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Mechanism of aggregation of UV-irradiated glycogen phosphorylase *b* at a low temperature in the presence of crowders and trimethylamine N-oxide

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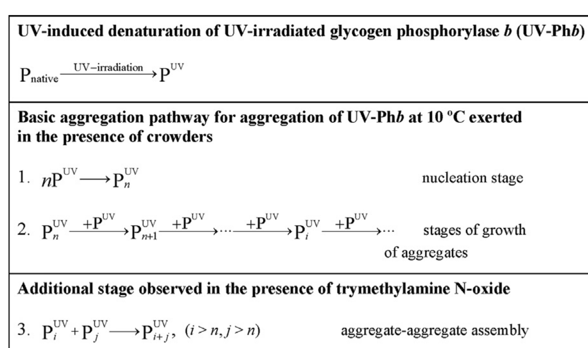
HIGHLIGHTS

- Kinetic regime of aggregation of UV-irradiated glycogen phosphorylase *b* at 10 °C has been established.
- Crowders (Ficoll-70 and polyethylene glycol) and osmolyte trimethylamine N-oxide (TMAO) stimulate protein aggregation.
- The initial rate of the stage of aggregate growth is proportional to the protein concentration squared.
- In contrast to crowders, TMAO induces sticking of the aggregates formed in the aggregation process.

GRAPHICAL ABSTRACT



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ABSTRACT

To characterize the initial stages of protein aggregation, the kinetics of aggregation of UV-irradiated glycogen phosphorylase *b* (UV-Ph*b*) was studied under conditions when the aggregation proceeded at a low rate (10 °C, 0.03 M Hepes buffer, pH 6.8, containing 0.1 M NaCl). Aggregation of UV-Ph*b* was induced by polyethylene glycol and Ficoll-70, acting as crowders, or a natural osmolyte trimethylamine N-oxide (TMAO). It has been shown that the initial rate of the stage of aggregate growth is proportional to the protein concentration squared, suggesting that the order of aggregation with respect to the protein is equal to two. It has been concluded that the aggregation mechanism of UV-Ph*b* at 10 °C in the presence of crowders includes the nucleation stage and stages of protein aggregate growth (the basic aggregation pathway). The aggregation mechanism is complicated in the presence of TMAO, and the stage of aggregate-aggregate assembly induced by TMAO should be added to the basic aggregation pathway. It has been shown that the ability of TMAO at a low concentration (0.05 M) to induce aggregation of UV-Ph*b* is due to the decrease in the absolute value of zeta potential of the protein in the presence of TMAO.

1. Introduction

All physiological media are very crowded owing to the presence of macromolecules taking up a significant part of the total cell volume (5–40%) [1–4], which becomes inaccessible for other macromolecules. Therefore, the available volume in the cell is reduced. The term “macromolecular crowding” means the non-specific effect of steric repulsions (excluded volume effect) of molecules on specific reactions

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that occur in media with a high degree of occupied volume [3,5].

Any reaction, which increases the available cellular volume, will be stimulated by crowded conditions [4]. Crowding stimulates more compact conformations of macromolecules, protein folding, association and supramolecular structure formation, protein aggregation and formation of amyloid inclusion bodies in some diseases [3–25]. On the contrary, crowding may interfere with protein denaturation [8,26–28]. To mimic crowded environment in vitro, macromolecules, such as polymers polyethylene glycol (PEG) and Ficoll, and neutral proteins are used.

Most living cells adapt to stress by accumulating high concentrations of osmolytes, which protect proteins under stress conditions. In many cases the protective stabilizing effect of natural osmolytes under stress conditions can be explained by the effect of excluded volume [29,30].

Natural osmolyte trimethylamine N-oxide (TMAO) is widely used as a crowding agent [6–9,11,21,27,31–33] in vitro at pH 6.0–8.0. In this range of pH, TMAO is almost neutral (zwitterionic form) [34] and it stabilizes protein structure [31,33]. Cho and coworkers have pointed to potential similarities between the action of TMAO on long polypeptide chains and entropic stabilization of proteins in a crowded environment and deduced that TMAO is a nanocrowding particle [32]. It should be noted that some researchers used TMAO as a chemical [35,36] or pharmaceutical [37] chaperone.

Generally, models of two classes are used to explain effects of osmolytes. The first class focuses on the binding between osmolytes and proteins. The second class focuses on the excluded volume effects arising from the steric repulsions between osmolytes and the protein. This class also includes preferential hydration [35,38–40], when osmolyte molecules are excluded from the layer close to the surface of the protein, and their place is taken by the water molecules whose properties in boundary (hydrated) layer differ from the water properties in free volume [41]. The thermodynamic equivalence of both approaches was shown by Wills and Winzor [42] and was discussed in a review [43]. However, Weatherly and Pielak [44] believe that a simple model is not appropriate to explain the interaction between osmolytes and proteins and propose to take into account both the effects of the excluded volume and those of osmolyte binding with protein.

In the previous work [45] we have demonstrated that heat-induced aggregation of UV-irradiated glycogen phosphorylase *b* (UV-Ph*b*) at 37 °C is an irreversible process. However, at 10 °C aggregation of UV-Ph*b* induced by crowders (PEG and Ficoll-70) results in the formation of reversible aggregates, which can be disrupted by dilution of the solution. A detailed study of the kinetics of UV-Ph*b* aggregation at 10 °C may provide an insight into the mechanism of the initial stages of protein aggregation.

The goal of the present work was to study the kinetics of UV-Ph*b* aggregation induced by crowders (PEG and Ficoll-70) or TMAO at 10 °C using dynamic light scattering (DLS) and to characterize the initial rate of the aggregate growth stage after termination of the nucleation stage as a function of protein concentration. It has been shown that the order of UV-Ph*b* aggregation with respect to protein is equal to 2. The obtained kinetic data have been interpreted in the frame of the mechanism of nucleation-dependent polymerization.

2. Materials and methods

2.1. Materials

Hepes, Ficoll with molecular mass of 70,000 Da (Ficoll-70) and trimethylamine N-oxide (TMAO) were purchased from “Sigma” (USA), polyethylene glycol with molecular mass of 20,000 Da (PEG) was purchased from “Ferak Berlin” (Germany). NaCl was purchased from “Reakhim” (Russia). All solutions for the experiments were prepared using deionized water obtained with the Easy-Pure II RF system (Barnstead, USA).

2.2. Phb isolation

Ph*b* was isolated from rabbit skeletal muscles as described earlier [46]. The preparations of Ph*b* were electrophoretically homogeneous. Prior to the experiments, Ph*b* was passed through a Sephadex G-15 column equilibrated with 0.03 M Hepes buffer, pH 6.8, containing 0.1 M NaCl. Ph*b* concentration was determined spectrophotometrically at 280 nm using the absorbance coefficient $A_{\mathrm{cm}}^{1\%}$ of 13.2 [47].

2.3. UV irradiation of Phb

UV irradiation of Ph*b* (1.5 mg·mL^{-1}) was carried out in 1 cm path quartz cell at 6 °C. The equipment with Hg-Xe Lamp L8252 (Hamamatsu Photonics, Japan) was used in the irradiation experiments. The power of incident light was 10.4 mW·cm^{-2}. The samples of UV-Ph*b* were centrifuged at 12,850 × *g* for 10 min at 4 °C.

2.4. Light scattering intensity measurements

For light scattering measurements a commercial instrument Photocor Complex (Photocor Instruments, Inc., USA) was used. A He-Ne laser (Coherent, USA, Model 31-2082, 632.8 nm, 10 mW) was used as a light source. The diffusion coefficient D of the particles is directly related to the decay rate τ_c of the measured time-dependent correlation function for the light scattering intensity fluctuations: $D = 1 / 2\tau_c k^2$. In this equation k is the wave number of the scattered light, $k = (4\pi n / \lambda) \sin(\theta/2)$, where n is the refractive index of the solvent, λ is the wavelength of the incident light in vacuum and θ is the scattering angle. The hydrodynamic radius of the particles, R_h, can then be calculated according to the Stokes–Einstein equation: $D = k_B T / 6\pi\eta R_h$, where k_B is Boltzmann's constant, T is the absolute temperature and η is the dynamic viscosity of the solvent.

DynaLS software (Alango, Israel) was employed for polydisperse analysis of DLS data. Effective regularization program allows us to solve the light scattering inverse problem and find multimodal distribution of disperse particles without any preliminary suggestions about form and number of components in polydisperse distribution. To analyze the aggregation processes, the experimentally measured intensity autocorrelation function, which corresponds to so-called z-average particle size, was used. Such an estimate of the particle size is essential for correct analysis of bimodal distributions because the additional recalculation into number or weight average could remarkably complicate and obscure the aggregation description due to a very strong dependence of the light scattering intensity on the particle size.

The kinetics of UV-Ph*b* aggregation was studied in 0.03 M Hepes buffer, pH 6.8, containing 0.1 M NaCl at 10 °C. The buffer was placed in a cylindrical cell with the internal diameter of 6.3 mm and pre-incubated for 10 min at 10 °C. To study the effects of Ficoll-70 and PEG on UV-Ph*b* aggregation, the solutions containing these compounds were pre-incubated in the cell for 10 min before the addition of an aliquot of the protein. When studying the kinetics of aggregation of UV-Ph*b*, the scattering light was collected at a 90° scattering angle. The aggregation kinetics was followed by an increase in the intensity of scattered light (I) in time. The values of the refractive index and dynamic viscosity of Ficoll-70 and PEG solutions, which were used for calculation of R_h values from DLS data, are given in Supplementary Information in Table S1.

It is generally accepted that the initial stage of aggregation of many proteins is that of association of non-native protein molecules with the formation of nuclei capable of further growth via the attachment of monomeric unfolded protein [48–50]. If the aggregation mechanism involves the nucleation stage, a lag phase is registered in the initial stage of the aggregation process. It is significant that the duration of the lag phase decreases with the increasing protein concentration [51].

In the aggregation mechanism under discussion the nucleation stage is followed by the stage of protein aggregate growth. To determine the

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