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Hemin is able to disaggregate lysozyme amyloid fibrils into monomers

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ABSTRACT

Lysozyme amyloidosis (ALys) is a disease of the gastrointestinal tract, liver and kidneys, which is caused by the accumulation of insoluble fibrils of lysozyme in the tissues of above organs. The ALys can be cured by disintegration and clearance of the fibrils from the affected tissues and organs. It is thought that protein fibrils are extremely stable. Consequently, small molecule-induced dissociation of fibrils under physiological conditions is really challenging. Here, we report kinetic and thermodynamic analyses of hemin-induced dissociation of hen egg white lysozyme amyloid fibrils. We examined the effect of hemin on the kinetics of dissociation of lysozyme fibrils. We observed that the hemin binding dissociates fibrils in a concentration dependent manner within a reasonable time. Studies of structural, morphological properties and gel filtration chromatography indicate that fibrils dissociate mainly into monomeric species. The conformational, hydrodynamic, unfolding and stability studies of the resolubilized proteins show that dissociated monomers possess characteristics of partially folded intermediate state of the protein. We also find that hemin-induced fibril dissociation mainly depends on the kinetic and thermodynamic stability of the fibrils. These results suggest that non-toxic derivatives of hemin and other porphyrins could pave a way for therapeutic intervention in amyloidosis and related pathologies.

1. Introduction

Amyloid fibril is an insoluble and filamentous protein aggregate, which is composed of repeating units of β-strands oriented perpendicular to the fibril axis. The deposition of amyloid fibrils in the brain is a hallmark of many neurodegenerative diseases such as Parkinson's and Alzheimer's diseases [1–3]. Apart from neurodegenerative diseases, the deposition of fibrils in various organs and tissues is also a cause of several other human pathologies collectively known as non-neurogenic amyloidosis [2,4]. Several reports suggest that soluble oligomers of proteins/peptides, as opposed to fibrils are the main cytotoxic species and cause the onset of the disease [1,2,5]. However, in many amyloidosis such as renal, lungs, heart and pancreas, etc., the buildup of fibril deposits is the main cause of damage of organs and tissues of the patient [4]. The fibrils deposit in large amount cause displacement of normal tissue structures and damage the affected organs [4]. Amyloid fibrils are thermodynamically very stable and the disaggregation of fibril into the monomeric state is a challenging problem of biophysical-chemistry and biomedical science.

Since these diseases affect millions of people every year, hence, there is a compelling demand to find drugs for their treatment. The complete treatment will require both prevention of fibrillation and dissolution of fibrils into monomer or smaller non-toxic species. Dissolution into smaller species is needed to clear the amyloid deposits from various organs and tissues. There has been an enormous amount of research to inhibit fibril formation [6–9] but there is little research on dissolution of amyloid fibrils using small molecules. It has been reported that low dose curcumin effectively disaggregates Aβ [10] and curcumin pyrazole and its derivatives are known to disaggregate α-synuclein fibril [11]. Indole 3-acetic acid has been found to enhance the disaggregation of the previously formed HEWL amyloid fibrils [12]. 4′-Iodo-4′-deoxydoxorubicin and tetracyclines are known to convert transthyretin amyloid fibrils in vitro into non-cytotoxic species [13]. Recently, Zhu et al. showed that ruthenium polypyridyl complexes disaggregates human islet amyloid polypeptide fibril into less toxic nano particles [14]. However, the mechanism of small molecules induced dissociation of the fibrils into monomer is not understood.

Hemin is an iron-containing protoporphyrin IX formed by the oxidation of heme. Heme is vital for the functioning of many proteins involved in electron transport and redox reactions [15,16]. The role of hemin in the prevention of fibril formation and disaggregation of preformed fibril is scarcely reported. Iannuzzi et al. have shown that hemin binding inhibits the fibrillation of amyloidogenic apomyoglobin and the resulting non-fibrillar aggregate was found to be non-cytotoxic [17].

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Another study has previously reported that hemin inhibited A-beta fibrillation and reduced the cytotoxicity of aggregated A-beta [18]. Yanqin et al. proposed that hemin is a generic inhibitor of protein aggregation [19]. They showed that hemin prevented the fibrillation of Aβ42, α-synuclein and RCM-κ-casein and reduced the cell toxicity of Aβ42 fibrils. They further reported that hemin is a potent inhibitor of amorphous aggregation of ADH, catalase and γs-crystalline [19]. They also investigated the ability of hemin to break down preformed amyloid fibrils of Aβ42 peptide and showed that hemin degraded the partially formed Aβ42 fibrils. However, a mechanistic study on hemin-induced disaggregation of fibrils has not been reported yet. The goals of this work are to examine (i) the kinetics of hemin-induced disaggregation of mature fibril (ii) interaction of hemin with protein in the fibril and native conformations (iii) subunit status, conformation and stability of the disaggregated protein and (iv) the stability of mature fibril. The above systematic analyses give a mechanistic insight into the hemin-induced disaggregation process of amyloid-like fibrils.

In this work, we have examined the fibril disaggregation ability of hemin at pH 7.4 and 37 °C. We checked the disaggregation properties of hemin on two kinds of fibrils of hen egg white lysozyme (HEWL), guanidine hydrochloride-induced fibrils (GIF) and low pH-induced fibril (LPF). Hen egg white lysozyme is not only structurally homologous to human lysozyme [20], but its unfolding/refolding and fibrillation behavior is also fundamentally similar to human lysozyme [21]. Therefore, HEWL can be used as a model system to understand the aggregation/disaggregation of human lysozyme. From our studies, we found that hemin binding to lysozyme under a fibril-like conformation assisted the disaggregation of fibrils into monomers as well as thinner and shorter fibrils. We also observed that more stable fibril was more resistant to disaggregation against denaturant GuHCl.

2. Materials and methods

2.1. Materials

Hen egg white lysozyme (HEWL), guanidine hydrochloride (GuHCl), Thioflavin T (ThT), Congo red (CR) and hemin were purchased from Sigma Aldrich Co., USA. All other chemicals and reagents used in this study were of analytical grade with purity > 99%. All solutions were filtered through a 0.2 μm Millex-LG syringe filter (Millipore, USA). The pH measurements were made with PICO$^+$ Benchtop pH-meter (Labindia Instruments Pvt. Ltd).

2.2. Methods

2.2.1. Concentration determination

Concentration of HEWL was determined by absorbance measurements on a UV/visible spectrophotometer (UV-1800, Shimadzu Corp., Kyoto, Japan) using the extinction coefficient of 2.65 mL mg^{-1} cm^{-1}(37,914 M^{-1} cm^{-1}) at 280 nm [22]. Concentrations of ThT and CR were determined spectrophotometrically using molar extinction coefficients of 36,000 M^{-1} cm^{-1} at 412 nm [23], 45,000 M^{-1} cm^{-1} at 495 nm [24],respectively.

Hemin solutions were freshly prepared as described previously [25]. Briefly, Hemin stock solution was prepared by dissolving hemin chloride in 25 mM NaOH and then diluted into 60 mM sodium phosphate buffer of pH 7.4. The stock hemin solution was filtered through a 0.2 μm Millex-LG syringe filter (Millipore, USA) and the concentration of hemin was determined using molar extinction coefficient of 58,400 M^{-1} cm^{-1} at 385 nm [26].

2.2.2. Purification of fibrils

The HEWL fibrils were prepared in two solution conditions as described previously [21,27]. Briefly, in the first condition, 140 μM lysozyme was incubated in 4 M GuHCl solution of pH 7.4 at 37 °C. In the second condition, 140 μM lysozyme was incubated at pH 1.7 and 65 °C. In both cases fibrils formed within 5 h upon moderate stirring at 230 rpm.

For disaggregation study, fibril samples were purified by repeated washing with 60 mM sodium-phosphate buffer of pH 7.4. In order to remove all the bound GuHCl, GuHCl-induced fibril sample was washed 4–5 times, while the low pH-induced fibril sample was washed 3–4 times. Each step of purification comprised of centrifugation of the samples containing fibrils at 13000 rpm for 15 min, throwing away the supernatant and resuspension of the pellets in 60 mM sodium-phosphate buffer of pH 7.4 for the next purification cycle. At the final step, the pellet was placed on a filter paper and air-dried to remove the remaining water. This produced flaky-white material, which was used for disaggregation, hemin binding and TEM etc. studies. Fibrils formation and morphology of the fibrils were verified by transmission electron microscopy (TEM).

2.2.3. Disaggregation kinetics

The disaggregation process was initiated by incubation of 0.12 mg/mL of fibrils with different concentrations of hemin at pH 7.4 and 37 °C. The disaggregation kinetics were measured by the absorbance (Abs) of the supernatants at 280 nm from centrifuged solutions of fibril samples that had been incubated with different molar ratios of hemin/lysozyme for various time periods. The Abs of the each sample was recorded against their respective blank. Each blank contains their respective concentrations of hemin.

All disaggregation kinetic traces were analyzed by a procedure of best fit, using the single exponential function.

$$A(t) = A(\infty) - a \times \exp(k \times t) \quad (1)$$

$A(t)$ and $A(\infty)$ are the absorbance of the supernatant at time t and ∞, respectively, a is the amplitude of the observed phase, and k is apparent rate constant of disaggregation.

2.2.4. Far-UV circular dichroism

Far-UV circular dichroism (far-UV CD) experiments were carried out on a Jasco J-815 spectropolarimeter (Tokyo, Japan). Far-UV CD spectrum of each sample (3 μM purified fibril or monomeric lysozyme) was recorded between 200 and 250 nm using a 1 mm cuvette and a slit width of 1 nm. All the spectra were background subtracted with their respective blank. The data were presented as mean residue ellipticity [θ] in deg cm^2 dmol^{-1}, which is defined as [θ] = CD / (10 × n × l × C), where the CD is in Milli degree, n is the number of amino acid residues, l is the path length of the cell in cm, and C is the molar concentration of the protein. The contents of α-helix and β-strand were determined using K2D3 deconvolution software [28].

2.2.5. ThT assay

6 μL of stock ThT (2.5 mM) were added to 1 mL of each purified fibril sample (3 μM) incubated for 200 h in the absence and presence of Hemin/fibril ratio of 2:1. The fluorescence of the resulting samples was measured on a Cary eclipse fluorescence spectrophotometer at 25 °C using a 1 cm path length cuvette. The emission spectra were recorded between 460 and 600 nm by exciting the samples at 450 nm. All the spectra are background subtracted with their respective blank. The data were presented as relative fluorescence in which fluorescence of fibril without hemin at λ_{max} was taken as 100.

2.2.6. Congo red assay

10 μL of stock CR (2.5 mM) were added to 1 mL of each purified fibril sample (5 μM) incubated for 200 h in the absence and presence of Hemin at Hemin/fibril ratio of 2:1. After 5–6 min of equilibration, optical absorption spectra were acquired from 450 to 700 nm by using a UV-1800 Shimadzu spectrophotometer. A 10-mm path length cuvette was used. The data were presented as difference spectra between 450 and 700 nm. Difference spectra were obtained by subtracting the spectra of the fibril alone and of CR alone from the spectra of the fibril