Biochemical and Biophysical Research Communications 450 (2014) 1339–1344

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

A comparative study on the aggregating effects of guanidine thiocyanate, guanidine hydrochloride and urea on lysozyme aggregation

Saeed Emadi *, Maliheh Behzadi

Department of Biological Sciences, Institute in Advanced Studies in Basic Sciences (IASBS), Zanjan, Iran

ARTICLE INFO

Article history:
Received 18 June 2014
Available online 5 July 2014

Keywords:
Lysozyme
Urea
Guanidine hydrochloride
Guanidine thiocyanate
Aggregation
Fibrillation

ABSTRACT

Protein aggregation and its subsequent deposition in different tissues culminate in a diverse range of diseases collectively known as amyloidoses. Aggregation of hen or human lysozyme depends on certain conditions, namely acidic pH or the presence of additives. In the present study, the effects on the aggregation of hen egg-white lysozyme via incubation in concentrated solutions of three different chaotropic agents namely guanidine thiocyanate, guanidine hydrochloride and urea were investigated. Here we used three different methods for the detection of the aggregates, thioflavin T fluorescence, circular dichroism spectroscopy and atomic force microscopy. Our results showed that upon incubation with different concentrations (0.5, 1.0, 2.0, 3.0, 4.0, 5.0 M) of the chemical denaturants, lysozyme was aggregated at low concentrations of guanidine thiocyanate (1.0 and 2.0 M) and at high concentrations of guanidine hydrochloride (4 and 5 M), although no fibril formation was detected. In the case of urea, no aggregation was observed at any concentration.

1. Introduction

Deposits of insoluble protein fibrils with cross β-sheet structure are the molecular hallmark of an increasing number of human disorders, including Alzheimer's disease, Parkinson's diseases and type II diabetes [1]. Protein aggregates can be generally considered as insoluble misfolded proteins that have lost their native forms and functions [2]. Aggregation of proteins is a nuisance in the biopharmaceutical applications where it can interfere with the production and characterization of therapeutic proteins/peptides [2,3].

Amyloid fibrils are known to possess specific structural and physicochemical features in common: presence of β-sheet rich secondary structure, fibrillar morphology, birefringence upon staining with Congo red dye and insolubility in most solvents [4].

Formation of amyloid is often explained by a nucleation-dependent kinetic mechanism. Once a critical nucleus is formed, the protein in question rapidly aggregates into amyloid fibrils and plaques. Massive fibril deposits can interfere with normal organ function. Amyloid lesions can induce inflammatory responses. Mature amyloid fibrils have also been found to induce cell death. However, other studies have shown amyloid fibrils to be relatively innocuous, leaving doubt about their impact [5].

In order to become aggregated and form fibrils, globular proteins are generally should become destabilized (e.g., by mutation, heat, high pressure, low pH, or chemical denaturants) [6].

Formation of amyloid fibrils by lysozyme has recently drawn the attention of many researchers. Effect of solution conditions such as denaturing compounds, e.g., SDS at alkaline conditions, has been studied recently by Moosavi-Movahedi et al. [7]. Lysozyme, whose concentration in mare's milk reaches hundreds of milligrams per liter, plays an important role in body defense from external bacterial infections [8]. Variant lysozyme was first discovered to be an amyloid fibril protein associated with hereditary systemic amyloidosis in 1993 [9].This disease is associated with point mutations in the lysozyme gene, causing the nonconservative substitutions Ile56Thr or Asp67His, and amyloid fibrils are widely deposited in a variety of tissues [10].

Hen Egg White Lysozyme (HEWL) is an archetypal protein which has been extensively used to understand the mechanism of protein folding, misfolding and amyloid formation [11]. HEWL is a 129-amino acid long enzyme which catalyzes the hydrolysis of the β-linkage between N-acetylmuramic acid and N-acetylglucosamine subunits in the peptidoglycan polymers of many bacterial cell walls and results in bacterial lysis and death. Its native form is cross-linked by four disulfide bridges and adopts mainly helical conformation (30% α-helix; 6% β-sheet) [2,12].

Wild-type human lysozyme was found to form amyloid fibrils that are similar to those extracted from pathological deposits when

* Corresponding author.
E-mail address: emadi@iasbs.ac.ir (S. Emadi).

http://dx.doi.org/10.1016/j.bbrc.2014.06.133
0006-291X/

incubated in vitro at pH 2.0 for several days. Krebs et al. obtained amyloid aggregates also from HEWL utilizing a variety of methods, such as incubation of the protein in acidic solution (pH 2.0–4.0) at elevated temperatures (37–65 °C) for several days [13].

Guanidine thiocyanate (GITC) is a powerful protein denaturant; both the guanidinium cation and thiocyanate anion are strong chaotropic agents [14]. Guanidine hydrochloride (GdnHCl) is also a strong denaturant which can coat the exterior of proteins. Above certain protein-specific concentrations, GdnHCl can fully denature a protein. While denatured, however, disulfide-containing proteins are never fully unfolded even by high levels of GdnHCl. A considerable degree of ordered structure remains due to the presence of disulfide bonds, which GdnHCl does not affect. Low levels of GdnHCl (<1 M) have been shown to assist in the correct refolding of lysozyme by inhibiting amorphous aggregation [15].

Urea has been widely used for protein folding or refolding studies [16]. Nielsen and co-workers showed that, as the concentration of urea was increased from 0 to 2.0 to 4.0 and to 6.0 M, lag times before growth decreased and growth rates increased. They concluded that urea promoted a population of partially folded species that decreased lag times and increased growth rates [15,17].

2. Materials and methods

2.1. Chemicals

Hen egg-white lysozyme was purchased from Sigma and used without further purification. Concentrations were determined spectrophotometrically using the extinction coefficient (at 280 nm) of 2.65 [L g^{-1} cm^{-1}] [18]. Guanidine hydrochloride, guanidine thiocyanate and urea were purchased from Sigma and Merck respectively. Thioflavin T (ThT) and all other reagents were also purchased from Sigma.

2.2. Fibril formation

A stock solution of lysozyme was prepared in 10 mM sodium phosphate (NaPhos), pH 7.0. Dilution with 7.0 M GITC, GdnHCl and urea in 10 mM NaPhos resulted in solutions of 2.0 mg/mL lysozyme with GITC, GdnHCl and urea concentrations ranging from 0, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 M. HEWL sample solutions (except sample without any denaturant that was incubated at 54 °C and pH 2.0 (control)) were incubated in shaker incubator with stirring at 150 rpm and 25 °C for 24 h to induce fibrillar species.

2.3. ThT fluorescence assay

Thirty five microliter of HEWL samples were mixed with 1965 μL of 13 μM ThT in phosphate-buffered saline (PBS). The increase in fluorescence was observed upon binding of amyloid fibrils to ThT which was employed to quantify fibril formation. ThT has been shown to selectively bind to ordered fibrillar aggregates without showing binding to native protein or amorphous aggregates. ThT fluorescence intensity measurements were performed by exciting samples at 440 nm and recording emission intensities at 480 nm using a Cary Eclips VARIAN fluorescence spectrophotometer. Slits were adjusted to 5 and 5 nm for both excitation and emission. The temperature was adjusted to 25 °C using a water bath heater. All measurements were done in triplicate.

2.4. Circular dichroism (CD)

CD spectra were measured using a Jasco 810 CD Spectrophotometer. Far-ultraviolet (UV) (190–260 nm) waves were used in 0.1 cm path length cell using a step size of 0.1 nm and band width of 1 nm. Each protein spectrum was corrected by subtracting the solvent spectrum. Spectra were recorded at 25 °C. For acquisition of spectra, the protein samples were diluted to 0.2 mg/mL just before the measurement. The results were plotted as ellipticity (deg. cm^2 $dmol^{-1}$) versus wavelength (nm).

2.5. Atomic force microscopy (AFM)

For AFM measurements, a 10 μL of each lysozyme sample was loaded onto a mica surface and allowed to bind to the mica by incubation for 30 min at room temperature. The mica surface was then rinsed one time with 100 μL deionized water and dried at room temperature. The AFM images were obtained using a Veeco AFM instrument operating in the noncontact mode. The diameters of aggregates were determined from the Z-heights in AFM images, using the profile option of WSXM.

3. Results and discussion

3.1. ThT fluorescence

The native monomeric form of HEWL (control) converts into insoluble amyloid-like aggregates upon incubation at pH 2.0 and 54 °C. To characterize the role of GITC, GdnHCl and urea in lysozyme fibril production, lysozyme was stirred and incubated at 25 °C in the presence of varying concentrations of these chaotropic agents, from 0.5 to 5.0 M (Fig. 1). At 3.0 M concentrations of GITC, low fluorescence intensity was observed, indicating minimal fibril formation. At 1.0 and 2.0 M GITC concentrations a dramatic increase in ThT fluorescence emission was obtained and the solution became visibly turbid. At 4.0 and 5.0 M GITC concentrations no signal was recorded at all (Fig. 1).

No signal was recorded at 0.5, 1.0, 2.0 and 3.0 M concentrations of GdnHCl but a dramatic increase in ThT fluorescence emission at 4.0 M GdnHCl and low ThT fluorescence intensity at 5.0 M GdnHCl can be seen in Fig. 1. No signal was observed in the presence of urea.

3.2. CD spectroscopy

Circular dichroism spectroscopy was used to analyze the secondary structures of lysozyme in solution. The CD spectra of lysozyme upon interaction in the absence and presence of GITC, GdnHCl and urea at above mentioned concentrations are shown in Fig. 2A–C respectively. The changes in the secondary structure of lysozyme upon addition of increasing concentrations of GITC, GdnHCl and urea can be observed as changes in shapes and intensities of the spectra.

The spectrum for 0.5 M GITC exhibited significant change at lysozyme structure. The changes in secondary structure of lysozyme continued upon addition of increasing concentrations of GITC. The CD spectra obtained from HEWL with 1.0 and 2.0 M concentrations of GITC were observed to possess a characteristic pattern of β-sheet-rich conformation. Our far-UV CD data suggested that the formation of β-sheet conformation and the HEWL fibrillation were strongly mitigated by the presence of 3.0, 4.0 and 5.0 M GITC. A plot of changes in intensity at 208 and 222 nm against the concentrations of GITC (Fig. 2D and E) shows that the intensity of these peaks dramatically decrease upon addition of 0.5 M GITC. This trend continued at 3.0 M GITC. Conversely, as shown in Fig. 2D and E intensity of these peaks at 208 and 222 nm exhibited an increase on the addition of GITC at 4.0 and 5.0 M that shows a large increase in helical content in this range of GITC (Table 1). Mason et al. [19] studied the hydration structure of guanidinium