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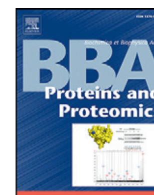

Curcumin and kaempferol prevent lysozyme fibril formation by modulating aggregation kinetic parameters

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Interaction of small molecule inhibitors with protein aggregates has been studied extensively, but how these inhibitors modulate aggregation kinetic parameters is little understood. In this work, we investigated the ability of two potential aggregation inhibiting drugs, curcumin and kaempferol, to control the kinetic parameters of aggregation reaction. Using thioflavin T fluorescence and static light scattering, the kinetic parameters such as amplitude, elongation rate constant and lag time of guanidine hydrochloride-induced aggregation reactions of hen egg white lysozyme were studied. We observed a contrasting effect of inhibitors on the kinetic parameters when aggregation reactions were measured by these two probes. The interactions of these inhibitors with hen egg white lysozyme were investigated using fluorescence quench titration method and molecular dynamics simulations coupled with binding free energy calculations. We conclude that both the inhibitors prolong nucleation of amyloid aggregation through binding to region of the protein which is known to form the core of the protein fibril, but once the nucleus is formed the rate of elongation is not affected by the inhibitors. This work would provide insight into the mechanism of aggregation inhibition by these potential drug molecules.

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1. Introduction

From a physicochemical perspective, the process of amyloid-like aggregation is a generic feature of polypeptide chains that needs to be fully understood for a thorough characterization of the nature of proteins [1,2]. Molecules that inhibit aggregation through either specific or non-specific interaction do so by modulating inter- and/or intra-molecular interactions between polypeptide chains. Therefore, investigation of inhibition of aggregation is useful for clarifying the mechanisms underlying protein aggregation [3]. Protein aggregation is linked to a large number of pathological conditions in humans [4] and is a major problem in the production of many recombinant therapeutic proteins [5]. Therefore, from a biomedical perspective, inhibition of aggregation is a public health priority.

Human lysozyme mutants have been known to form huge amyloid deposits in the livers and kidneys of individuals affected by hereditary systemic amyloidosis. Currently, there are no clinical treatments available to prevent or reverse the formation of such amyloid deposits. It is also known that destabilized lysozyme mutants aggregate faster than wild type lysozyme both in vitro and in vivo [6,7]. Lysozymes from various sources are also known to form amyloid-like aggregates in a variety of destabilizing conditions [8–10]. Moreover, despite a large amount of work supporting the importance of the small molecule inhibitors in preventing amyloidogenesis [11,12], little is known about the precise mechanism by which they inhibit aggregation and the effect that these inhibitors have on different kinetic parameters of the aggregation process, on interaction with native state and aggregation precursor state, on unfolding pathway and on the overall aggregation pathway [13].

In this work, we investigated the effect of two small polyphenolic molecules, curcumin and kaempferol, on different kinetic parameters such as amplitude, elongation rate constant and lag time of guanidine hydrochloride-induced aggregation process of hen egg white lysozyme (HEWL). We have also shown the effect of interaction of these molecules on the unfolding pathway and on the structure of HEWL. Curcumin, a polyphenol found in the spice turmeric, has been shown to have strong antioxidant property and inhibit aggregation of many proteins and peptides [13–15]. On the contrary, kaempferol, which is also a natural flavonol found in many plant species and commonly used in traditional medicine, is a weak antioxidant and its aggregation inhibition properties are not known [16]. We show how the interaction of these molecules with the HEWL affects the kinetic parameters of aggregation reactions and equilibrium unfolding pathway of protein. On

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the basis of these findings, we emphasize that a full understanding of the kinetic parameters is essential for deducing the correct mechanism of aggregation inhibition by potential aggregation inhibition drugs.

2. Materials and methods

2.1. Materials

Hen egg white lysozyme (HEWL), curcumin, kaempferol, guanidine hydrochloride (GuHCl), 1-anilinonapthalene-8-sulfonic acid (ANS), thioflavin T (ThT), and Congo red (CR) were purchased from Sigma-Aldrich Co. All other reagents were of analytical grade with purity > 99%. The concentrations of HEWL were determined by UV absorbance (at 280 nm, with extinction coefficients ($\varepsilon^{1\%}$) of 2.63) [17].

2.2. Aggregation inhibition kinetic studies (ThT and light scattering assays)

The aggregation of HEWL in the absence and presence of various concentrations of inhibitors was initiated by continuous stirring of the protein (140 μM) at 230 rpm (rotation per minute) in 4 M GuHCl at 37.0 ± 0.1 °C and pH 6.3. Kinetics of aggregation was monitored using ThT and 90° light scattering assays. 100 μL of aggregation reaction mixtures at different time intervals was added to 1.9 mL of 25 μM ThT solution and pH 7.4 Na-phosphate buffer for ThT assay and light scattering measurements, respectively. The fluorescence of the resulting samples was measured at room temperature with excitation wavelength fixed at 440 nm. The 90° light scattering of the resulting sample was measured at room temperature with excitation wavelength and emission wavelength fixed at 350 nm. In each case, slit width for excitation and emission was 3 nm each.

All the kinetic traces were satisfactorily fitted to a sigmoidal function as described by the following equation.

$$F = F_0 + m_0 x + \frac{F_1 + m_1 x}{1 + e^{-[(x - x_{1/2})/\tau]}} \tag{1}$$

where F_0 is the observed ThT fluorescence ratio; x and $x_{1/2}$ are time and time to 50% of maximum fluorescence, respectively. Therefore, the apparent rate constant (k_{app}) for the aggregation is given by $1/\tau$, and the lag time is given by $x_0 - 2\tau$.

2.3. Dye binding assays of final aggregates

To induce aggregation, HEWL solutions (140 μM) in the absence and presence of inhibitors were subjected to stirring at 230 rpm for 3 h in 4 M GuHCl at 37.0 ± 1.0 °C and pH 6.3. ThT, ANS and CR binding assays were performed as described previously [18]. Briefly, 100 μL of final aggregate samples in the absence and presence of inhibitors was added to 1.9 mL of respective solutions prepared in 25 mM Na-phosphate buffer at pH 7.4. The ThT fluorescence and ANS fluorescence of the resulting samples were measured at room temperature with excitation wavelength fixed at 440 nm and 380 nm, respectively. The light scattering of the resulting samples was measured with excitation wavelength and emission wavelength fixed at 350 nm. The CR assay was performed by incubating various aggregate samples with CR for 2–3 min and optical absorption was monitored between 400 and 700 nm. Samples without CR and without protein were used as control to obtain difference spectrum.

2.4. Amyloid intrinsic fluorescence

GuHCl-induced HEWL aggregates prepared as described above were also characterized by amyloid intrinsic fluorescence. 100 μL of final aggregate samples in the absence and presence of inhibitors was added to 1.9 mL of 25 mM Na-phosphate buffer (pH 7.4) and fluorescence spectra between 365 nm and 500 nm were monitored by excitation of the sample at 357 nm with both excitation and emission slit widths kept at 10 nm [19].

2.5. Circular dichroism studies

Far-UV circular dichroism (far-UV CD) spectra of various aggregates in the absence and presence of inhibitors was measured between 200 and 250 nm using Jasco 810 Spectropolarimeter. Far-UV CD spectra of native HEWL and 4 M GuHCl unfolded protein without and with different concentrations of inhibitors were also measured. For all measurements, a protein concentration of 2.8 μM, slit width of 1 nm and a cell of 1 mm path length were used. The data were presented as mean residue ellipticity [θ] in deg $cm^2 dmol^{-1}$, which is defined as $[\theta] = CD / (10 \times n \times l \times C_p)$, where CD is in milli-degree, n is the number of amino acid residues (129), l is the path length of the cell in cm, and C_p is the molar concentration of the protein in monomeric form. The amount of secondary structures (%α-helix) of native HEWL in the absence and presence of inhibitors was determined as described in Supplementary information [20].

2.6. Protein intrinsic fluorescence

All aggregate samples (140 μM) were diluted 100 fold in 25 mM sodium phosphate buffer at pH 7.4 immediately before fluorescence measurements. Native, 4 M and 6 M GuHCl-incubated HEWL samples in the absence and presence of different concentrations of inhibitors were also prepared at the protein concentration of 1.4 μM. Intrinsic fluorescence spectra of all these samples were acquired between 300 and 400 nm upon exciting the protein at 280 nm.

2.7. Scanning electron microscopy (SEM)

SEM images of various aggregates with and without inhibitors were obtained as described previously [21]. Aggregate sample was diluted to 35 μM using distilled water. A drop of the resulting solution was deposited on aluminum foil and was subsequently air dried. Samples of protein aggregate without inhibitors and those of 2:1 and 5:1 inhibitor to protein ratios were analyzed at low vacuum using Quanta 200 SEM at different magnifications.

2.8. GuHCl-induced unfolding studies

Solutions for unfolding experiment in the absence and presence of inhibitors were prepared as described previously [22]. The unfolding/refolding of the protein was followed by measurements of intrinsic fluorescence of the protein. The protein sample was excited at 280 nm and emission was monitored between 300 and 450 nm. All data points were subtracted with their respective blank. The unfolding profiles were analyzed according to two and three state models as described by the following equations [23].

$$F = \frac{F_N + F_U e^{-(\Delta G_0 - m[GuHCl])/RT}}{1 + e^{-(\Delta G_0 - m[GuHCl])/RT}} \tag{2}$$

$$F = \frac{F_N + F_I \exp\{-(G_I - m_1[Gu])/RT\} + F_U \exp\{-(G_1 - m_1[Gu])/RT\} \exp\{-(G_2 - m_2[Gu])/RT\}}{1 + \exp\{-(G_I - m_1[Gu])/RT\} + \exp\{-(G_I - m_1[Gu])/RT\} \exp\{-(G_2 - m_2[Gu])/RT\}} \tag{3}$$

where, F is the observed fluorescence intensity at 340 nm. F_N, F_I and F_U are the fluorescence intensity of native, intermediate and unfolded protein states, respectively. R and T are the gas constant and temperature in Kelvin (298 K). ΔG_0 is the change in free energy of unfolding of HEWL in the presence of inhibitors. ΔG_1 and ΔG_2 are the changes in free energies corresponding to N ↔ I and I ↔ U transitions, respectively.

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