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Investigating the effects of erythrosine B on amyloid fibril formation derived from lysozyme

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ABSTRACT

Formation of amyloid fibrils has been associated with at least 30 different protein aggregation diseases. The 129-residue polypeptide hen lysozyme, which is structurally homologous to human lysozyme, has been demonstrated to exhibit amyloid fibril-forming propensity *in vitro*. This study is aimed at exploring the influence of erythrosine B on the *in vitro* amyloid fibril formation of hen lysozyme at pH 2.0 and 55 °C using ThT binding assay, transmission electron microscopy, far-UV circular dichroism absorption spectroscopy, 1-anilinonaphthalene-8-sulfonic acid fluorescence spectroscopy, and synchronous fluorescence study. We found that lysozyme fibrillogenesis was dose-dependently suppressed by erythrosine B. In addition, our far-UV CD and ANS fluorescence data showed that, as compared with the untreated lysozyme control, the α-to-ß transition and exposure of hydrophobic clusters in lysozyme were reduced upon treatment with erythrosine B. Moreover, it could be inferred that the binding of erythrosine B occurred in the vicinity of the tryptophan residues. Finally, molecular docking and molecular dynamics simulations were further employed to gain some insights into the possible binding site(s) and interactions between lysozyme and erythrosine B. We believe the results obtained here may contribute to the development of potential strategies/approaches for the suppression of amyloid fibrillogenesis, which is implicated in amyloid pathology.

1. Introduction

Protein aggregates in general can be categorized into two classes: amyloid fibrils, the ones with highly ordered structure, and amorphous aggregates, the ones with no long-range order. Formation of amyloid fibrils has been recognized to play a significant role in the development of a vast array of debilitating and yet incurable neuropathic or non-neuropathic disorders in humans, including hemodialysis amyloidosis, type II diabetes, Parkinson disease, Huntington's disease, and Alzheimer's disease [1–4]. Amyloid fibrils derived from different proteins/peptides exhibit several tinctorial and physicochemical features in common, such as β-sheet rich secondary structure, fibrillar morphology, birefringence to polarized light, insolubility in most solvents, and protease-resistance [1,2]. Given these morphological similarities between amyloid fibrils from different protein building blocks, it has been hypothesized that different proteins/peptides may likely follow similar fibril formation routes [2,5]. However, the exact molecular mechanism of amyloid fibril formation remains to be deciphered in full [1–3].

Several therapeutic approaches have been put forth to prevent or to treat the amyloid-associated diseases [6–10]; however, neither definite diagnostic tool and nor proper treatment is currently available toward tackling these diseases. Inhibition of amyloid fibrillogenesis and disruption of fibrillar assemblies have been by far envisaged as two promising therapeutic strategies. A variety of synthesized or natural molecules/compounds have been reported to

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retard or prevent amyloid fibril formation both *in vitro* and *in vivo* [11,12]. These inhibitory molecules/compounds include drugs (e.g., statins, aspirin), antibodies, antibiotics (e.g., rifampicin, tetracycline), small dyes (e.g., Congo red, thioflavin T), natural phenols (curcumin) and phenolic disaccharides [8,13].

Compelling evidence has indicated that amyloid fibrillogenesis is not only restricted to disease-related proteins/peptides. Amyloid fibrils and/or amyloid-like fibrils, under certain favorable conditions, can also be produced from the self-assembly of disease-unrelated proteins/peptides *in vitro* [2,14–16]. The aforesaid findings have brought to the idea that the potential to form amyloid/amyloid-like structure is an inherent property of polypeptide chains. Investigations of the amyloid fibrillogenesis using non-disease associated proteins could thus aid in our understanding on the modes of amyloid fibrillogenesis and the possible ways to inhibit them. Hen egg-white lysozyme, a globular protein composed of 129 residues, possesses four disulfide bonds and adopts predominantly helical conformation [17]. Hen lysozyme exhibits nice structural stability, unique folding/unfolding mechanism(s), and thermodynamic properties [18–20]. In addition, studies showed that hen lysozyme is able to self-assemble into fibrillar species under certain conditions (e.g., low pH, elevated temperature, and in the presence of additives) [21–25]. Also, hen lysozyme has high sequence identity with human lysozyme, which is responsible for non-neuropathic hereditary systemic amyloidosis disease [26,27]. Moreover, the fibrillar species of human lysozyme formed were found to resemble the hen lysozyme fibrils. The above-mentioned reasons have enabled hen lysozyme to serve as a suitable model template with which to examine the *in vitro* fibril formation-relevant phenomena [19,28].

In the present study, using hen lysozyme as a model system to induce amyloid fibrils, we set out to investigate the influence of erythrosine B on the fibrillogenic behavior of lysozyme and to further characterize the relationship between the presence of erythrosine B and protein misfolding resulting in the eventual amyloid formation. Via several spectroscopic techniques and transmission electron microscopy, our results demonstrated that erythrosine B exhibited an anti-amyloidogenic activity, which was found to be dependent upon erythrosine B concentration and incubation time. Also, we found that the treatment with erythrosine B led to marked conformational changes in the lysozyme sample as compared with the untreated lysozyme control. In addition, the predicted docking pose along with the possible interactions associated with the binding of lysozyme with erythrosine B was revealed by molecular docking and molecular dynamics simulations. Our results suggested that the inhibition of lysozyme fibril formation by erythrsoine B may be attributed mainly to the binding of erythrosine B to lysozyme's potential amyloidogenic prone region. We believe the results reported here could have implications for the rational design of potential therapeutics for the diseases associated with amyloid fibrils.

2. Materials and methods

2.1. Materials

Hen egg-white lysozyme (EC 3.2.1.17) with a purity of ≥90% was obtained from Merck (Germany) and was used for the experiments without further purification. Erythrosine B was purchased from Sigma-Aldrich (USA). Hydrochloric acid, sodium chloride, Na_2HPO_4, 1.76 mM NaH_2PO_4, and 1.54 mM NaN_3 were acquired from Nacalai Tesque (Japan). Glycine, and sodium dodecyl sulfate were from Bio Basic (Canada). Tris was purchased from USB (USA). 2-propanol and ethanol were acquired from J.T. Baker (USA). Protein markers were obtained from GenMark (USA). All other chemicals, unless otherwise specified, were purchased from Sigma-Aldrich (USA).

2.2. Preparation of lysozyme sample solutions and fibril formation conditions

Lysozyme samples (without and with erythrosine B) at 0.5 mg/mL were prepared by dissolving appropriate amount of lyophilized lysozyme and erythrosine in the buffer (100 mM glycine, 100 mM NaCl, and 1.54 mM NaN_3, pH 2.0). The molar ratios of erythrosine to lysozyme used in this study were 0:1, 0.1:1, 1:1, and 5:1. All the samples were incubated at 55 °C with stirring at 470 rpm. Experiments in which erythrosine B was added in lysozyme sample (except the control) during the incubation period for fibril formation were conducted to investigate the inhibitory potency of erythrosine B against lysozyme fibrillogenesis.

2.3. Thioflavin t (ThT) binding assay

Thioflavin T (ThT) can rapidly and specifically bind to the antiparallel β-sheet structure contained in amyloid fibrils, and the binding gives rise to the elevated fluorescence intensity. 5 mg ThT dye were dissolved in 10 mL ethanol to a concentration of 1.57 mM, the solution was then diluted by phosphate buffer (10 mM Na_2HPO_4, 1.76 mM NaH_2PO_4, 1.54 mM NaN_3) to a final concentration of 10 μM. 40 μL of the untreated lysozyme control or the samples with different molar ratios of erythrosine B to lysozyme (e.g., 0.1:1, 1:1, and 5:1) were mixed with 960 μL of 10 μM ThT to form 25X diluted lysozyme samples and then placed in a 1 cm light path quartz cuvette. ThT fluorescence intensity was detected using a Cary Eclipse fluorescence spectrometer (Varian, USA) at an excitation wavelength of 440 nm and an emission wavelength of 490 nm. The excitation and emission slits were both 5 nm. The photomultiplier tube voltage was 600 V. All measurements were triplicated.

2.4. 1-Anilinonaphthalene-8-sulfonic acid (ANS) binding spectroscopy

ANS is a fluorescent dye that can bind to the hydrophobic clusters of proteins. 100 μL of the untreated lysozyme control or the samples with different molar ratios of erythrosine B to lysozyme (e.g., 0.1:1, 1:1, and 5:1) were added to 900 μL of 20 μM ANS in phosphate buffer (10 mM Na_2HPO_4, 1.76 mM NaH_2PO_4, and 1.54 mM NaN_3). The resultant mixtures (10X diluted samples) were placed in 1 cm light path quartz cuvettes and measured by a Cary Eclipse 300 fluorescence spectrophotometer (Varian, USA). The fluorescence emission spectra spanning from 420 to 580 nm were collected upon excitation of the samples at 380 nm and the fluorescence intensity of solvent blanks was subtracted from the sample solutions. All measurements were repeated at least three times and averaged.

2.5. Far-UV circular dichroism (CD) absorption spectroscopy

Far-UV circular dichroism spectra of the (10X diluted) untreated lysozyme control and the samples with molar ratios of erythrosine B to lysozyme at 0.1:1, 1:1, and 5:1 were monitored on a JASCO J-815 spectrometer (JASCO Corporation, Japan) in the far-UV region (200–260 nm) at 25 °C. The parameters were as follows: bandwidth of 2 nm, response time of 2 s, step interval of 0.1 nm, and scanning speed of 50 nm/min in continuous scan mode. The path length of quartz cuvette was 0.2 cm. The baseline was determined by use of the control buffer and was subtracted from the results of each lysozyme sample. The ellipticity (millidegree or mdeg) was plotted against the wavelength (nm). Using the software provided by the