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Investigating the inhibitory effects of zinc ions on amyloid fibril formation of hen egg-white lysozyme

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ABSTRACT

The amyloid fibrils derived from protein and peptide self-assembly have been studied in many diseases. In the present study, in combination with Thioflavin T(ThT) assay, Congo red(CR),transmission electron microscopy and cell cytotoxicity assay, we investigated the influence of zinc ions on amyloid fibril formation using hen egg white lysozyme (HEWL) as a model protein under high temperature and acidic pH conditions. We observed that HEWL tended to form the amyloid fibrils at pH 2.0 and 60 °C, which was consistent with the previous studies. However, as the concentrations of zinc ions increased, the amounts of amyloid fibrils of HWEL gradually reduced, but the overall morphology of individual amyloid fibril was not significantly altered whether or not zinc ions were present. Moreover, by using circular dichroism (CD), ANS and intrinsic fluorescence spectra, we illustrated that zinc ions inhibited the formation of β-sheet and exposure of hydrophobic regions of HEWL. This work would help to understand the molecular mechanism of amyloid fibril formation.

1. Introduction

The intermolecular self-assembly of partly unfolded proteins to form highly ordered amyloid fibrillary structure is correlated with a variety of human neurodegenerative diseases, including Alzheimer's, Parkinson's, Huntington's and Creutzfeldt-Jakob diseases [1–3]. Until now over 30 different proteins and peptides *in vivo* including huntingtin, human lysozyme, and human islet amyloid polypeptide have been found to form amyloid aggregates leading to severe pathologies [4,5]. Although these diseases-associated proteins are not related in sequence and structure, they all form long, straight and unbranched proteinaceous fibrils with several common properties, such as exhibition of β-sheet-rich secondary structure, birefringence upon staining with Congo red, increased surface hydrophobicity, and fluorescence upon binding to thioflavin T (ThT) [6–10]. Moreover, recent studies also showed that many proteins not associated with diseases also formed the amyloid fibrils under certain conditions.

Hen egg white lysozyme (HEWL) is a 129-amino acid enzyme to break down the cell wall of bacteria. It has been extensively studied as a model system for protein folding and amyloid aggregation [11–13]. Its native form is cross-linked by four disulfide bonds with mainly helical conformation (~30% α-helix; ~6% β-sheet) [Fig. 1]. Furthermore, HEWL is highly homologous to human lysozyme which is related to fatal hereditary systemic lysozyme amyloidosis. It has been demonstrated that HEWL tended to form the amyloid fibrils under stressed conditions when partly folded intermediates of HEWL were highly populated. Such conditions include acidic pH, high temperature, denaturant and high pressure [14–16]. Hence, HEWL has become one of the well studied model proteins for understanding the amyloid fibril formation of globular proteins.

Many external factors, including pH, temperature, vitamin and various small molecules, affected amyloid fibril formation [17–19]. In addition, metal ions have also been demonstrated to play a significant role in the amyloid fibril formation [20,21]. Some previous studies showed that transition metal ions, such as Zn^{2+} and Cu^{2+}, accelerated the fibrillation of proteins [22–25]. It has been demonstrated that transition metal ions caused an electrostatic screening between protein molecules by binding with the specific binding sites of proteins, which resulted in the accelerant effects. However, other studies showed that metal ions inhibited the amyloid fibril formation [26,27]. Thus, more studies on the roles of metal ions in protein aggregation are important to understand the molecular mechanisms of amyloid fibril formation.

Interactions between metal ions and proteins are highly diverse and have received much attention because of their influences on the amyloid aggregation [28,29]. In contrast to other amyloid proteins,

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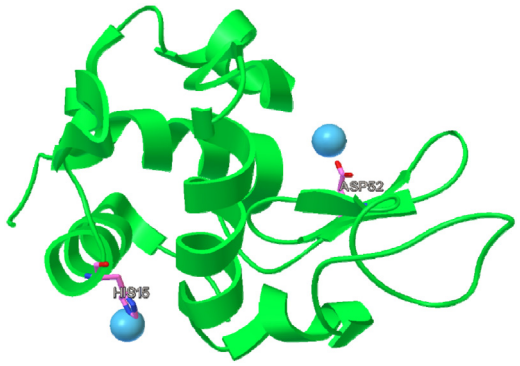

Fig. 1. Schematic representation of HEWL structure.

for which the roles of metal ions have been well studied, the influences of metal binding on HEWL amyloid formation are not clear. Here, the effects of zinc ions on the conformation and amyloid fibril formation of HEWL were studied. Our results clearly demonstrated that zinc ions inhibited the conversion of α-helix to β-sheet and retarded the fibril formation of HEWL.

2. Materials and methods

2.1. Meterials

Hen egg white lysozyme (HEWL) was purchased from Sigma (Sigma-Aldrich Co, St. Louis, Mo). Thioflavin T (ThT), 1-Anilinonaphthalene-8-sulfonic Acid (ANS) and Congo Red (CR) were purchased from Sigma. $ZnCl_2$ was purchased from the Research and Development Center of Guangdong Fine Chemical Engineering Technology Inc. All the chemical regents were of analytical grade.

2.2. Fibril formation

5 mg/ml HEWL was prepared in the absence or presence of zinc ions. HEWL (50 mg) was dissolved in 10 mL hydrochloric acid (10 mM, pH 2.0). The HEWL solutions were incubated for 150–200 h at 60 °C in water path with agitation of 150 rpm. To investigate the effects of zinc ions, $ZnCl_2$ was added to HEWL solutions to the final concentrations 0, 50, 100, 150 and 200 μM, respectively. The pH was readjusted when $ZnCl_2$ was added into the solutions.

2.3. ThT assay

To monitor the procedure of fibril formation, 100 μL aliquots of protein aggregates were taken at different time intervals with addition of 20 μM ThT for fluorescence measurements. ThT fluorescence was excited at 450 nm and recorded at 482 nm using a Hitachi F-4600 Florescence Spectrophotometer. All measurements were taken in triplicate. The data from ThT fluorescence measurements were fitted against sigmoidal curves described by the following equation

$$I = \frac{I_{max} - I_0}{1 + e^{(t - t_{1/2})/k]}}. \quad (1)$$

Where I_0 and I_{max} are the initial and maximum fluorescence values, $t_{1/2}$ is the time required to reach half intensity, and k is apparent first-order constant for the growth of fibrils. The lag time t, the time predicted by nucleation dependent polymerization theory before detectable amyloid formation occurs, is given by '$t_{1/2} - 2/k$ [30].

2.4. CR binding assay

CR solution (200 μM) was prepared by dissolving the dye in 10 mM sodium phosphate buffer. Five microliter of each well-mixed sample was added to 500 μL of the CR solution (final concentration 20 μM) and incubated for 30 min. Spectral measurements were recorded from 400 to 600 nm by an ultraviolet-visible spectrometer (Shimadzu UV-1800, Japan). All samples were measured in triplicate.

2.5. Intrinsic fluorescence assay

Steady state intrinsic fluorescence spectra were monitored with a Hitachi F-4600 Florescence Spectrophotometer using a quartz cuvette with a path length of 1 cm. The measurements were recorded between 300 and 420 nm by exciting the samples at 280 nm 80 μL lysozyme samples (0.5 mg/ml) with various concentrations of zinc ions were mixed with 1920 μL hydrophobic acid.

2.6. ANS fluorescence assay

ANS dye was dissolved in the sodium phosphate buffer (10 mM Na_2HPO_4, 1.76 mM NaH_2PO_4, pH 7.4) to a final concentration of 20 μM. The HEWL solutions with different concentrations of zinc ions were mixed with the ANS dye in a volume ratio of 1:9 and incubated at room temperature for 30 min. ANS fluorescence spectra were made on an F-4600 Fluorescence Spectrophotometer (Hitachi, Japan) using 1 cm light path quartz cuvette. A slit width of 5 nm was used on both excitation and emission wavelengths, respectively. The fluorescence was excited at 380 nm and emission spectra were measured between 400 and 600 nm. The fluorescence of the solvent was subtracted. All measurements were performed in triplicate.

2.7. Circular dichroism (CD) measurement

CD spectra were performed on a Jasco-815 spectrophotometer. The path length is 1 mm. Spectra were recorded from 195 to 250 nm with a step size of 1.0 nm at a bandwidth of 1.5 nm and averaging time of 10s. To be accurate, five scans were averaged. CD spectra of the appropriate buffers were recorded and subtracted from the protein spectra as blank.

2.8. Cell viability assay

SH-SY5Y (human neuroblastoma cell line) cells were cultured at a density 5×10^4cells/ml DMEM medium supplemented with 10% FBS and 100U/ml penicillin and incubated in humidified 5% (v/v) CO_2/air at 37 °C. The viability of SH-SY5Y cells was determined using the MTT assay. The cells were seeded in the 96-well plates for 24 h. For the MTT reduction assays, sample solutions of HEWL in the absence and presence of zinc ions were added to wells incubated for 24 h and cell viability was assessed. MTT was added to the culture medium to yield a final concentration of 0.5 mg/ml and incubated for 4 h at 37 °C in CO_2 incubator then removed supernatant carefully. 200 μL of DMSO was added and mixed. After 20 h of incubation in a humidified CO_2 incubator, the absorbance at 570 nm was read using a Micro plate absorbance reader (Bio-Rad Instruments, iMarkTM). Cell viability was compared to control cells without prior exposure to the fibril solutions.

2.9. Transmission electron microscopy (TEM)

A 10 μL HEWL sample was placed on formvar-coated copper grids and left at room temperature for 5 min. The grids were stained with 2% (w/v) phosphotungstic acid solution for another 90 s before