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Physical basis for the ofloxacin-induced acceleration of lysozyme aggregation and polymorphism in amyloid fibrils

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ABSTRACT

Aggregation of globular proteins is an intractable problem which generally originates from partially folded structures. The partially folded structures first collapse non-specifically and then reorganize into amyloid-like fibrils via one or more oligomeric intermediates. The fibrils and their on/off pathway intermediates may be toxic to cells and form toxic deposits in different human organs. To understand the basis of origins of the aggregation diseases, it is vital to study in details the conformational properties of the amyloidogenic partially folded structures of the protein. In this work, we examined the effects of ofloxacin, a synthetic fluoroquinolone compound on the fibrillar aggregation of hen egg-white lysozyme. Using two aggregation conditions (4M GuHCl at pH 7.0 and 37 °C; and pH 1.7 at 65 °C) and a number of biophysical techniques, we illustrate that ofloxacin accelerates fibril formation of lysozyme by binding to partially folded structures and modulating their secondary, tertiary structures and surface hydrophobicity. We also demonstrate that Ofloxacin-induced fibrils show polymorphism of morphology, tinctorial properties and hydrophobic surface exposure. This study will assist in understanding the determinant of fibril formation and it also indicates that caution should be exercised in the use of ofloxacin in patients susceptible to various aggregation diseases.

1. Introduction

The understanding of conversion of proteins and peptides into amyloid fibrils is a fundamental problem of interdisciplinary sciences. Amyloid fibril refers to insoluble fibrillar aggregates of proteins and peptides and is structurally characterized by an extended cross-β structure [1–3]. The deposition of amyloid fibrils in tissues or extracellular spaces is linked up with a group of human diseases, including type II diabetes, Alzheimer, Parkinson and several systemic amyloid diseases [1,3]. It is likewise a major problem in therapeutic proteins production as the large-scale production of these proteins often aggregates and form inclusion bodies with amyloid-like structural features [4,5]. In contrast, they have also been found to play important functional roles in many organisms ranging from bacteria to mammals. They are involved in biofilm formation, cell/cell and cell/substrate adhesion in bacteria and polymerization of the pigment melanin in human [1]. Moreover, the amyloid fibril has been increasingly investigated for its possible role in nanotube formation for nano-biotechnology [6].

It is well recognized that initiation of aggregation reaction mainly requires accumulation of partially folded/unfolded intermediate states [1,7,8]. The fate of its assembly into oligomers, fibrils or amorphous aggregates is largely determined by the conformation of the partially unfolded states [9]. The fibril morphology is determined by aggregation conditions, such as pH, temperature, agitation, salts or small molecules ligand. These solution conditions also control the conformation of partially folded state. Therefore, not only the formation of different type of aggregates, but the polymorphism in fibrils of the same sequence is also determined by the conformation of the partially folded state [9,10]. Thus, the search of partially folded conformations capable to accelerate/decelerate the aggregation process is essential to understand the basis of aggregation process and its prevention.

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A large number of compounds have been investigated for their ability to inhibit aggregation. The inhibitor compounds comprise of classes of drugs (statins, aspirin), antibodies, antibiotics (rifampicin, tetracycline), small dyes (Congo Red, Thioflavin T), natural phenols (curcumin) and phenolic disaccharides etc. They have been described and reviewed in considerable detail [11,12]. In particular, the antibiotic class of compounds greatly interests us as different antibiotics have shown conflicting results. Antibiotics such as rifampicins, tetracyclines, geldanamycin, deoxydoxorubicin and clioquinol are known to prevent amyloid aggregation of several proteins [13–17]. A contradictory result about rifampicin is also reported [18]. On the other hand aminoglycoside antibiotics are reported to promote protein aggregation in bacteria [19]. The macrolide antibiotics like erythromycin and azithromycin have an inhibitory activity on two major intracellular protein degradation systems and can induce nonspecific protein aggregation [20]. The β-lactum antibiotics have no effect on protein aggregation in bacteria [21]. The effect of quinolones antibiotics on the amyloid fibril formation is yet not recognized.

Fluoroquinolone drug ofloxacin (Ofx) is commonly practiced to treat infection of renal system, lung, spleen and liver, the deposition sites in systemic amyloidosis. In this work, we looked into the effects of Ofx on the aggregation of model protein hen egg white lysozyme (HEWL) in two well characterized aggregating conditions. HEWL is known to convert into fibrils through partially folded aggregation precursor states in 4M guanidinium hydrochloride (GuHCl) solution of pH 6.3 at 37 °C [7,22] and pH 1.7 at 65 °C [23]. We found that Ofx interacted with both native state and aggregation precursor states and significantly modulated their conformations and stability. The partially folded intermediate states accumulated in the presence of Ofx were found to accelerate the aggregate processes and generate fibrils of different conformations compared to partially folded states formed in the absence of the drug.

2. Material and methods

2.1. Materials

Hen Egg White Lysozyme (HEWL L6876), Guanidine hydrochloride (GuHCl G3272), 1-Anilino 8-naphthalene sulphonic acid (ANS A1028), Thioflavin T (ThT 3516) and Congo red (CR C6277) were purchased from Sigma–Aldrich, Co., USA. Ofloxacin (Ofx) was obtained from Cipla. All other chemicals and reagents used were of Analytical grade with purity >99%. All buffer solutions were filtered through a 0.2 μm Millex-LG syringe filter (Millipore, USA). The pH was checked with PICO^{+} Benchtop pH-meter (Labindia Instruments Pvt Ltd).

2.2. Aggregation kinetics

HEWL 20 mg/mL was prepared in distilled water and its concentration was determined spectrophotometrically on a UV–visible spectrophotometer (UV–1800, Shimadzu Corp., Kyoto, Japan) using $A^{1\%}_{1cm} = 26.4$ (2.64 mL mg^{-1} cm^{-1}) [23]. To examine the effect of Ofx on HEWL aggregation, fibrillar aggregation of HEWL was investigated in two solvent conditions in the presence of Ofx/HEWL molar ratio of 0:1, 1:1 and 2:1. In the first condition, the aggregation was initiated by continuous stirring of 140 μM HEWL at 230 rpm in 4 M GuHCl at 37.0 ± 0.1 °C and pH 7.0 [7]. In the second condition, 20 μM of HEWL was aggregated at pH 1.7 at 65 °C and 230 rpm.

Kinetics of aggregation reactions were followed by ThT and ANS fluorescence on Fluoromax-4 spectrofluorometer, Horiba Scientific, Horiba Jobin Yvon Inc., USA. 2 μM protein aggregates from each sample were taken at different time intervals and added to 10 μM ThT and ANS solutions at pH 7.0 for ThT and ANS fluorescence measurements, respectively. Samples were excited at 444 nm and 380 nm for the ThT and ANS fluorescence measurements, respectively. In both cases, kinetics profiles were obtained by plotting fluorescence emission at 480 nm as a function of time in minutes.

The apparent rate constant ($k_{app} = 1/\tau$) and lag time ($t_{lag} = t_{1/2} - 2\tau$) for the aggregation reactions were calculated by fitting kinetic traces using following sigmoidal equation (1) [24].

$$F = F_0 + m_0 t + \frac{F_1 + m_1 t}{1 + e^{-[(t - t_{1/2})/\tau]}} \quad (1)$$

where F_0 and F are the observed ThT fluorescence ratio at time zero and t minutes, respectively. $t_{1/2}$ is the time taken to reach 50% of maximum fluorescence. m_0 and m_1 are the slopes of pre- and post-transition regions of the aggregation profiles, respectively.

2.3. Morphological, tinctorial and structural properties of the aggregates

To characterize the aggregates formed in the absence and presence of Ofx, the aggregate samples were washed with double distilled water several times to remove GuHCl and other solutes. Washing involved centrifugation of the sample at 13,000 rpm for 15 min, discarding the supernatant, followed by re-suspension in double distilled water. Samples were then air-dried at 37 °C, which produced white materials. To check if the washing altered the properties of aggregates, we measured the far-UV circular dichroism (far-UV CD) (SI Fig. 1A and SI Fig 1B) and Thioflavin T (ThT) fluorescence (SI Fig. 1C and SI Fig. 1D) spectra of the aggregates with and without washing. We found that both far-UV CD and ThT fluorescence spectra are essentially similar in spectral features, indicating that washing did not significantly alter the properties of the aggregates.

2.3.1. Morphological analysis

The morphology of the aggregates was analyzed using Transmission Electron Microscope (TEM). A 10 mg/mL suspension of each washed, dried fibrils sample was prepared in deionised (Milli-Q) water and then diluted 50-fold. 5 μL of each aggregate sample were placed on a 200 mesh carbon coated copper grid. After adsorption for 2 min, the grids were washed with deionised water, air dried and then stained with 2% (w/v) uranyl acetate for 1 min. The grids were again air dried. The images of the stained samples were taken by Philips Transmission Electron Microscope CM200 operating at 20–200 kv.

2.3.2. Secondary structure analysis

Secondary structure analysis of the aggregates was done by far-UV circular dichroism (Far-UV CD) measurements on a Jasco 815 Spectropolarimeter. Far-UV CD spectrum of each aggregate sample (0.1 mg/mL) was recorded between 200 and 250 nm using a 1 mm cuvette and a slit width of 1 nm. All the spectra are background subtracted with their respective blank.

2.3.3. Tinctorial properties analysis

Dyes such as ThT, ANS and CR binding assays were done as reported previously [18]. Briefly, 100 μL of final aggregate samples in the absence and presence of Ofx were added to 2.9 mL of respective solutions prepared in 60 mM Na-phosphate buffer at pH 7.0. The ThT and ANS fluorescence spectra of the resulting solution were measured at room temperature with excitation wavelength fixed at 444 nm and 380 nm, respectively. The CR assay was performed by monitoring absorbance of the samples from 400 nm–700 nm. Differential absorption spectrum of CR-fibril complex was obtained