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Binding of hen egg white lysozyme fibrils with nucleic acids

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ABSTRACT

Non proteinaceous substances are found to be associated with toxic protein aggregates commonly known as fibrils. Hen egg white lysozyme (HEWL) is able to form fibrillar species under various conditions. Here for the first time we report concentration dependent binding affinities of preformed HEWL fibrils towards DNA and RNA at physiological pH (pH 7.4). We have found that HEWL fibrils bind with DNA and RNA that is distinctly different when compared to native HEWL. The association constant (K_a) of native HEWL and ct-DNA at pH 7.4 is 6.8×10^5 M^{-1}. We have also investigated the conformational alterations of DNA that occur on binding with HEWL fibrils. Our study has demonstrated dominant electrostatic interactions between oppositely charged polyelectrolytes which accounts for the binding of nucleic acids with fibrils. The affinity between the moieties could lead to disruption in the functions of cellular components that might be attributed to the toxicity of the aggregates formed *in vivo*.

1. Introduction

Self assembly of proteins/polypeptides leads to formation of toxic deposits commonly known as amyloid fibrils. These fibrils share a common structural feature responsible for distinct pathological disorders such as Alzheimer's, Parkinson's, Huntington's and Prion diseases [1,2]. The nature of these deposits is mainly proteinaceous [3] though several reports have revealed the presence of other non-proteinaceous substrates such as polysaccharides, nucleic acids along with these aggregates [4–6]. Nucleic acids such as RNA were found to affect the aggregation of tau and prion proteins [6,7]. There are reports which have illustrated that DNA enhanced fibrillation of α-synuclein [8]. DNA was also found to influence the binding capability with curly fibrils of *Escherichia coli* [9]. The tight binding of DNA with human lysozyme and acylphosphatase fibrils is non specific in nature [10]. Association of human lysozyme and acylphosphatase fibrils with DNA is of high affinity and low specificity where structured fibrils act as suitable templates for binding with DNA [10]. RNA was found to facilitate aggregation of the tau protein [7,11] as well as the conversion of the prion protein [6]. In addition to this, amyloid fibrils can promote polymerization of nucleotides [12]. Binding of these polyanions (nucleic acids) with amyloid fibrils has encouraged researchers to study the nature, affinity and specificity of the binding characteristics in detail as they can in turn contribute to the modulation of cellular function.

Hen egg white lysozyme (HEWL) is a globular protein having 129 amino acid residues containing two different domains which are cross linked by four disulfide bonds [13]. Fibrillation of HEWL occurs under varying conditions such as elevated temperature, change in pH, and presence of several additives [14–24]. Human lysozyme, a structural homologue of HEWL is responsible for the origin of a hereditary systemic amyloidosis disease. The fibrils of both species resemblance one another enabling the use of HEWL as an appropriate model to study *in vitro* [15,25–27].

Understanding DNA–protein interactions is crucial and a primary requirement in chemical biology. It has been observed earlier that native HEWL binds with DNA in a non specific manner initiated primarily by electrostatic interactions [28]. Existence of three types of DNA–HEWL complexes were found depending on the degree of association and temperature [29]. Recently, it has been illustrated that apart from morphological variation, toxicity of amyloid fibrils is attributed to their interaction with membranes [30]. On the other hand, it has been reported that higher net charge of the aggregates as a result of codeposition with charged species might be responsible for better interaction with the plasma membrane [10]. With this background information and considering the fact that the nature of interactions between amyloid fibril and polyanions is still unclear, we have investigated the comparative binding affinity of native HEWL and HEWL fibrils with the nucleic acids. We have monitored the binding of DNA and RNA with both native HEWL and HEWL fibrils using an agarose gel based assay. Spectroscopic techniques such as Tryptophan (Trp) fluorescence, three dimensional fluorescence and circular dichroism (CD) spectroscopy were employed to monitor structural and conformational changes in DNA and HEWL. Our study reveals a differential binding pattern of native (functional) HEWL and fibrillar (dysfunctional) HEWL towards both DNA and RNA. This has significant biological relevance in terms of regulation of biological functions of both DNA and RNA.

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2. Materials and methods

2.1. Materials

Hen egg white lysozyme (HEWL), RNA (torula utilis), and Ribonuclease A (RNase A) were purchased from Sigma Chemical Co. (St. Louis, USA) and used as received. Ethidium bromide (EB), calf thymus DNA (ct-DNA), agarose superior grade type I and all other chemicals were obtained from SRL, India. Supercoiled circular *pBR322* DNA was obtained from Bangalore (Genei).

2.2. Preparation of HEWL fibrils

HEWL (150 μM) was incubated in the presence of 80% (*v*/*v*) ethanol at pH 7 (20 mM phosphate buffer in the presence of 20 mM NaCl) at ~60 °C for 6 h followed by incubation at room temperature as described earlier [31].

2.3. Agarose gel electrophoresis

Prior to the agarose gel based assay, preformed HEWL fibrils of varying concentration (20–120 μM) were incubated with *pBR322* DNA (supercoiled circular) keeping the DNA concentration at 18 μg/ml in 20 mM phosphate buffer of pH 7.4 in each case for 1 h at room temperature. Native HEWL was also incubated in a similar manner (20–120 μM) with *pBR322* DNA (18 μg/ml). Control experiments were performed in the absence of both native HEWL and HEWL fibrils. Agarose gel (0.5%) was prepared in Tris–acetic acid-EDTA (TAE) buffer (1X, pH 7.4) and stained with ethidium bromide solution for visualization. To determine the RNA binding property of HEWL fibrils, preformed HEWL fibrils of concentrations ranging from 20 to 120 μM were incubated with RNA (0.5 mg/ml) in 20 mM phosphate buffer of pH 7.4 in each case for 30 min at room temperature. Native HEWL was also incubated in a similar manner (20–120 μM) with RNA (0.5 mg/ml). Control experiments were carried out in the absence of both native HEWL and HEWL fibrils. Agarose gel (1.1%) was prepared in Tris acetic acid EDTA (TAE) buffer (1X, pH 7.4) and stained with ethidium bromide. To investigate whether binding of RNA with native or fibrillar HEWL can affect the degrading property of RNase A, we have performed agarose gel based assay using RNA (2 mg/ml) and RNase A (0.1 μM) in the presence of both native HEWL and HEWL fibrils (20 μM and 40 μM) separately. Native/fibrillar form of HEWL was first incubated with RNA at room temperature for 30 min followed by incubation with RNase A for another 30 min and then gels were run. The conditions have been kept the same as that of the RNA binding assay and control experiments were carried out in the absence of RNase A.

2.4. Intrinsic fluorescence measurements

Native HEWL (120 μM) and HEWL fibrils (120 μM) were incubated with ct-DNA (60 μM) in 20 mM phosphate buffer (pH 7.4) for 1 h at room temperature. Samples were diluted 24-fold to final protein and ct-DNA concentrations of 5 and 2.5 μM respectively. Trp fluorescence was monitored keeping the excitation maximum at 295 nm (to avoid any contribution from Tyr residues) in the scanning range of 315–550 nm in a Horiba Jobin Yvon Fluoromax 4 spectrofluorimeter. Slit width and integration time were kept at 5 nm and 0.2 s respectively. Each spectrum was corrected with respect to the corresponding blank. The apparent dissociation constant $(K_d)_{app}$ was determined assuming a one to one binding interaction for native HEWL and ct-DNA as has been suggested by earlier reports [32].

$$K_d = \frac{[\mathrm{HEWL}][\mathrm{DNA}]}{[\mathrm{HEWL}-\mathrm{DNA}]} \tag{1}$$

$$f = \frac{[\mathrm{HEWL}-\mathrm{DNA}]}{[\mathrm{HEWL}]_t} \tag{2}$$

$$f = \frac{(\mathrm{HEWL}_t + \mathrm{DNA}_t + K_d) - \sqrt{(\mathrm{HEWL}_t + \mathrm{DNA}_t + K_d)^2 - 4\mathrm{HEWL}_t DNA_t}}{2\mathrm{HEWL}_t} \tag{3}$$

t = total concentration of respective species.

Here,

$$f = \frac{\Delta F}{\Delta F_{\max}} \tag{4}$$

where $\Delta F = F_o - F_t$; $\Delta F_{max} = F_o - F_f$; F_o, F_t, F_f are Trp fluorescence intensity (at 344 nm) of HEWL in the absence of DNA, in the presence of varying concentration of DNA (0–3.225 μM) and when HEWL is fully bound to DNA.

The association constant (K_a) for HEWL and DNA binding can then be obtained as follows:

$$K_a = \frac{1}{K_d} \tag{5}$$

The binding studies were performed keeping the initial concentration of native HEWL at 2 μM (3 ml) with successive addition of 10 μl of ct-DNA (100 μM) with other parameters similar to those mentioned above. Binding parameters were optimized using nonlinear least square curve fitting in Origin Pro 7.5.

2.5. Circular dichroism (CD) spectroscopy

Far-UV and near-UV circular dichroism (CD) spectra were acquired using a Jasco-810 spectrophotometer. A quartz cuvette of 0.1 cm path length was used. CD spectra were accumulated keeping protein/fibril and ct-DNA concentrations at 40 μM and 20 μM respectively and scanned between 190 and 350 nm at 25 °C at a scan rate of 50 nm/min. In each case, samples (protein/fibril and/or ct-DNA) were incubated for 1 h prior to each scan at room temperature. Protein secondary structure content was estimated using an online server, DICHROWEB [33]. Spectra were corrected with respect to the appropriate blank.

2.6. Three-dimensional fluorescence

Three-dimensional spectra of proteins were acquired in a Hitachi F-7000 spectrofluorimeter by scanning the excitation wavelength in the region of 200–350 nm and emission wavelength from 210 to 500 nm at an interval of 10 nm and slit width of 5 nm. Native HEWL (120 μM) and HEWL fibrils (120 μM) were incubated separately with ct-DNA (60 μM) for 1 h at room temperature prior to scanning. Samples were diluted using 20 mM phosphate buffer (pH 7.4) to accomplish a final protein/fibril and ct-DNA concentration of 10 μM and 5 μM respectively.

3. Results and discussion

Co-deposition of non-proteinaceous substances such as nucleic acids with fibrillar species in case of patients suffering from Alzheimer's disease has been found to occur [34]. Protein aggregates often serve as biological polyelectrolytes bearing opposite charge to that of the other non-proteinaceous substrates and undergo strong electrostatic interactions. This results in an assembly formation between oppositely charged polyelectrolytes in most of the cases that usually occur with high affinity but low specificity.