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Visible light-induced insulin aggregation on surfaces via photoexcitation of bound thioflavin T

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ABSTRACT

Insulin is known to form amyloid aggregates when agitated in a hydrophobic container. Amyloid aggregation is routinely measured by the fluorescence of the conformational dye thioflavin T, which, when incorporated into amyloid fibers, fluoresces at 480 nm. The kinetics of amyloid aggregation in general is characterized by an initial lag-phase, during which aggregative nuclei form on the hydrophobic surface. These nuclei then lead to the formation of fibrils presenting a rapid growth during the elongation phase. Here we describe a novel mechanism of insulin amyloid aggregation which is surprisingly devoid of a lag-time for nucleation. The excitation of thioflavin T by visible light at 440 nm induces the aggregation of thioflavin T-positive insulin fibrils on hydrophobic surfaces in the presence of strong agitation and at physiological pH. This process is material surface-induced and depends on the fact that surface-adsorbed insulin can bind thioflavin T. Light-induced insulin aggregation kinetics is thioflavin T-mediated and is based on an energy transfer from visible light to the protein via thioflavin T. It relies on a constant supply of thioflavin T and insulin from the solution to the aggregate. The growth rate increases with the irradiance and with the concentration of thioflavin T. The supply of insulin seems to be the limiting factor of aggregate growth. This light-induced aggregation process allows the formation of local surface-bound aggregation patterns.

1. Introduction

In solution, the folding and conformational stability of proteins are mainly affected by temperature, pH, ionic strength, intermolecular interactions, agitation and exposure to electromagnetic radiation [1]. Protein aggregation usually results from conformational transition states or chemical degradations promoting a structural stabilization by inter-protein interactions [2].

Ultraviolet radiations for instance can directly excite aromatic amino acids, inducing protein denaturation. In particular, it has been shown for cutinase that an UV-excited tryptophan can disrupt an adjacent disulfide bridge via the ejection of an electron [3]. Moreover, the release of the energy of an absorbed UV photon as vibrational energy has been proposed as the mechanisms of reversible denaturation and subsequent aggregation of β-crystallin [4].

By contrast, the effect of visible light on protein stability is seldom described in the scientific literature, because most soluble proteins do not absorb radiations from this part of the spectrum. Light-induced functions in proteins are therefore based on the excitation of a bound chromophore and are mediated either by a change in the chromophore structure (e.g. rhodopsin [5]) or via a chemical reaction of the chromophore with the protein (e.g. LOV domain [6]). In addition, porphyrins, among other molecules, are known to be able to bind to proteins and oxidize them photochemically, directly or indirectly, via reactive oxygen species production [7,8].

Amyloid fibers are a class of highly ordered aggregates [9] in which the proto-filaments are stabilized by inter-protein β-sheets and the bundling of proto filaments into fibrils is in turn stabilized by the hydrophobic effect. Thioflavin T (ThT) undergoes a fluorescence shift when bound to amyloid fibrils, a state referred to as ThT-positive. Due to their high activation energy, the amyloid fibril formation usually follows a nucleated aggregation kinetic characterized by a lag-time [10].

Insulin, the most used protein drug, forms such amyloid aggregates at low pH and high temperature [11]. In addition amyloid-like aggregates can be formed at physiological conditions upon agitation by nucleation on hydrophobic surfaces, which raises formulation as well as possible iatrogenic issues [12,13]. Previous studies have shown that the rapid adsorption of an insulin layer on hydrophobic surfaces was triggering the slow conformational transition towards its amyloid state

Abbreviation: AOI, Area Of Interest; HI, Human Insulin; LIA, Light-Induced Aggregation; ND, Neutral Density; ThT, Thioflavin T; ThT_F, fluorescent Thioflavin T inside the aggregates

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[14–16]. Heterogeneous nucleation of insulin on hydrophobic and rough surfaces presents a much higher rate than homogeneous nucleation in solution [17].

Here we present a setup allowing the constant agitation of a protein solution above a continuously illuminated surface in the presence of ThT. We demonstrate, for the first time, that the energy transfer from visible light to insulin, mediated by ThT, triggers light-induced aggregation (LIA) of human insulin at a hydrophobic surface. The formation of these ThT-positive aggregates is restricted to the illumination area, which could be used for patterning strategies. Interestingly the aggregation kinetics follows a quasi-exponential growth without any observable lag-time which is unusual for amyloid-like aggregation [18].

2. Materials and Methods

2.1. Insulin and Thioflavin T Preparation

Recombinant human insulin (HI) produced in *Saccharomyces cerevisiae* was purchased from Sigma-Aldrich (ref: I2643). HI solutions were prepared in TN buffer (25 mM Tris-HCl pH 7.4, 125 mM NaCl), using Milli-Q® deonized water (resistivity = 18 MΩ·cm at 25 °C). In order to fully solubilize insulin, the pH of the solution was first lowered to 3.2 using 1 M HCl then adjusted to pH 7.4 with 1 M NaOH using a pH electrode. The HI concentration was then precisely adjusted to 86 μM, using the 280 nm absorbance (ε_{280nm} = 5.53 $mM^{-1}{\cdot}cm^{-1}$). Once adjusted at the proper concentration, the HI solution was filtered through a 0.22 μm Millex-GV filter unit (ref: SLGV033SS). The solutions were stored at T = 4 °C for < 2 weeks and filtered again before use.

Upon binding to amyloid fibers, Thioflavin T (ThT) undergoes a fluorescence shift from (λex = 342 nm; λem = 430 nm) to (λex = 450 nm; λem = 482 nm). This property allows the direct quantification of amyloid fibers by fluorescence since the fluorescence of bound ThT is proportional to the amount of amyloid fibers. Thioflavin T (from Sigma-Aldrich, ref.: T3516) was solubilized in TN buffer. The ThT concentration was then adjusted to 1 mM by absorbance measurement (ε_{416nm} = 26.6 $mM^{-1}{\cdot}cm^{-1}$ in ethanol). Finally, ThT solutions were filtered through a 0.22 μm Millex-GV filter and stored at 4 °C protected from light for < 2 weeks.

2.2. Material surface treatment

Borosilicate glass coverslips (VWR international 631-0162) were used either without surface treatment (water contact angle = 60°) or after etching in 14 M NaOH for 30 min (water contact angle = 30°). As the same rotating glass rod was used for every experiment, it was cleaned with sodium hypochlorite to remove any adsorbed protein, then etched in the same manner as described before to prevent protein adsorption and aggregation on its surface.

2.3. Experimental setup

The experimental setup consists of a home-made chamber installed on the microscope stage of an inverted microscope (Fig. 1A). The microscope provides both the illumination for the photoactivation process and the monitoring of ThT fluorescence. The chamber was built using a 5 mm diameter tube cut from the top of a 1200 μL polypropylene pipette tip (Sorenson 34000), sealed over a borosilicate glass coverslip using melted Parafilm M®. It was filled with 750 μL of a protein and ThT solution, continuously stirred with a 1.5 mm diameter cylindrical glass rod rotating at 1200 rpm thanks to a 6 mm diameter DC gear motor (Nano Planetary 16 mm Type ref.: 206-102). The chamber was then placed on the stage of an Olympus IX71 inverted microscope and illuminated by episcopic illumination at the ThT excitation wavelength through a 60 x magnification objective (1.25 numerical aperture). The microscope is fitted with a mercury short arc bulb (Osram cb1915239) and a Chroma DEAC filter cube (ref. U-N31036v2 C108867). The spectroscopic parameters of the illumination and the fluorescence recording were: excitation wavelength λ_{ex} = 436 nm, bandwidth 20 nm, long-pass dichroic mirror cutoff wavelength λ_{cutoff} = 455 nm, emission wavelength λ_{em} = 480 nm, bandwidth 30 nm. The illuminating light intensity was modulated using neutral density (ND) filters and the area of illumination was delimited using the field diaphragm (Fig. 1C). The time lapse imaging of the ThT fluorescence distribution at the surface was performed with an Olympus DP30BW camera (256 grey levels). The exposure time is set depending on the illumination irradiance.

The illumination irradiance was determined in the following manner. The radiant flux falling on the illuminated area (1725 μm^2) was measured at 440 nm using a photodiode detector (18I27 Lasermate Q, Coherent, USA) with each ND filter used in this study. From these measurements we determined that the radiant flux (P) within the total illuminated area was given by $P = 3.3187{\cdot}\tau$ (mW), where τ is the ND filter transmittance. This corresponds to an irradiance $I = 1920{\cdot}\tau$ nW·μm^{-2}.

For insulin aggregation assays on plain surfaces, the protein solution contained 86 μM HI and 20 μM ThT in TN buffer (unless otherwise specified). The field diaphragm aperture was set to its minimum resulting in an octagonal illumination area (50 μm diameter, Fig. 1C). The illumination was continuous, at different light intensities, and the surface fluorescence was measured every 30 s.

2.4. Light-induced aggregation at bead surfaces

For insulin aggregation assays on beads, red fluorescent carboxylate-modified polystyrene beads (0.5 μm diameter, $4.87{\cdot}10^7$ beads·mL^{-1}) were purchased from Sigma-Aldrich (L3280) and diluted 100 times in TN buffer. Then 10 μL of the diluted suspension were added to the protein solution containing 86 μM HI and 20 μM ThT in TN buffer. The suspension was observed under red fluorescence conditions (λ_{ex} = 575 nm; λ_{em} = 610 nm) for 10 min, until about 10 beads had been stably adsorbed on the surface in the field of view. The illumination was then switched to the ThT fluorescence conditions and the aggregation kinetics was recorded as described above.

2.5. Peptide addition during exponential growth of the aggregates

In order to test the effect of peptides in solution on the kinetic of light-induced aggregation, we constructed a chamber fitted with an injection hole allowing the direct addition of small volumes to the solution during the imaging process. We tested the effect of peptides on the growth rate via the addition of 5 μM $(LK)_5L$ peptide and observed peptide localization via the addition of 0.42 μM TAMRA-$(LK)_5L$ peptide. The two peptide solutions were prepared as described in [19].

2.6. Image processing and data analysis

We defined an area of interest (AOI) located in the center of the illumination field (Fig. 1C). The fluorescence intensity of each image was obtained by averaging the value of the pixels in this region: average raw fluorescence intensity. The saturation of the CCD camera limits the linear range of fluorescence recordings. We therefore excluded from analysis images presenting saturated pixels. In addition, we observed, after sufficient time, that ThT-positive objects grow rapidly extending in height above the coverslip surface. This results in defocusing of the fluorescent zone and therefore a reduction of its measured fluorescence intensity. Images that were not well focused were therefore removed from the quantitative analysis.

To compare fluorescence intensities measured with different exposure times t_{exp} (s), at different irradiances $I_0 = 1920{\cdot}\tau$ (nW·μm^{-2}), where τ is the ND filter transmittance, and at different ThT concentrations [ThT] (μM), the average raw fluorescence intensity is divided by the following coefficient $H = \tau{\cdot}t_{exp}{\cdot}[ThT]/0.6\ s^{-1}{\cdot}\mu M^{-1}$, resulting in F, the normalized surface fluorescence intensity. The