernatant was collected after centrifugation at 30,000 rpm, 4 °C for 30 min. Additionally, quick release of monomer was tested by mixing the buffer followed by immediate separation of solution and fibrils by centrifugation. The sample volume was kept constant throughout the experiment. The concentration of released hPTH(1–84) in the supernatant was monitored by absorption spectroscopy at 280 nm.

2.6. Mass spectrometry and fibril core identification

The fibrils were prepared as mentioned in the previous section. These fibrils were mixed with chymotrypsin in the 1:100, enzyme to fibril (*w*/*w*) [27] ratio in 25 mM Tris–Cl, 50 mM NaCl at pH 7.8. Tosyllysine chloromethyl ketone hydrochloride (TLCK) treated MS grade chymotrypsin was used. The mixture was incubated at 37 °C and samples were taken at different time points. The fibrils were dissociated into monomers adding hexafluoro-2-propanol in 1:1 ratio by volume. Samples were analyzed by MALDI-TOF or ESI-MS-MS. For the mass spectrometry, samples were desalted by Pierce C18 Tips prior to analysis.

2.7. Transmission electron microscopy (TEM)

The samples were diluted to 50 μM and a 5 μl droplet of hPTH(1–84) fibril samples was pipetted onto a formvar carbon-coated copper grid (Ted Pella Inc.) and washed three times with 50 μl of water drops. The water was carefully removed with filter paper between washes. Grids were then stained with 50 μl of 2% (*w*/*v*) uranyl acetate which was then removed and air dried. Specimens were examined using Zeiss 900 transmission electron microscope. The microscope was operated at an acceleration voltage of 80 kV.

2.8. Inhibition of fibril growth and solution NMR spectroscopy

The inhibition of hPTH(1–84) fibril growth was monitored by electron microscopy using 3000–30,000 magnification. For the EM analysis, 10 mg/ml of hPTH(1–84) was dissolved with and without EGCG in 50 mM borated buffer to the molar ratio of 1:2.4 and 1:10 (protein: EGCG). Inhibition was examined by microscopy at different time points from 1 to 48 h. Fluorescence titration was carried in the same buffer, while intrinsic fluorescence of Trp23 was utilized for binding analysis. The sample was excited at 280 nm with an addition of 40 μM of EGCG at each step. For the solution NMR analysis, 50 μM of ^{15}N-hPTH(1–84) was titrated with EGCG to the molar ratio of 1:1 and 1:5, respectively, and monitored by 2D ^{1}H-^{15}N HSQC experiments. The NMR titration was carried out in 10 mM BisTris, 300 mM Na_2SO_4, 0.02% NaN_3, pH 5.3 and at 25 °C.

2.9. Solid-state NMR spectroscopy

hPTH(1–84) fibril solutions were ultracentrifuged at 86,000 ×*g* for 2 h at 4 °C. The pellets were lyophilized, rehydrated to 50 wt.% H_2O, homogenized by freezing the sample in liquid nitrogen and thawing it at 37 °C and centrifuged into 4 mm MAS rotors. MAS NMR spectra were acquired on a Bruker Avance III 600 MHz NMR spectrometer (BrukerBioSpin GmbH, Rheinstetten, Germany) at a resonance frequency of 600.1 MHz for ^{1}H, 150.9 MHz for ^{13}C and 60.8 MHz for ^{15}N. A double channel 4 mm MAS probe was used in all NMR experiments and the temperature was set to 30 °C. NMR spectra were acquired using direct polarization, INEPT or CP excitation schemes. The typical ^{1}H and ^{13}C 90° pulse length were 4 μs. For ^{13}C CP MAS experiments the contact times were 20 μs, 100 μs, 400 μs, 700 μs and 1500 μs and for ^{15}N CP MAS experiments contact times were 100 μs, 500 μs, 1000 μs and 2000 μs with a relaxation delay of 2.5 s and a MAS frequency of 7 kHz. ^{1}H dipolar decoupling during acquisition with an rf amplitude of 65 Hz was applied using Spinal64. Two dimensional ^{13}C-^{13}C DARR