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Slow aggregation of lysozyme in alkaline pH monitored in real time employing the fluorescence anisotropy of covalently labelled dansyl probe

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Abstract The onset of hen egg white lysozyme aggregation on exposure to alkaline pH of 12.2 and subsequent slow growth of soluble lysozyme aggregates (at 298 K) was directly monitored by steady-state and time-resolved fluorescence anisotropy of covalently attached dansyl probe over a period of 24 h. The rotational correlation time accounting for tumbling of lysozyme in solution (40 μM) increased from ~3.6 ns (in pH 7) to ~40 ns on exposure to pH 12.2 over a period of 6 h and remained stable thereafter. The growth of aggregates was strongly concentration dependent, irreversible after 60 min and inhibited by the presence of 0.9 M L-arginine in the medium. The day old aggregates were resistant to denaturation by 6 M guanidine · HCl. Our results reveal slow segmental motion of the dansyl probe in day old aggregates in the absence of L-arginine (0.9 M), but a much faster motion in its presence, when growth of aggregates is halted.

Keywords: Lysozyme aggregation; Fluorescence anisotropy; Dansyl; Arginine

1. Introduction

Investigating the molecular basis of protein aggregation is important owing to its role in a number of neurodegenerative diseases like Alzheimer's and Parkinson's disease [1]. Recent findings indicate that soluble oligomers of proteins which are precursors in the pathway leading to insoluble amyloid fibrils are the toxic species [2–4]. Also, in most cases where protein aggregation and disease associated phenotypes were separated in time, fibrous proteinacious inclusions were observed well after the onset of the behavioural or neuropathological symptoms [5]. Clearly, the soluble aggregates formed early in the aggregation pathway seem to play a major role in the neurodegenerative diseases.

Early diagnosis of amyloid disease has proved difficult because monitoring the early growth of protein aggregates has not been possible for two reasons: (i) a simple and sensitive method to specifically detect protein aggregates in solution is not available, and (ii) the rapid rate at which proteins aggregate in vitro (e.g., barstar, a ribonuclease inhibitor, aggregates almost instantaneously when transferred to pH 3 from pH 7 [6]) makes tracking difficult. A consequence of the latter is that, structural snapshots of the intervening steps of the aggregation pathway, which are important to develop a molecular level understanding, have remained inadequate. This report addresses both the above issues in protein aggregation. Once the method to monitor growth of aggregates was established, therapeutic strategies to inhibit the growth are easily tested and identified as shown by us in the case of L-arginine.

2. Materials and methods

2.1. Materials

Hen egg white lysozyme (HEWL) (L-6876) and L-arginine hydrochloride (A-5131) used in our studies was procured from Sigma–Aldrich Company Pvt. Ltd. 2-Dimethylaminonaphthalene-6-sulfonyl chloride (D-23), was obtained from Molecular Probes (Eugene, Oregon, USA).

2.2. Labelling with dansyl probe

For labelling, the procedure recommended by Molecular Probes was followed. To a 3 mM lysozyme solution in 1 ml of 0.1 M, pH 9 sodium bicarbonate buffer, 50 μl of 75 mM dansyl chloride in DMF was added. The reaction mixture was kept at 4 °C with constant stirring for 3 h. Subsequently, 1.5 ml buffer was added to the reaction mixture. The unlabelled fluorophores were separated from the labelled protein solution using an Amersham PD-10 desalting column previously eluted with 50 mM, pH 7 sodium phosphate buffer. Protein and dye concentrations were determined by using absorbance at 280 and 339 nm, respectively. The molar extinction coefficient for the labelled probe at 339 nm is 3370 M^{-1} cm^{-1} [14,15]. The molar ratio of the protein to dye in the dye conjugated protein was calculated to be ~1:1. For aggregation experiments, stocks of the labelled protein in pH 7 buffer were diluted at least tenfold in 50 mM, pH 12.2 phosphate buffer.

2.3. Steady-state fluorescence

Steady-state fluorescence anisotropy measurements (*G* factor corrected) were made in Jobin-Yvon Fluoromax-3 spectrofluorometer equipped with automated Glan-Thompson polarisers and a PMT operating at a voltage of 950 V in photon counting mode. Slit widths for excitation at 370 nm were 1 nm and for emission at 444 nm between 5 and 10 nm. The background intensity from Raman scatter or buffer was negligible (<5%) compared to sample emission intensity under identical conditions as observed with blank samples.

2.4. Time-resolved fluorescence

Nanosecond time-resolved fluorescence intensity and anisotropy decay measurements were carried out in Fluorocube (IBH, Glasgow) using the time-correlated single photon counting method. The system was equipped with motorized Glan-Thompson polarisers and an IBH TBX04 photon-detection module. For excitation an IBH 370 nm NanoLED with a FWHM of 1.3 ns and a repetition rate of 1 MHz was used. Excitation light was attenuated, whenever required, using neutral density filters (OD = 3). A Schott UG-1 filter was also

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used at the excitation end to cut out long wavelength glow from the 370 nm light source. Emission was detected after passing it through a Schott 420 nm long pass filter in order to block excitation photons. Fluorescence intensity decay was collected in 1024 channels, with a temporal resolution of 0.113 ns/channel. Peak counts were typically between 10 and 15 k. All measurements were done at 298 K.

The fluorescence intensity decay data were fit to a multi-exponential model using the IBH DAS6 fluorescence decay analysis software. The anisotropy decay data were fitted to multi-exponential decay model, as described earlier [6]. In both cases above, the goodness-of-fit was determined by reduced χ^2 and the randomness of the residuals. For anisotropy decay, the fits were additionally constrained to yield a calculated steady-state anisotropy value that is close to that observed independently using steady-state fluorimeter [6,17].

3. Results and discussion

Earlier reports using equilibrium sedimentation, have shown that HEWL (at 1 mM concentration) forms dimers in the pH range 5–9 and higher oligomers at pH 10–11 [8,9]. There are also reports on the formation of fibrils by hen egg white lysozyme at acidic pH and elevated temperatures [10,11]. Our results showed that the tendency to aggregate (at μM concentrations) was most pronounced at pH 12.2 (see supplementary material). Thus, exposure to alkaline pH of 12.2 served as a convenient approach to initiate the aggregation of HEWL.

Fluorescence anisotropy is an excellent technique to study the rotational motion of fluorophores in the excited state following their excitation using plane polarized light [7]. Steady-state fluorescence anisotropy (r_{ss}) gives us a time integrated average value of the rotational motion of molecules in the excited state. This parameter is however dependent both on the fluorescence lifetime and rotational correlation time of the fluorescent probe. Time-resolved anisotropy decay observations can reveal the multiple rotational motions experienced by the fluorophore in the excited state in terms of their rotational correlation time(s), ϕ_i. As ϕ arising from whole protein tumbling, is directly related to the size (volume) of the fluorophore conjugated protein from the Stokes–Einstein equation, any event, like protein aggregation, which changes the size of the protein can be directly monitored by measuring ϕ. In this work, HEWL was covalently tagged with the fluorescent probe, 2-dimethylaminonaphthalene-6-sulfonyl chloride (dansyl chloride from now on) in order to specifically monitor the rotational motion of lysozyme in solution under two different conditions: (a) pH 12.2 (aggregation prone condition); and (b) pH 7 (native state).

Fig. 1 shows the variation in fluorescence steady-state anisotropy (r_{ss}) of dansyl labelled lysozyme with time at pH 12.2 and pH 7 (control). At pH 7, no significant change in r_{ss} (~0.06) of lysozyme is observed for over a day, indicating that the overall structure of native protein is intact and unperturbed in this time span. At pH 12.2, immediately on exposure to alkaline pH, the r_{ss} increased to ~0.08. Subsequently, we see a gradual increase in r_{ss} during the first 360 min, followed by saturation over a period of 24 h in contrast to pH 7 (native state). Importantly, the magnitude of this increase is dependent on the concentration of lysozyme from 4 to 200 μM (Fig. 1). At 4 μM, the r_{ss} saturates at 0.13, while with 40 μM it settles down at 0.15 and finally at 200 μM, it stabilises at 0.20. Time-resolved fluorescence intensity decays with 40 μM lysozyme have revealed the following: (a) the mean fluorescence

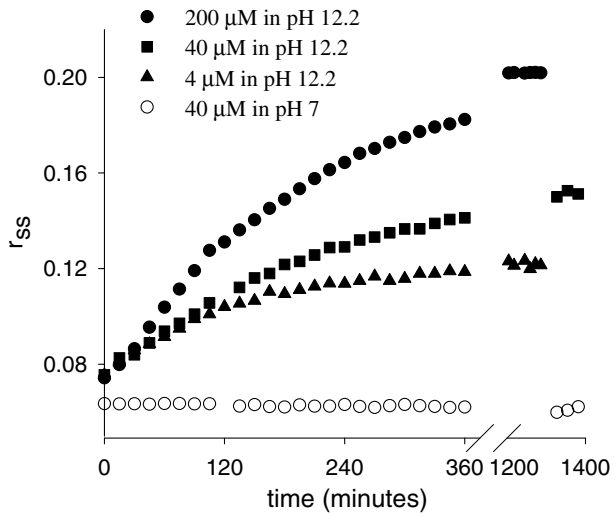

Fig. 1. Change in steady-state anisotropy of dansyl labelled lysozyme with time. pH 7, 40 μM, open circles; pH 12.2, 4 μM, filled triangles; pH 12.2, 40 μM, filled squares; and pH 12.2, 200 μM (only 40 μM was dansyl labelled, rest are unlabelled), filled circles. All measurements were done at 298 K.

lifetime of dansyl conjugated HEWL (Fig. 2) was constant over the period of first 360 min shown in Fig. 1, and (b) the mean fluorescence lifetime at pH 12.2 showed no significant variation compared to that at pH 7 (Fig. 2). Therefore, the quantum yield of the dansyl probe is fairly constant with time and unaffected by change in pH. Covalent linkage of the dansyl chromophore to a solvent exposed lysine residue on the surface can account for this insensitivity of dansyl quantum yield to lysozyme aggregation. Therefore, the observed increase in r_{ss} at pH 12.2, can only occur owing to a large increase in average rotational correlation time of dansyl probe. This would

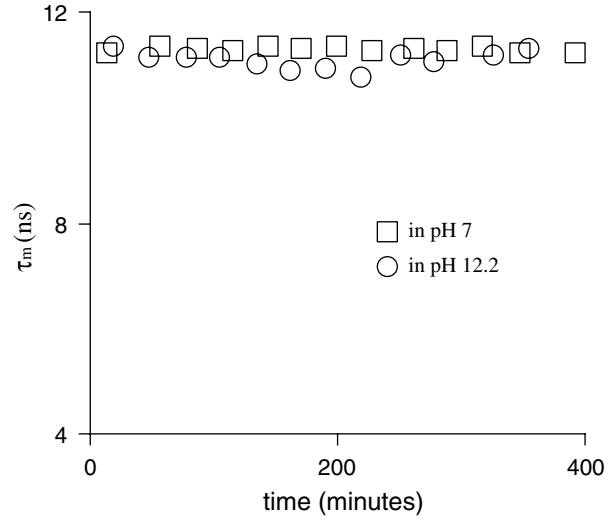

Fig. 2. The mean fluorescence lifetime, τ_m, of 40 μM dansyl labelled lysozyme was calculated using the formula $\tau_m = \sum_{i=1}^{3} \alpha_i \tau_i$, where τ_i and α_i correspond to the ith lifetime component and its amplitude, respectively. The intensity decays (see supplementary material) were monitored over a period of ~360 min. The mean fluorescence lifetimes (τ_m) were determined in pH 7 (open squares) and in pH 12.2 buffer (open circles).