Estimation of Chaperone-Like Activity Using Test Systems Based on Protein Amyloid Aggregation

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Introduction

The main function of small heat shock proteins (sHsps) is suppression of accumulation of protein aggregates in the cell [1-3]. A classic example of protective action of human sHsps is sustaining transparence of lens through whole life [4]. This function in lens is fulfilled by α-crystallin capable of binding the proteins that have lost their native structure. To characterize anti-aggregation (chaperone-like) activity of sHsps, different test systems based on amorphous or amyloid aggregation of protein substrates are used. The quantitative estimation of anti-aggregation activity of chaperones allows the strict comparison of the protective effects of chaperones of different classes to be carried out. Besides, such quantitative approaches provide the necessary background to the elucidation of the mechanism of chaperone functioning.

The methods of quantification of the chaperone-like activity using test systems based on amorphous aggregation of target proteins have elaborated in the articles by Kurganov and coworkers [5-8]. When the kinetics of aggregation of the target protein is followed by the increase of the light scattering intensity (I) or apparent optical absorbance (A), the initial part of the kinetic curve can be described by a quadratic equation [5]:

\[ I = I_0 + v(t-t_0)^2 \text{ or } A = A_0 + v(t-t_0)^2, \quad (t > t_0) \quad (1) \]

where \( I_0 \) is the initial value of the light scattering intensity, \( A_0 \) is the initial value of the apparent optical absorbance (at \( t=0 \)), \( t_0 \) is the duration of the lag phase and \( v \) is a constant. Suppose that the apparent optical absorbance (or the light scattering intensity) is proportional to the amount of the protein in the aggregated form. Such a proportionality between absorbance and the amount of the aggregated protein was experimentally demonstrated, for example, by Finke et al. [9] for the dithiothreitol (DTT)-induced aggregation of bovine serum albumin (BSA) [8] and α-lactalbumin [12] and thermal aggregation of apoglycogen phosphorylase b at 37°C [11]. It should be noted that the dependence of \( v/v_0 \) on \( [\text{target substrate}] \) ratio follows the quadratic law.

The reciprocal value of the length cut off on the abscissa axis by \( v/v_0 \) on \( [\text{target substrate}] \) ratio gives the AC value which can be considered as a measure of the anti-aggregation activity of the protein chaperone. The chaperone-like activity of α-crystallin was estimated by such a way in [8,11,12] where the following test systems were used: dithiothreitol (DTT)-induced aggregation of bovine serum albumin (BSA) [8] and α-lactalbumin [12] and thermal aggregation of apoglycogen phosphorylase b at 37°C [11]. It should be noted that the dependence of \( v/v_0 \) on \( [\alpha\text{-crystallin}]/[\text{target substrate}] \) ratio was nonlinear indicating that the decrease in the adsorption capacity of α-crystallin occurred with increasing the \([\alpha\text{-crystallin}] / [\text{target substrate}]\) ratio. Such a nonlinearity can be interpreted as an evidence for dynamic α-crystallin structure and polydispersity of α-crystallin-target protein complexes. From the initial parts of the dependences of \( v/v_0 \) on \( [\alpha\text{-crystallin}] / [\text{target substrate}] \) ratio the AC values were calculated:

\[ AC_0=1.18 \text{ molecules of } \alpha\text{-lactalbumin per one } -\text{crystallin subunit} \quad [8] \]

\[ AC_0=2.5 \text{ BSA monomers per one } -\text{crystallin subunit} \quad [8] \]

The linear dependence of \( 1/v_0 \) on \( [\alpha\text{-crystallin}] \) was used in [8] for comparison of the chaperone-like activities of intact -crystallin and α-crystallin cross-linked by glutaraldehyde. BSA unfolded by disruption of disulphide bonds under the action of DTT was used as a target protein in this work. It was shown that cross-linking resulted in 12-fold decrease in the AC value.

In the case of low-molecular-weight chemical chaperones forming readily dissociable complexes with the target protein the analysis of the dependence of the initial rate of aggregation on the chemical chaperone concentration (\([\text{chaperone}]\)) allows determining the semi-saturation concentration (\([\text{chaperone}]_{1/2}\)). The following equation can be used for the description of the dependence of \( v/v_0 \) on \( [\text{chaperone}] \) [8]:

\[ \frac{v}{v_0} = \frac{1}{1 + ([\text{chaperone}]/[\text{chaperone}]_{1/2})^2}. \quad (3) \]

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where $h$ is the Hill coefficient and $[L]_{0.5}$ is the [L] value at which $v = 0.5$. Parameter $[L]_{0.5}$ can be used as a measure of the anti-aggregation activity of the chemical chaperone. When studying the suppression of DTT-induced aggregation of BSA by arginine [8], the following values of parameters $[L]_{0.5}$ and $h$ were found: $[L]_{0.5} = 116 \text{ mM}$ and $h = 1.6$ (0.1 M Na-phosphate buffer, pH 7.0; 45°C). The fact that the Hill coefficient is higher than unity is indicative of the positive cooperative interactions upon binding of arginine to the unfolded protein. Such a cooperative binding can be due to clustering of arginine molecules on the surface of the unfolded protein molecule. The formation of arginine clusters on the surface of the protein molecule has been proved experimentally by Vagenende et al. [13].

Protein aggregation is associated with many neurodegenerative diseases including Alzheimer’s, Parkinson’s and Type II diabetes. The natively structured or unstructured proteins adopt partially folded conformation and subsequently self-associate through nucleation dependent polymerization to form amyloid fibrils which are characterized by cross-$\beta$-sheet rich structure and capable of binding the amyloidophilic fluorophores thioflavin T (ThT) and Congo red [14-18]. These fibrils are very stable, resistant to proteases and to harsh environmental conditions. Many natural polypeptide chains are able to form amyloid fibrils in vivo or in vitro, and this ability has been suggested to represent an inherent consequence of the chemical structure of the polypeptide chain.

In the works by Kamihira et al. [19] and Sabaté et al. [20] the fibrillation process was considered as an autocatalytic process with a nucleation reaction followed by an elongation one with the rate constants $k_0$ and $k_2$, respectively. The following equation was used, for example, for the description of the kinetics of aggregation of $\beta$-amyloid peptide registered by the increase in the apparent optical absorbance at 400 nm (37°C) [20]. To analyze the dependence of the apparent optical absorbance on time, Equation (4) can be modified as follows [21]:

$$A = A_0 + K_1 \frac{\exp(K_1 t) - 1}{1 + K_2 \exp(K_1 t)},$$

where $A$ and $A_0$ are the current and initial values of the apparent optical absorbance, $K_1$, $K_2$ and $K_3$ are constants. Parameter $K_3$ is the total increase in the A value in course of aggregation.

To characterize the shape of the kinetic curve, we can use the following parameters: parameter $(dA/dt)_{\text{max}}$, which is the slope of the tangent to the kinetic curve at the inflexion point and parameter $t_1$, which is the length cut off by this tangent at the horizontal line $A=A_0$ (Figure 1). Following determination of constants $K_1$, $K_2$, and $K_3$ by fitting Equation (5) to the experimental data, parameters $(dA/dt)_{\text{max}}$ and $t_1$ can be calculated using the following formulæ:

$$\left(\frac{dA}{dt}\right)_{\text{max}} = \frac{K_1 K_3 (1 + K_2)}{4},$$

$$t_1 = \frac{1}{K_1} \ln \left( \frac{1}{K_2} - \frac{2(1 - K_3)}{1 + K_2} \right).$$

Parameter $(dA/dt)_{\text{max}}$ may be considered as a measure of the aggregate growth rate. Parameter $t_1$ is usually called a lag-period (see, for example, the analysis of the correlation between lag time and growth rate in the spontaneous formation of amyloid-like aggregates and fibrils [22]).

It is significant that the use of Equation (5) for description of the full kinetic curve means that the initial increase in the apparent optical absorbance proceeds at $t=0$. In other words, it is beyond reason to believe that the kinetic curve begins with a lag phase characterizing by the $t_1$ value different from zero.

Caramelo et al., were first to demonstrate the applicability of the quadratic equation for the description of the initial parts of the kinetic curves for aggregation processes leading to the formation of amyloid fibrils using lysozyme fibrillation as an example [23]. The kinetics of lysozyme fibrillation was monitored by the measurement of the increment of ThT fluorescence ($F$). The increase in the fluorescence intensity at 482 nm is proportional the amount of amyloid fibrils [24]. The following modified form of Equation (1) can be used for the analysis of the fibrillation kinetics:

$$F = F_0 + v(t - t_0)^2, \quad (t > t_0)$$

where $F_0$ is the initial value of ThT fluorescence, $t_0$ is the lag period and $v$ is a parameter characterizing the initial rate of fibrillation.

Figure 2 shows the application of Equation (8) to the description of the initial part of the kinetic curve of lysozyme fibrillation registered by the increase in ThT fluorescence ([lysozyme]=2.5 mg/ml). The fitting procedure gives the following values of parameters: $F_0 = 0.028$, $t_0 = 21.6 \pm 0.2 \text{ min}$ and $v = (1.43 \pm 0.02) \cdot 10^{-3} \text{ min}^{-2}$.

The applicability of the quadratic law to the description of the initial part of the kinetic curve of amyloid fibril formation was discussed also for fibrillation of insulin (25 mM HCl, 0.1 M NaCl, 60°C) [25].

The use of Equation allows performing the quantitative estimation of the effects of different agents on the rate of the fibrillation process. The fibrillation process can be controlled by binding of the specific ligands to the initial native form of the target protein or by binding of the protein or chemical chaperones to the unfolded protein forms. Consider, for example, the retardation of lysozyme fibrillation by natural inhibitors, namely by N,N′-diacetylactylchitobiose and N,N′,N′-triacetylchitobiose ([lysozyme]=0.5 mg/ml) [23]. The effect of these
inhibitors is demonstrated in Figure 3. Fitting of Equation (8) to the experimental data gave the following values of parameters $t_0$ and $v$: $t_0 = 52.2 \pm 0.1$ min and $v = (4.24 \pm 0.04) \times 10^{-5}$ min$^{-1}$ in the absence of inhibitors, $t_0 = 90.8 \pm 0.4$ min and $v = (2.13 \pm 0.01) \times 10^{-5}$ min$^{-1}$ in the presence of N,N'-diacetylchitobiose and $t_0 = 161.3 \pm 0.2$ min and $v = (3.05 \pm 0.03) \times 10^{-5}$ min$^{-1}$ in the presence of N,N',N''-triacetylchitobiose. Thus, the protective effect of these natural inhibitors of lysozyme is due to the decrease in the initial rate of aggregation expressed by parameter $v$ and the increase in the lag period (parameter $t_0$).

In conclusion, it should be emphasized that the quantitative approaches to the estimation of the initial rate of the aggregation process and the duration of the lag phase provide the task-oriented screening of the agents which act as efficient suppressors of protein aggregation and are of interest to biotechnologists and specialists in medicinal chemistry elaborating the drugs for treatment of neurodegenerative diseases.

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