International Journal of Biological Macromolecules 95 (2017) 713–718

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Attenuation of amyloid fibrillation in presence of Warfarin: A biophysical investigation

Mohammad Khursheed Siddiqi[a], Parvez Alam[a], Sumit Kumar Chaturvedi[a], Saima Nusrat[a], Yasser E. Shahein[b], Rizwan Hasan Khan[a,*]

[a] Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh, 202002, Uttar Pradesh, India
[b] Molecular Biology Department, Genetic Engineering and Biotechnology Division, National Research Centre, Dokki, Cairo, Egypt

ARTICLE INFO

Article history:
Received 15 October 2016
Received in revised form
26 November 2016
Accepted 28 November 2016
Available online 2 December 2016

Keywords:
Amyloidosis
ThT binding
Aggregation inhibition
Congo red binding
Beta sheet conformation
Amyloid fibrils

ABSTRACT

Protein misfolding and aggregation are associated with more than twenty diseases, such as neurodegenerative diseases. The amyloid oligomers and fibrils may induce cell membrane disruption and lead to cell apoptosis. A great number of studies have focused on discovery of amyloid inhibitors which may prevent or treat amyloidosis. In this study, we used human serum albumin (HSA) as an amyloid model to test the anti-amyloid effects of warfarin (WFN), a very well-known drug for treatment of thrombosis and also used by biophysicists to characterize the specific binding site on HSA (site I of subdomain IIA). We have used a combination of different biophysical, spectroscopic and imaging techniques to prove the anti-amyloidogenic behavior of WFN. Our results demonstrated that WFN is capable enough to inhibit the HSA fibrillation. Exposed HSA surface hydrophobicity was decreased by 50% as judged by ANS analysis. Moreover, anti-amyloidegenic behavior of WFN was found to be concentration dependent as supported by decreased ThT fluorescence by 22.4% and 46% at WFN concentrations of 500 and 1000 μM, respectively. Circular dichroism technique showed the change in secondary structure of native HSA as well as in presence of WFN. These results suggests that WFN is capable of inhibiting amyloid aggregation, hence, WFN related compounds may thus be further explored for designing effective anti-amyloidosis compounds.

1. Introduction

Protein misfolding and their aberrant assembly into amyloid fibrils attracted the attention of several researchers because of their association with amyloid associated diseases – commonly known as amyloidoses [1]. Amyloids are characterized by the presence of characteristic cross β sheet structure where β sheets run perpendicular to the fibril axis; and are common feature of systemic and neurodegenerative diseases ranging from Alzheimer's, Parkinson's, Huntington's diseases, type II diabetes, and the systemic condition of dialysis related amyloidosis [2–4]. It has been reported that proteins which are not responsible for any amyloidoses, may also form amyloid fibrils under different conditions which dictates that inherent ability of polypeptide to amyloid fibril but their propensity to form amyloid fibrils depend on their amino acid sequence [5,6]. Moreover, it is believed that amyloid fibril formation provides an alternative path to the native protein to form a structure with global energy minima (nonnative structure usually amyloids) [7]. Therefore, more emphasis is directed towards the stabilization of the native structure of protein. Since, amyloids fibrils are rich in cross β-sheet structural content would suggest that the strategies with potential to inhibit the packing of β-sheet could be beneficial for the development of therapeutics against the amyloid associated pathogenesis.

Warfarin (also known as Coumadin) is widely known for its anticoagulant activity and useful in treatment of thromboembolic strokes in common clinical conditions like atrial fibrillation, prosthetic heart valves, venous thromboembolism, and coronary artery disease [8–10]. Herein, we have tested the effect of WFN against HSA fibrillation. Human serum consists of variety of proteins, of which albumin is the most abundant one [11,12]. Under physiological conditions, HSA acts as a carrier of endogenous as well as exogenous drugs [13]. It also regulates the osmolarity of blood and helps in regulation of blood pressure [12,14]. Apart from its physiological function, HSA is also known for its readability to aggregate [15]. Therefore, HSA is exploited as a model protein to study mechanisms of aggregation [16] and inhibition which will help in getting

* Corresponding author.
E-mail addresses: rizwanhkhan1@yahoo.com, rizwanhkhan1@gmail.com (R.H. Khan).

http://dx.doi.org/10.1016/j.ijbiomac.2016.11.110
0141-8130/

better insight into the etiology involved in amyloid associated diseases and their possible intervention.

HSA is a globular protein with single polypeptide chain. It consists of 585 amino acid residues and molecular weight of 66411 kD. The crystal structure of albumin reveals a heart-shaped molecule that contains an equilateral triangle with sides of ~8 nm and a depth of 3 nm [17]. It has three major domains which are divided into six subdomains. HSA has 35 SH groups in total, of which 34 are involved in making 17 disulfide bonds and one SH group remains free [18–20]. HSA performs other functions such as sequestering oxygen free radicals and inactivating various toxic lipophilic metabolites [21]. HSA predominantly exists in α helical conformation because more than 60% of its sequence are involved in forming α helix [22–25]. Such properties of HSA do not reflect any features that make them more susceptible to aggregate. Therefore, HSA is induced to form amyloid under different destabilizing conditions [15].

In the present study, we have evaluated the effect of WFN on HSA amyloid formation by using different biophysical as well as imaging techniques. Thioflavin T (ThT) fluorescence assay was performed to monitor the formation of amyloid aggregate and their inhibition by WFN. Change in surface hydrophobicity was observed by 1-anilino 8-naphthalene sulfonate (ANS) fluorescence measurement. Further, congo red assay was performed to detect the presence of amyloid fibrils and circular dichroism spectroscopy was used to observe the changes in secondary structure of HSA. Moreover, size and morphology of aggregate were monitored by Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM), respectively.

2. Materials and methods

2.1. Preparation of HSA solution

The stock solution of HSA (500 μM) was prepared by dissolving it in 20 mM sodium phosphate buffer of pH 7.4 at 25 °C; and extensively dialyzed against the same buffer. Protein concentration was determined using a UV–vis spectrophotometer (Perkin Elmer Lambda25) $E^{1\%}_{cm}$ = 5.3 at 280 nm. HSA amyloid aggregates were prepared by incubating 20 μM of HSA at 65 °C for 120 h in a circulating shaking water bath [15].

2.2. pH measurement

pH was determined using Mettler Toledo Seven Easy pH meter (model S20) which was routinely calibrated with standard buffers. The experiments were performed in the 20 mM pH 7.4 sodium phosphate buffer. All preparations used in the experiments were filtered through 0.45 μm Millipore Millex-HV PVDF filter.

2.3. Thioflavin T binding assay

ThT was prepared by dissolving in double distilled water. The concentration of ThT was determined using a molar extinction coefficient $\varepsilon = 36000\ M^{-1}\ cm^{-1}$ at 412 nm. The protein samples (20 μM), was incubated at pH 7.4 for 120 h at 65 °C, with and without the addition of WFN (500 and 1000 μM). Post incubation, 20 μM of ThT solution was added to each sample and incubated for 30 min in the dark. The spectra were recorded in the wavelength range of 450–650 nm and the excitation wavelength was 440 nm. The excitation and emission slit widths were set at 10 nm. Appropriate blank corrections were done prior to all measurements.

2.4. Congo red binding assay

Congo red was prepared by dissolving in 20 mM phosphate buffer (pH 7.4) containing 50 mM NaCl. Concentration of Congo red was determined spectrophotometrically using $\varepsilon = 45\,000\ M^{-1}\ cm^{-1}$ at 498 nm 20 μM congo red was mixed with the protein samples in the absence and presence of WFN (incubated for 120 h at 65 °C) and then kept in dark for 15 min. The protein concentration was fixed at 20 μM. The absorption spectra (400–600 nm) of the samples were recorded on a UV–vis spectrophotometer (Perkin Elmer Lambda 25) in a 1 cm path length cuvette.

2.5. ANS fluorescence measurements

ANS binding assay was performed on Shimadzu spectrofluorophotometer (RF-5301 PC). ANS was mixed with the protein samples (incubated at 65 °C for 120 h in the absence and presence of WFN in 20 mM phosphate buffer pH 7.4 buffer) at a final protein/dye molar ratio of 1:50. Post incubation, samples were kept for 30 min at 25 °C in dark. Both excitation and emission slits were set at 5 and 3 nm, respectively. Emission spectra were recorded in the wavelength range of 400–650 nm by exciting ANS at 380 nm.

2.6. Circular dichroism measurements

Far-UV CD spectra were obtained using a JASCO-J 815 spectro polarimeter equipped with a Peltier-type temperature controller at 298 K using a quartz cell of path length of 0.1 cm. Spectra were recorded in the wavelength range of 202–250 nm at 25 °C. Three scans were accumulated at a scan speed of 100 nm min^{-1}. 5 μM of protein solution was used for far-UV CD spectra measurement in the absence and presence of WFN.

2.7. Transmission electron microscopy (TEM)

TEM images were taken on Philips CM-10 transmission electron microscope operating at an accelerating voltage of 200 kV. The amyloid fibril formation was assessed by applying 6 μl of HSA (20 μM) incubated for 120 h at 65 °C alone and with 500 and 1000 μM of WFN on 200-mesh copper grid covered by carbon-stabilized formvar film. Excess of fluid was removed after 2 min and the grids were then negatively stained with 2% (w/v) uranyl acetate. Images were viewed at 10000X. Before taking the image all the samples were incubated overnight.

2.8. Statistical analysis

All data were presented as mean ± standard error of the mean (SEM) from 3 independent determinations. The statistical analysis was made by performing one-way Analysis of variance (ANOVA) for 3 independent determinations. The significance of difference was determined by ANOVA analysis and/or a paired Student *t*-test. The difference was considered to be significant when the P value was less than 0.05.

3. Results and discussions

3.1. Aggregation kinetics by ThT fluorescence measurement

ThT is an extrinsic fluorophore that exhibits fluorescence upon binding with characteristic cross β sheet structure [26–29]. This property of ThT enables it to be exploited as a tool to characterize the amyloid aggregates. Here, we have utilized ThT dye to monitor the aggregation kinetics and their suppression with respect to ThT fluorescence intensity [27]. Fig. 1 shows the aggregation kinetics of HSA with respect to incubation time. Fig. 1 shows an increase in