Biochimica et Biophysica Acta 1865 (2017) 652–663

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Very rapid amyloid fibril formation by a bacterial lipase in the absence of a detectable lag phase

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ARTICLE INFO

Article history:
Received 23 December 2016
Received in revised form 3 March 2017
Accepted 14 March 2017
Available online 16 March 2017

Keywords:
Alzheimer's disease
Amyloidosis
Protein aggregation
Protein misfolding
Rapid aggregation

ABSTRACT

The conversion of proteins from their soluble states into well-organized amyloid fibrils has received abundant attention. This process typically consists of three stages: lag, growth and plateau phases. In this study, the process of amyloid fibril formation by lipase from *Pseudomonas* sp. after diluting out urea was examined by Thioflavin T (ThT) fluorescence, Congo red (CR) binding, 8-anilinonaphthalene-1-sulfonic acid (ANS) binding, dynamic light scattering (DLS), circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopies, X-ray diffraction (XRD) and transmission electron microscopy (TEM). To exclude the presence of preformed aggregates in the pure lipase sample, aforementioned assays were also performed for the protein unfolded in urea before dilution. The aggregates formed immediately after dilution were found to bind to ThT and CR and contain a significant amount of β-sheet structure, as determined by far-UV CD and FTIR spectroscopies, as well as XRD analysis. Moreover, these aggregates present, at least in part, a fibrillar morphology, as deduced with TEM. This examination showed that lipase fibril formation proceeds quickly after dilution, within a few seconds, without a detectable lag phase. We also investigated bacterial inclusion bodies formed after expression of lipase in *E. coli*, providing evidence for the existence of rapidly formed amyloid-like structural and tinctorial properties in the lipase-containing inclusion bodies.

1. Introduction

The conversion of proteins from their soluble states into well-organized aggregates known as amyloid fibrils has received a great deal of attention. The pathology of a great number of human diseases is associated with amyloid fibrils [1]. Moreover, protein aggregation is a challenge in biotechnology and pharmacy, since many of the newly designed proteins and peptides tend to aggregate into amyloid-like assemblies [2]. On the other hand, this phenomenon is occurring naturally in some proteins that have functional properties, so they are called as functional amyloids [1,2]. Amyloid fibrils also have a great potential application in material science because of their heterogeneous and peculiar characteristics [3].

The numerous studies on the kinetics of amyloid fibril formation conducted so far have revealed three distinct phases, including a *lag phase*, an *exponential phase* (also called *elongation* or *growth phase*) and an *equilibrium phase* (also called *plateau* or *saturation phase*) [4–9]. As a consequence, a sigmoidal curve will appear after tracing amyloid development with time [8,10]. In the lag phase soluble monomeric species associate to form the nuclei; alternatively monomeric species aggregate rapidly to form unstructured aggregates that later convert into nuclei [8]. In the past few years, however, the lag phase has been shown to be a process in which a few fibrils may elongate rapidly from the nuclei and may present, even at the early stages, a significant population of fibrils [11]. A vast number of intrinsic and extrinsic factors such as mutations, pH, temperature, salts, co-solvents, surfaces, polymers, small molecules and agitation influence the length of the lag phase [10–14].

The formation of highly organized amyloid fibrils is a generic property of proteins, since under certain conditions many proteins unrelated to diseases have been shown to assemble to form amyloid-like fibrils [15] [16]. One remarkable example is lipase from *Pseudomonas* sp.

Abbreviations: ANS, 8-anilinonaphthalene-1-sulfonic acid; CD, circular dichroism; CR, Congo red; DTT, dithiothreitol; DLS, dynamic light scattering; FTIR, Fourier transform infrared; HEWL, hen egg white lysozyme; IBs, inclusion bodies; IGEPAL® CA-630, octylphenoxypoly(ethyleneoxy)ethanol branched; IPTG, isopropyl-β-D-1-thiogalactopyranoside; LB, Luria Broth; PK, proteinase K; PMSF, phenylmethylsulfonyl fluoride; TEM, transmission electron microscopy; ThT, Thioflavin T; XRD, X-ray diffraction.

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http://dx.doi.org/10.1016/j.bbapap.2017.03.004
1570-9639/

[17]. This protein can be readily purified to high yields and is stable in a buffer containing 8.0 M urea at pH 8.0 [18], facilitating any experimental study of amyloid fibril formation by this protein following removal of the denaturant [17]. In fact, the conversion of this protein into amyloid fibrils was found to occur from a sample containing urea and pure lipase, after removal of urea by dialysis down to a solution condition close to physiological, namely in the absence of denaturants and also in the absence of agitation [17].

Nevertheless, our previous investigation based on slow dialysis did not allow the detection of the early aggregated species of lipase [17]. In the current study we investigated the kinetics of amyloid fibril formation following a dilution method, where the formation of the early aggregates could be monitored at the start of the process, following urea removal. The results showed that dilution of the unfolded protein to a solution condition containing a small concentration of the denaturant at neutral pH leads to a very rapid aggregate production that could not be retarded by changing the dilution conditions. The aggregates of lipase were characterized with a number of biophysical methods indicating that amyloid fibril formation by this protein is a very rapid, instantaneous process, with a significant number of fibrils present immediately after dilution, with an extremely rapid lag phase that remains undetectable on our time scale and with a rapidity that is independent of the time of incubation under the various conditions tested.

We will also show that the rapid amyloid aggregation process of lipase from *Pseudomonas* sp. occurs *in vivo*, after expression of the protein in *E. coli*, where the protein is localized in inclusion bodies (IBs) and adopts amyloid-like structural and tinctorial characteristics, in agreement with other studies that have provided compelling evidence for the existence of highly ordered, amyloid-like structures in bacterial IBs [19–26].

2. Materials and methods

2.1. Protein expression and purification

E. coli BL-21 (DE3) cells previously transformed with the pET-28a vector containing the gene coding for lipase from *Pseudomonas* sp. were cultivated in Luria Broth (LB) medium under vigorous shaking at 37 °C with 50 μg/ml kanamycin. When OD_{600} reached a value of 0.6, protein expression was induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). After 5 h of induction at 30 °C, bacterial cells were separated from the culture medium with centrifugation at 2800*g* for 20 min. Then, bacterial cells were resuspended in lysis buffer containing 50 mM Tris-HCl, 300 mM NaCl, pH 8.0. Cell disruption was carried out with sonication in ice by ten cycles of 30 s bursts at a frequency of 50 Hz with 30 s cooling intervals. Inclusion bodies (IBs) were collected with centrifugation at 9450*g* for 20 min at 4 °C. IBs were solubilized in Tris-HCl buffer, pH 8.0, containing 8.0 M urea and 1 mM dithiothreitol (DTT) and were then injected to a Nickel Agarose purification column, pre-equilibrated at room temperature with 50 mM Tris-HCl, pH 8.0, containing 8.0 M urea and 300 mM NaCl. The bound protein was washed with 50 mM Tris-HCl, pH 8.0, containing 8.0 M urea and 5 mM imidazole, and then eluted with the same buffer containing 250 mM imidazole. Purity of the eluted fractions was checked with SDS-PAGE, using the Laemmli method [27]. Protein concentration was determined by UV absorption using an ε_{280} value of 27,515 M^{-1} cm^{-1}.

2.2. Thioflavin T (ThT) assay

The lipase stock solution (4 mg/ml lipase in Tris-HCl buffer, pH 8.0, containing 8.0 M urea) was centrifuged for 5 min at 14300 *g* and then filtered with 0.22 μm cut-off filter. It was 10-fold diluted in either Tris-HCl buffer, pH 8.0, containing 8.0 M urea or Tris-HCl buffer, pH 8.0 without urea. Aliquots of the lipase samples (60 μl) were withdrawn at regular time intervals and mixed with 440 μl of 25 mM phosphate buffer, pH 6.0, containing 25 μM ThT, 25 °C with or without 8.0 M urea, depending on whether or not urea was present in the lipase sample. The fluorescence of the resulting samples was measured at 25 °C using a 2 × 10-mm path length cuvette and a PerkinElmer Life Sciences LS-55 fluorimeter (Waltham, MA, USA) equipped with a thermostated cell compartment attached to a Haake F8 water bath (Karlsruhe, Germany). The excitation wavelength was 440 nm and the emission ranged from 470 to 520 nm. The plots of ThT fluorescence at 485 nm versus time were fitted to a single exponential function of the form:

$$F(t) = F_{eq} + A \exp(-k\,t) \quad (1)$$

where F(t) is the ThT fluorescence at time t, F_{eq} is the maximum ThT fluorescence obtained at the end of the observed exponential phase, A is the amplitude of the fluorescence change, and k is the apparent rate constant.

In another experimental set, the duly centrifuged and filtered lipase stock solution was 10-fold diluted in Tris-HCl buffers to reach final pH values of 8.5, 9.0, 9.5, 10.0 (in 0.8 M urea) or final urea concentrations of 1.2 M, 1.6 M, 2.0 M and 2.4 M (at pH 8.5). The ThT assay was then performed as described above.

2.3. Dynamic light scattering (DLS) analysis

The lipase stock solution (4 mg/ml lipase in Tris-HCl buffer, pH 8.0, containing 8.0 M urea) was filtered with a 20 nm syringe filter (Whatman, Maidstone, UK). The concentration of the protein stock solution was determined after filtration. It was then diluted as described in the previous section using buffers filtered with a 0.22 μm filter (Sarstedt, Nümbrecht, Germany). The DLS measurements were obtained using the Malvern Zetasizer Nano S instrument (Malvern, Worcestershire, UK) setting the appropriate viscosity and refractive index parameters for each solution. Disposable polystyrene cuvettes having a 1-cm path length were used. Every sample was measured three times and the average distributions were reported. The average and the standard deviation values of the sizes corresponding to the peak of interest in each of these three distributions represent the apparent hydrodynamic diameter and the experimental error for each sample, respectively.

2.4. 8-Anilinonaphthalene-1-sulfonic acid (ANS) fluorescence

Protein samples were prepared as described in the ThT fluorescence assay subsection. 60 μl of the protein samples at time 0 min and 1500 min after dilution were added to 440 μl of 50 mM Tris-HCl buffer, pH 8.5, containing 55 μM ANS, 25 °C with or without 8.0 M urea, depending on whether or not urea was present in the lipase sample. The ANS solution was prepared fresh for all experiments. ANS concentration was determined spectrophotometrically using an ε_{375} value of 8000 M^{-1} cm^{-1}. The excitation wavelength was 380 nm and the emission was recorded from 400 to 600 nm. Fluorescence spectra were acquired at 25 °C, using the same cell and equipment described above. ANS fluorescence spectra were also acquired under corresponding conditions in the absence of protein (blank). The ratio between the total area (from 400 to 600 nm) of the spectrum acquired in the presence of lipase (F) and that of the spectrum acquired in the absence of the protein (F_0) was calculated in each case.

2.5. Congo red (CR) binding assay

Protein samples were prepared as described in the ThT fluorescence assay subsection. 60 μl of the protein samples at time 0 min and 1500 min after dilution were mixed with 440 μl of 20 μM CR in 5 mM phosphate buffer, 150 mM NaCl, pH 7.4, 25 °C, with or without 8.0 M urea, depending on whether or not urea was present in the lipase sample. CR absorption spectra were recorded in the 400–700 nm wavelength range using a Jasco V-630 spectrophotometer (Tokyo, Japan)