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Kinetics of heat- and acidification-induced aggregation of firefly luciferase

Keyang Wang[a], Boris I. Kurganov[b,*]

[a]*Department of Ophthalmology, Columbia University, 630 West 168th Street, New York, NY 10032, USA*
[b]*Bach Institute of Biochemistry, Russian Academy of Sciences, Leninsky prospekt, 33, Moscow 119071, Russia*

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Abstract

The general approach to analysis of the kinetics of protein aggregation registered by the turbidimetric method has been elaborated. The terminal part of the kinetic curves is analyzed using a theoretical equation connecting the derivative of the apparent absorbance (A) with respect to time (dA/dt) and A (t is time). This analysis allows the limiting value of A at $t \rightarrow \infty$ (A_{lim}) and the order of aggregation with respect to protein (n) to be calculated. Approach proposed was applied to analysis of thermal and acidification-induced aggregation of firefly luciferase. In both cases the A_{lim} value is a linear function of the protein concentration. The terminal part of the kinetic curves of thermal aggregation follows the first-order kinetics ($n=1$), whereas the kinetics of acidification-induced aggregation are characterized by the value of n higher than unity ($n=1.29$). The mechanism of nucleation-dependent aggregation has been discussed.

Keywords: Luciferase; Aggregation; Kinetics; Nucleation

1. Introduction

Molecular chaperones have one property in common, suppression of aggregation of non-native proteins [1–7]. To characterize the chaperone-like activity, the test-systems are used where aggregation of the protein substrate is induced by the action of elevated temperatures or acidification of the protein solution. In the case of proteins containing disulfide bonds (insulin, lysozyme, α-lactalbumin, bovine serum albumin and ovotransferrin), aggregation may be induced by the addition of the reducing agents (for example, dithiothreitol). In the experiments on renaturation of proteins denatured by guanidine hydrochloride or urea the diminishing of the concentration of the denaturing agent by dilution is accompanied by aggregation of the protein. Such systems may be used as test-systems for estimation of the chaperone-like activity.

Self-assembly or aggregation of protein molecules may be easily registered by the turbidimetric method. The limitations and criteria of validity of this technique were discussed by Andreu and Timasheff [8]. In order to use the turbidimetric

*Corresponding author. Tel.: +7-95-952-5641; fax: +7-95-954-2732.
E-mail address: kurganov@gagarinclub.ru (B.I. Kurganov).

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method for the quantitative characterization of the rate of self-assembly or aggregation, we should document the proportionality between measured value of the light-scattering intensity or apparent absorbance and the amount of the polymerized (aggregated) protein. When studying reversible polymerization of tubulin–colchicine complex, Andreu and Timasheff observed the correlation between the limiting value of absorbance at 450 nm at $t \to \infty$ (t is time) and the amount of protein sedimented during centrifugation at $30\,000 \times g$ for 15 min [8,9]. Such a correlation permitted the use of turbidity as a strictly empiric tool for following polymerization in the case under discussion. If we consider irreversible aggregation of proteins, we should take into account that at $t \to \infty$ all the protein converts to the aggregated state. Therefore, the application of the turbidimetric method for registration of the kinetics of protein aggregation is justified only in the case when the limiting value of absorbance is proportional to the total concentration of the protein.

Firefly luciferase is one of the protein substrates used for testing the ability of molecular chaperones to suppress protein aggregation [10–18]. The understanding of the mechanisms of aggregation of the protein substrates is required to interpret the experimental data on testing the chaperone-like activity in the test-systems based on suppression of aggregation of the protein substrates. In this article to elucidate the mechanism of aggregation of the protein substrate under discussion, we studied aggregation of firefly luciferase induced by the action of elevated temperature or acidification of the solution at various concentrations of the protein.

2. Materials and methods

2.1. Materials

The recombinant firefly luciferase was obtained from Promega Corp. (Madison, WI). Tricine and phosphoric acid were purchased from Sigma Chemical Company (St. Louis, MO).

2.2. Aggregation of luciferase

Various amounts of luciferase were dissolved in 25 mM tricine, pH 7.5, in covered quartz cuvettes. Total volume was 1.0 ml. The heat-induced aggregation of luciferase was started at 42 °C. The acidification-induced luciferase aggregation was initiated by adjusting the pH of the enzyme solution to 5.5 with 0.5 M H_3PO_4 at 30 °C. Aggregation of luciferase was followed by measuring the relative turbidity at 360 nm in a Beckman DU-70 spectrophotometer equipped with a six-cell-holder accessory and a Fisher Scientific IsoTemp 1006S temperature-control circulator. The temperature of the sample in the cells was measured by inserting a small, short thermometer inside one of the six cells in the holder. The turbidity in each cell was recorded automatically every minute.

2.3. Analysis of the kinetic curves of aggregation

Aggregation is considered as an irreversible reaction proceeding with the participation of n molecules of the non-aggregated protein P:

$$n\mathrm{P} \xrightarrow{k_n} \mathrm{P_{agg}}, \tag{1}$$

where $\mathrm{P_{agg}}$ is the aggregated form of the protein, k_n is the rate constant of the nth order. The rate of aggregation (v_{agg}) may be written as the diminishing of the concentration of the non-aggregated protein in time:

$$v_{agg} = -\mathrm{d[P]}/\mathrm{d}t = nk_n[\mathrm{P}]^n, \tag{2}$$

where n is the order of aggregation with respect to protein. Let A_{lim} be the limiting value of absorbance (A) at $t \to \infty$. If the A value is proportional to amount of the aggregated protein, the portions of the aggregated and non-aggregated protein are equal to A/A_{lim} and $(1-A/A_{lim})$, respectively. On these assumptions, the current value of the molar concentration of the non-aggregated protein [P] is equal to $(1-A/A_{lim})[\mathrm{P}]_0$ ($[\mathrm{P}]_0$ is the value of [P] at $t=0$). The rate of the change in absorbance has the following form:

$$\frac{\mathrm{d}A}{\mathrm{d}t} = \frac{nk_n[\mathrm{P}]_0^{n-1}}{A_{lim}^{n-1}}(A_{lim}-A)^n = k(A_{lim}-A)^n, \tag{3}$$

where $k = nk_n[\mathrm{P}]_0^{n-1}/A_{lim}^{n-1}$.

Consider the case when $n=1$ (the first-order of aggregation with respect to protein). Eq. (3) is transformed into the following equation:

$$dA/dt = k_I(A_{lim} - A), \quad (4)$$

where k_I is the rate constant of the first order. Integration of Eq. (4) gives the expression describing the dependence of A on t:

$$A = A_{lim}\{1 - \exp[-k_I(t - t_0)]\}, \quad (5)$$

where t_0 is the value of t, at which $A=0$. Knappik and Plückthun [19] pioneered in applying such an equation to analysis of the kinetics curves of protein aggregation registered by the turbidimetric method.

2.4. Calculations

All the calculations in this article were carried out using the program Scientist (MicroMath, Inc., USA). In order to characterize the degree of agreement between the experimental data and calculated values, we used the determination coefficient R^2 (without considering the statistical weight of the results of measurements) [20]:

$$R^2 = \frac{\sum_{i=1}^{i=n}\left(Y_i^{obs} - \bar{Y}^{obs}\right)^2 - \sum_{i=1}^{i=n}\left(Y_i^{obs} - Y_i^{calc}\right)^2}{\sum_{i=1}^{i=n}\left(Y_i^{obs} - \bar{Y}^{obs}\right)^2}, \quad (6)$$

where $\bar{Y}^{obs} = \frac{1}{n}\sum_{i=1}^{i=n} Y_i$ is the average of the experimental data (Y_i^{obs}), Y_i^{calc} is the theoretically calculated value of the function Y, and n is the number of measurements.

3. Results

3.1. Thermal aggregation of luciferase

Fig. 1 shows the kinetic curves of thermal aggregation of luciferase. The enzyme concentration was varied in the range from 0.2 to 4.0 μM. Analysis of the kinetics of aggregation shows that

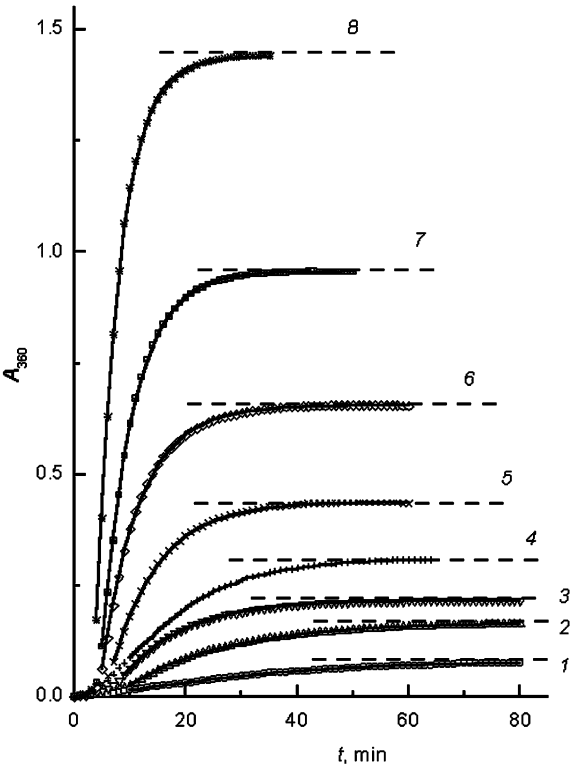

Fig. 1. Thermal aggregation of luciferase (25 mM Tricine, pH 7.5; 42 °C). Kinetics of aggregation were registered by the increase in absorbance at 360 nm (A_{360}). The protein concentration: 0.2 (1), 0.5 (2), 0.6 (3), 1.0 (4), 1.5 (5) 2.0 (6), 3.0 (7) and 4.0 μM (8). The solid curves calculated from Eq. (3). The horizontal dotted lines correspond to the A_{lim} values.

terminal phase of the kinetic curves (from $A/A_{lim} \approx 0.2$ to higher) may be satisfactorily described by Eq. (3) with $R^2 \geqslant 0.9990$. The values of parameters A/A_{lim}, k and n are presented in Fig. 2 as functions of the protein concentration. The A/A_{lim} value is proportional to $[P]_0$ (Fig. 2a). The k value is a linear function of $[P]_0$, the intercept of the ordinate axis being nonzero value (Fig. 2b). The n value remains constant with variation of $[P]_0$ (Fig. 2c). The average value of n is equal to 0.98 ± 0.01.

The fact that the A_{lim} value is proportional to the protein concentration allows us to consider the value of absorbance (A) as a measure of the amount of the aggregated protein. Thus, the kinetic curves of increase in absorbance may be used for

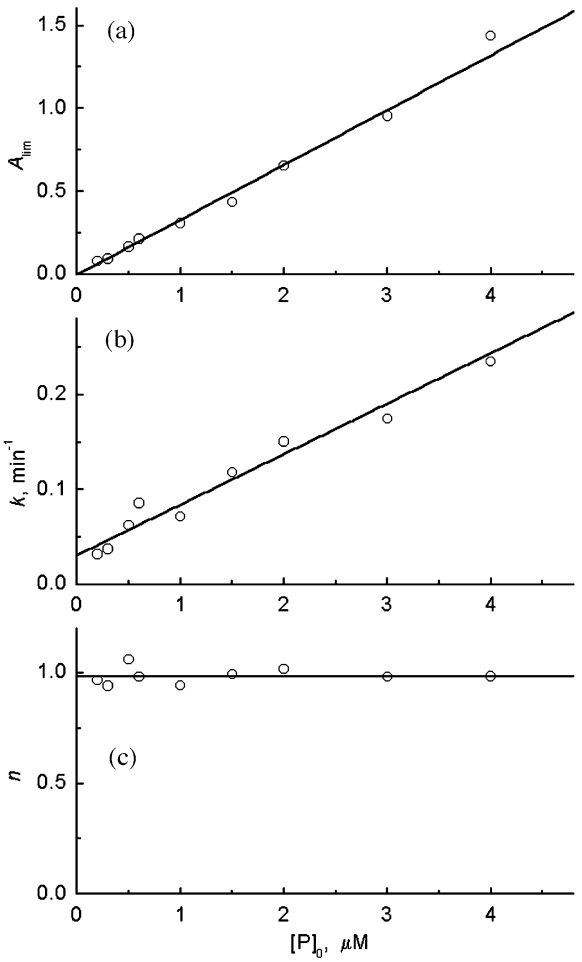

Fig. 2. Analysis of thermal aggregation of luciferase. The dependences of parameters A_{lim} (a); k (b) and n (c) on the protein concentration, $[P]_0$.

characterization of the kinetic mechanism of the aggregation process. Since the value of n was found to be unity, we should conclude that the terminal phase of aggregation follows the first-order kinetics and the parameter k is the rate constant of the first order (k_I). Strictly speaking, the rate constant k is a linear function of the protein concentration. Therefore, aggregation is a process of *the pseudo-first order* and k_I is the rate constant of the pseudo-first order.

In the case of the first-order kinetics the integral form of the kinetic equation, namely Eq. (5), may be used for the quantitative description of the kinetic curve. Fig. 3 shows the application of Eq. (5) to analysis of the kinetic curve of aggregation obtained at $[P]_0 = 2.0$ μM. As can be seen from this figure, the main part of the kinetic curve ($A/A_{lim} = 0.1$ to higher) is satisfactorily described by the equation corresponding to the first-order kinetics ($R^2 = 0.9998$). Apart from parameters A_{lim} and k_I, this procedure allows parameter t_0 to be determined. t_0 is a segment on the abscissa axis intercepted by a continuation of the theoretical curve, i.e. the curve calculated from Eq. (5) (curve 2 in Fig. 3). Parameter t_0 characterizes the duration of the lag period. The value of t_0 decreases with increasing the protein concentration (Fig. 4).

Over the lag period the rate of aggregation is very low. Therefore, it is reasonable to make estimations of the rate of aggregation only after passing the lag period. If one assumes that at $t \geqslant t_0$ aggregation follows strictly the first-order kinetics, the initial rate of aggregation (v_0^{agg}) may be calculated as a slope of a tangent to the curve at $t = t_0$ (straight line 4 in Fig. 3). The value of the initial rate of aggregation is equal to the product $k_I \cdot A_{lim}$. It should be noted that the dimension of the $k_I \cdot A_{lim}$ product is (absorbance unit)/

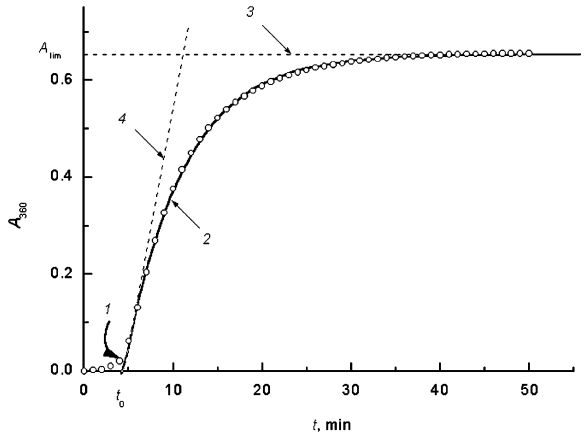

Fig. 3. Applicability of Eq. (5) for description of the terminal phase of thermal aggregation: (1) the experimental dependence of absorbance (A_{360}) on time ($[P]_0 = 2.0$ μM); (2) the theoretical dependence corresponding to Eq. (5); (3) the limiting value of A corresponding to A_{lim}; (4) a tangent to the curve 2 in the point with coordinates $t = t_0$ and $A = 0$.

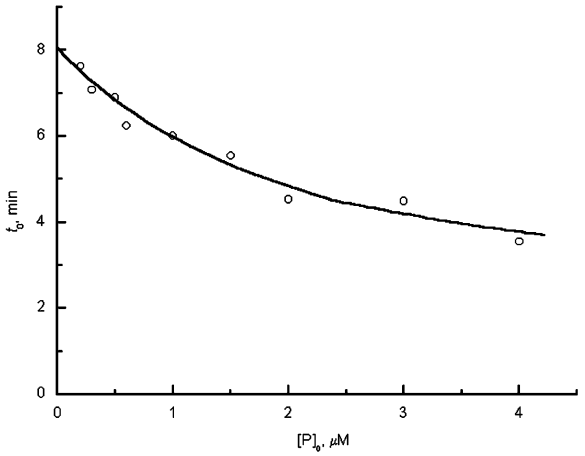

Fig. 4. The dependence of the parameter t_0 on the concentration of luciferase.

min. This dimension may be transformed into a more familiar dimension, namely M/min, if one takes into account that there is proportionality between absorbance and the protein concentration. As can be seen from Fig. 5, the initial rate of aggregation is a linear function of the protein concentration squared. This means that the order of aggregation with respect to protein calculated from the dependence of v_0^{agg} on $[P]_0$ is equal to 2.

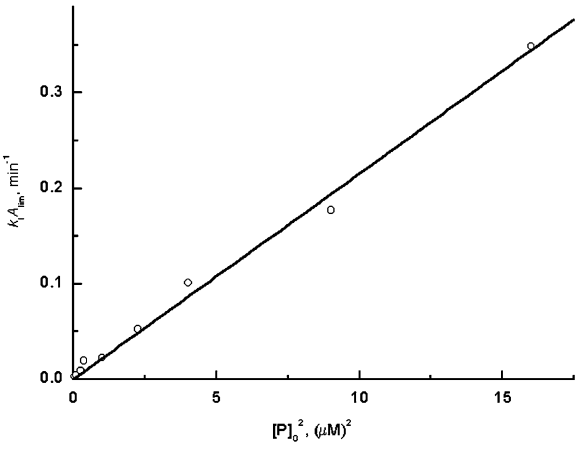

Fig. 5. The dependence of the $k_1 \cdot A_{lim}$ magnitude on the concentration of luciferase squared.

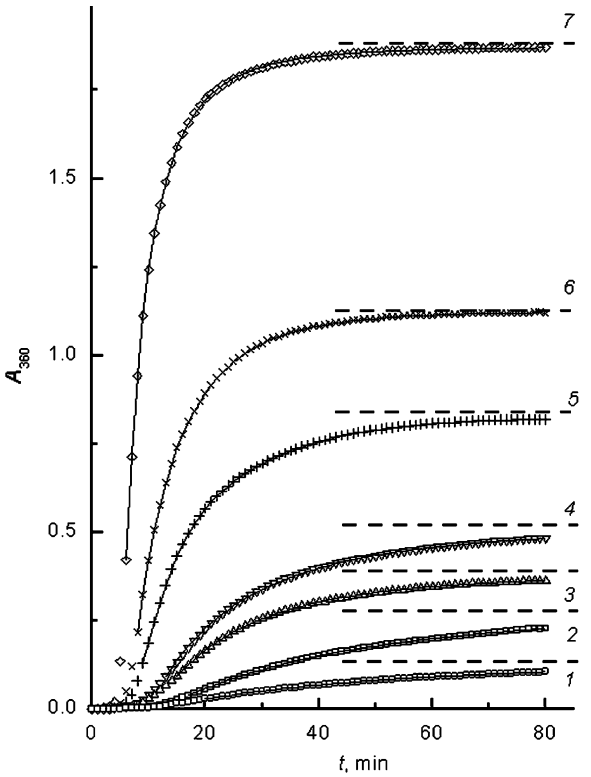

Fig. 6. Acidification-induced aggregation of luciferase (pH 5.5; 30 °C). Kinetics of aggregation were registered by the increase in absorbance at 360 nm (A_{360}). The protein concentration: 0.5 (1), 1.0 (2), 1.5 (3), 2.0 (4), 3.0 (5) 4.0 (6) and 6.0 μM (7). The solid curves calculated from Eq. (3). The horizontal dotted lines correspond to the A_{lim} values.

3.2. Acidification-induced aggregation of luciferase

Fig. 6 shows the kinetic curves of aggregation luciferase induced by acidification of the solution (pH 5.5). The enzyme concentration was varied in the range from 0.5 to 6.0 μM. Analysis of the kinetics of aggregation shows that the terminal phase of the kinetic curves (from $A/A_{lim} \approx 0.2$ to higher) may be satisfactorily described by Eq. (3) with $R^2 \geqslant 0.9996$. The values of parameters A/A_{lim}, k and n are presented in Fig. 7 as functions of the protein concentration. The A_{lim} value is proportional to $[P]_0$ (Fig. 7a). The k value is a linear function of $[P]_0$, the intercept of the ordinate axis being nonzero value (Fig. 7b). The n value

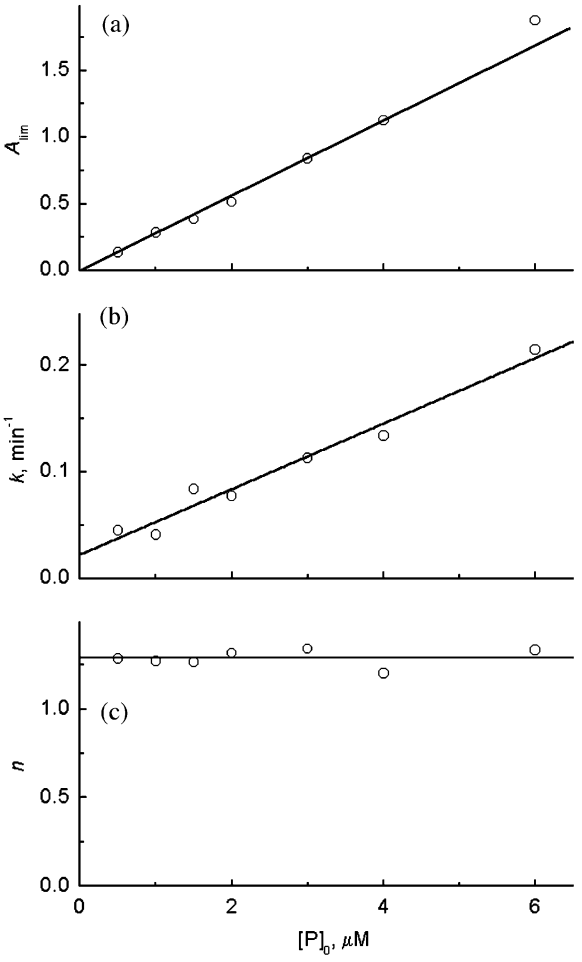

Fig. 7. Analysis of acidification-induced aggregation of luciferase. The dependences of parameters A_{lim} (a); k (b) and n (c) on the protein concentration, $[P]_0$.

remains constant with variation of $[P]_0$ (Fig. 7c). The average value of n is equal to 1.29 ± 0.02.

4. Discussion

The distinctive characteristics of thermal aggregation of luciferase are the following: (1) the kinetic curves have a lag period, (2) the duration of the lag period decreases with increasing protein concentration, (3) the terminal part of the kinetic curves ($A/A_{lim} \geqslant 0.2$) follows the first-order kinetics ($n=1$), (4) the order of aggregation with respect to protein calculated form the dependence of the initial rate of aggregation on the protein concentration is equal to 2.

It is generally accepted that the process of aggregation of proteins has the second (or higher) order with respect to protein [21–30]. Actually, second-order of aggregation was established experimentally in the case of aggregation accompanying refolding of proteins. Zettlmeissl et al. [31] studied the kinetics of aggregation of lactate dehydrogenase from pig muscle after dilution of the enzyme denatured by 6 M guanidine hydrochloride. The order of aggregation with respect to protein calculated from the dependence of the initial rate of aggregation on the protein concentration (n_c) was found to be 2.5 ± 0.1. Martineau and Betton [32] calculated the value of n_c for aggregation of a mutant of a human anti-β-galactosidase single chain antibody fragment scFv13R4c after dilution of the protein denatured by 7.5 M urea: $n_c = 2.0 \pm 0.1$. Intensity of light-scattering was measured in both cases for following the kinetics of aggregation. Schokker et al. [33] studied the kinetics of heat-induced aggregation of β-lactoglobulin at 80 °C (pH 2.5; 0.1 M NaCl). The time-course of aggregation was followed by measuring the decrease in the amount of native-like β-lactoglobulin by SE-HPLC combined with MALS. The value of n_c was found to be 2.1 ± 0.4.

It is expected that the time course of protein aggregation will follow the second-order kinetics. However, analysis of the kinetic curves of aggregation shows that, as a rule, the first-order kinetics is fulfilled at the terminal part of the process. Table 1 summarizes the results of analysis of the data on thermal aggregation of proteins. The kinetics of aggregation were registered using the turbidimetric method. Parameters n and k were calculated from Eq. (3). The values of n close to unity are indicative of the fulfillment of the first-order kinetics. The values of t_0 calculated from Eq. (5) are also given in Table 1.

To explain the kinetic features of thermal aggregation of firefly luciferase and other proteins the idea of nucleation-dependent aggregation may be used. Some authors assume that the process of aggregation include the stage of nucleation of denatured protein molecules and the stage of growth of aggregate [39,42–45]. It is evident that

Table 1
Analysis of the kinetics of thermal aggregation of proteins

Protein (source)	Conditions	Protein concentration	Interval of A/A_{lim}	n	k (1/min)	R^2	t_0 (min)	Reference
Alcohol dehydrogenase holoform (yeast)	50 mM K phosphate buffer pH 7.8, 50 °C	25 μg/ml	0.14–0.83	1.1±0.1	0.182±0.004	0.9994	2.24±0.05	[34]
Alcohol dehydrogenase (equine liver)	50 mM Na phosphate buffer pH 7.0, 0.1 M NaCl, 37 °C	1.25 mg/ml	0.04–0.992	1.05±0.07	0.122±0.005	0.9967	6.4±0.2	[35]
Carbonic anhydrase B (bovine erythroctes)	63 °C	0.06 mg/ml	0.23–0.98	1.02±0.01	0.146±0.001	0.9997	6.63±0.01	[36]
Catalase from (bovine liver)	50 mM Na phosphate pH 7.0, 55 °C	249 μg/ml	0.19–0.995	0.96±0.01	0.074±0.001	0.9991	7.55±0.02	[37]
Citrate synthase (pig heart mitochondria)	40 mM Hepes pH 7.5, 40 °C	3 μM	0.02–0.97	0.99±0.06	0.26±0.06	0.9991	1.29±0.03	[38]
Coat protein (tobacco mosaic virus)	100 mM phosphate buffer pH 8.0; 42 °C	0.03 mg/ml	0.01–0.98	0.95±0.01	0.27±0.01	0.9987	0.13±0.01	[39]
βL-Crystallin (lenses of rats)	50 mM phosphate buffer pH 7.2, 65 °C	0.3 mg/ml	0.25–1.0	1.01±0.01	0.183±0.001	0.9981	4.92±0.02	[40]
γ-Crystallin (lenses of rats)	50 mM phosphate buffer pH 7.2, 65 °C	0.3 mg/ml	0.20–0.98	1.06±0.01	0.110±0.001	0.9998	3.86±0.02	[40]
Malic dehydrogenase (porcine heart mitochondria)	50 mM Hepes/KOH pH 7.5, 45 °C	12 μg/ml	0.27–0.94	0.92±0.01	0.071±0.003	0.9997	3.94±0.04	[41]

Parameters k and n were calculated from Eq. (3). Parameter t_0 was calculated from Eq. (5).

such a mechanism of aggregation is identical to nucleation-dependent self-assembly [46] (or cooperative association [47,48]). It is worthy to note that nucleation-dependent amyloid-fibril formation is studied in sufficient detail [49–56].

The mechanism of thermal aggregation including the nucleation stage may written as follows. The first stage is unfolding of the protein molecule:

$$\mathrm{N} \rightarrow \mathrm{D}, \tag{7}$$

where N and D are the native and denatured states of the protein molecule. Association of some molecules D results to the formation of a nucleus:

$$n\mathrm{D} \rightarrow \mathrm{R}, \tag{8}$$

where R is a nucleus. Growth of aggregate proceeds as a bimolecular reaction of attachment of D to the nucleus R:

$$\mathrm{R} + \mathrm{D} \overset{k_{\mathrm{II}}^{\mathrm{macro}}}{\rightarrow} \mathrm{R}', \tag{9}$$

where R′ is a nucleus containing an additional molecule D. In this expression $k_{\mathrm{II}}^{\mathrm{macro}}$ is the macroscopic rate constant of the second order.

In general case the nucleus R contains some points of growth where the attachment of molecule D takes place. Let Ω be the point of growth and j be the number of the points of growth in the nucleus. If [R] is the molar concentration of nuclei, the molar concentration of the points of growth is equal to j[R]. Assume that the attachment of the molecule D to the point of growth Ω is not accompanied by the abolishment of the latter:

$$\mathrm{R} + \Omega \overset{k_{\mathrm{II}}^{\mathrm{micro}}}{\rightarrow} \Omega, \tag{10}$$

where $k_{\mathrm{II}}^{\mathrm{micro}}$ is the microscopic rate constant of the second order. The macroscopic and microscopic rate constants of the second order are connected by the following relationship:

$$k_{\mathrm{II}}^{\mathrm{macro}} = j k_{\mathrm{II}}^{\mathrm{micro}}. \tag{11}$$

The stage of growth of aggregate is a bimolecular reaction and the stage of nucleation involves probably a series of bimolecular reactions, whereas the stage of unfolding of the protein molecule is a monomolecular reaction. According to the laws of the chemical kinetics, the rate of the monomolecular reaction (A → B) is proportional to the concentration of the reagent A (c_{A}) to the first power ($v \sim c_{\mathrm{A}}$). The rate of the bimolecular reaction (A + B → C) is proportional the product of the concentrations of the reagents A and B (in the case of the reaction 2A → B the rate of the reaction is proportional to c_{A}^2). Such a situation means that in the region of relatively low concentrations of the protein the rate-limiting stages of the process of protein aggregation will be the stages of nucleation and growth of aggregate.

The appearance of a lag period on the kinetic curves is connected with the stage of nucleation. Ben-Zvi and Goloubinoff [57] showed that substoichiometric amounts of one fast co-aggregating protein can significantly increase thermal aggregation kinetics of otherwise soluble, slow-aggregating protein. The ability of aggregates to seed and propagate aggregation of other proteins is consistent with the idea that thermal aggregation of proteins includes the stage of nucleation.

One can expect that increasing the protein concentration will favor the formation of a nucleus and, consequently, decrease the duration of a lag period. The dependence of parameter t_0 on the protein concentration in the case of thermal aggregation of luciferase is just the one predicted by the mechanism of nucleation-dependent of aggregation (Fig. 4).

After the completion of the stage of nucleation the growth of aggregate proceeds as a reaction of pseudo-first order if the concentration of nuclei remains constant in the course of aggregation. The rate of aggregation expressed as a decline of the concentration of the D molecules has the following form:

$$v_{\mathrm{agg}} = -\mathrm{d}[\mathrm{D}]/\mathrm{d}t = k_{\mathrm{II}}^{\mathrm{macro}}[\mathrm{R}][\mathrm{D}]. \tag{12}$$

When [R] = const, the product $k_{\mathrm{II}}^{\mathrm{macro}}$[R] is the rate constant of the pseudo-first order:

$$k_{\mathrm{I}} = k_{\mathrm{II}}^{\mathrm{macro}}[\mathrm{R}]. \tag{13}$$

The constancy of the concentration of the nuclei is equivalent to a prerequisite of the indestructibility of the growth points or their constancy. If $[\Omega]=\text{const}$, the rate constant of the pseudo-first order may be written as follows:

$$k_{\mathrm{I}}=k_{\mathrm{II}}^{\mathrm{micro}}[\Omega]. \quad (14)$$

The indestructibility of the points of growth of aggregate means that the process of protein aggregation is closely analogous to the chemical chain reactions discovered by Semenov [58]. The basis of the chain reactions is the indestructibility of the free valency. The chain mechanism is typical of some important classes of the chemical reactions such as oxidation by molecular oxygen, chlorination and bromination of many compounds, the reactions of thermal decomposition and the reactions of chain polymerization. The free radicals participating in the chain reaction are called the active sites of the chain reactions. In our terminology the active sites of the chain reactions are the points of growth of aggregate. The main stages of the chemical chain reaction are the stage of the generation of the chains, the stage of propagation, and the stage of the ending of the chain. The generation of the chains is the stage of the chain reaction resulting in the formation of free radicals from the compounds in which all valencies are saturated. The analog of this stage in the process of protein aggregation is unfolding of the protein molecule followed by the stage of nucleation. The reactions of propagation of the chain are the elementary stages of the chain reaction proceeding with the conservation of free valency and resulting in the expenditure of the initial reagents and formation of the reaction products. In the case of protein aggregation the stage of growth of aggregate proceeding with the conservation of the growth point corresponds to these stages of the chain reaction. The ending of the chains are the stages of the chain reaction resulting in disappearance of free valency. The reason of the ending of the chain may be interaction of two free radicals. As for protein aggregation, interaction of nuclei possessing the points of growth of aggregate brings about not only the disappearance of certain number of the points of growth but also the violation of the proportionality between absorbance and the amount of the aggregated protein. The interactions of such a type resulting ultimately in precipitation of the aggregated protein become significant with increasing the initial concentration of the protein.

The chain reactions proceeding without the branching of the chains are called the unbranched chain reactions. In this case the disappearance of the active site at the stage of the propagation of the chain is accompanied by the formation of a new active site. When comparing aggregation of proteins and the chemical chain reactions, we can say that *protein aggregation is a unbranched chain reaction*. Strictly speaking, such a definition of protein aggregation is valid only for the stage of growth of aggregate. As for the stage of nucleation, this stage is evidently a branching-chain reaction, since the essence of the stage of nucleation is the formation of a nucleus having some the points of growth (some active sites). Moreover, such an approach to the stage of nucleation allows the duration of this stage to be more strictly assessed. Growth of the original nucleolus is accompanied by an increase in the number of the points of growth in the nucleolus. Starting with the definite size of the nucleolus, the number of the points of growth reaches the limiting value implying that the process of nucleation for this nucleolus is completed. Then the nucleus is growing by attachment of 'monomers' (the denatured molecules of the protein) with no change of the number of the points of growth in the nucleus.

Some substances added to the running chain reaction replace the active free radicals driving the chain with low-active free radicals, which are incapable of propagating the chains. Such substances are called the inhibitors of the chain reactions. Thus, chaperones suppressing aggregation of the protein substrates may be called the inhibitors of the chain reaction of protein aggregation. Chaperone has the function of completely blocking the points of growth of protein aggregate (the active sites of the chain reaction).

The main kinetic feature of thermal aggregation of firefly luciferase is the fact the terminal part of the kinetic curves (after the completion of a lag period) follows the first-order kinetics. This fact may be explained in the frames of the model of

nucleation-dependent aggregation provided that the concentration of nuclei (or, more strictly, the concentration of the points of growth of aggregate $[\Omega]$) remains constant in the process of aggregation. The linear character of the dependence of the rate constant of the first order k_I on the protein concentration means that the $[\Omega]$ value is proportional to $[P]_0$ and explains the second-order of aggregation with respect to protein calculated from the dependence of v_0^{agg} on $[P]_0$. It should be noted that the following experiments may be carried out to prove the model of aggregation under discussion. In the terminal phase of aggregation the mass of aggregate (M) should be enhanced in accordance with the law: $M=M_{lim}\{1-\exp[-k_I(t-t_0)]\}$ (M_{lim} is the limiting value of M at $t\rightarrow\infty$). An important point is that the value of M_{lim} should be the same at various concentrations of the protein. The data on heat-induced aggregation of β-lactoglobulin AB (0.1 M NaCl; pH 2.5; 80 °C) obtained by Schokker et al. [33] agree with this prediction. These investigators showed that the protein concentration had no effect on aggregate size.

Turning back to the dependence of the rate constant of pseudo-first order on the protein concentration (Fig. 2b), we see that intercept on the ordinate axis is distinct from zero. It is worthy noting that, when the protein concentration is relatively low, adsorption of the protein onto the surface of vessel where the protein solution is placed plays a leading role in protein denaturation (see, for example, [59]). Since in the frames of the model of nucleation-dependent protein aggregation the magnitude of the rate constant of pseudo-first order is proportional to the concentration of the sites where growth of aggregate occurs, it can be assumed that nonzero value of k_I at $[P]_0\rightarrow 0$ is connected with the formation of the points of growth of aggregate on the surface of the vessel (adsorption of lysozyme onto mica resulting in surface aggregation was discussed recently by Kim et al. [60]).

In the case of acid-induced aggregation of firefly luciferase the deviations from the first-order kinetics are observed: $n=1.29$. The reason of such deviations may be the change in the number of points of growth, as aggregate size increases. Taking into account that after the completion of the nucleation stage the concentration of the denatured molecules D is proportional to $[P]_0(1-A/A_{lim})$, the change in dA/dt in time may be written as follows:

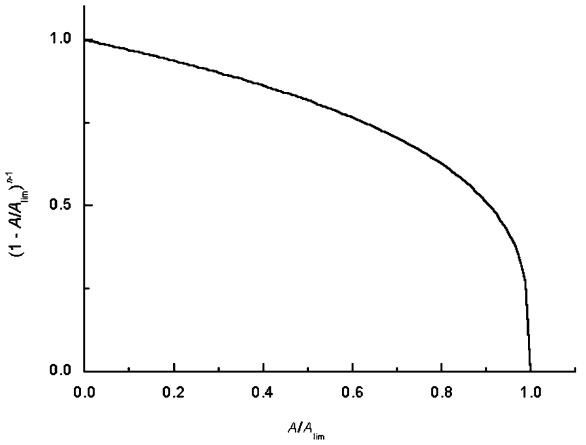

Fig. 8. The dependence of $(A_{lim}-A)^{n-1}$ on the A/A_{lim} ratio calculated from Eq. (16) at $n=1.29$.

$$dA/dt=k_{II}^{micro}(A_{lim}-A). \quad (15)$$

Combining Eqs. (3) and (15), we obtain the expression for the concentration of the points of growth of aggregate:

$$[\Omega]=(k/k_{II}^{micro})(A_{lim}-A)^{n-1}. \quad (16)$$

Thus, the multiplier $(A_{lim}-A)^{n-1}$ determines the character of changing the $[\Omega]$ value in the course of aggregation. Fig. 8 shows the dependence of the multiplier $(A_{lim}-A)^{n-1}$ on the A/A_{lim} ratio characterizing the degree of aggregation. This dependence is calculated from Eq. (16) at $n=1.29$, k_{II}^{macro} at value of n obtained for the case of acidification-induced aggregation. The character of the dependence of the multiplier $(A_{lim}-A)^{n-1}$, which is proportional to the concentration of the points of growth of aggregate, on the A/A_{lim} ratio suggests that the deviation from the first-order kinetics ($n>1$) is due to diminishing the number of the points of growth, as size of aggregate increases.

In conclusion, it may be said that the systems of aggregation of the protein substrates where the

A_{lim} value is proportional to the protein concentration may be used for the quantitative estimation of the chaperone-like activity [6,7,61]. The dependence of the A_{lim} value on the concentration of chaperone allows the stoichiometry of the complex between the denatured protein and chaperone to be determined. If the terminal part of the kinetic curves of aggregation follows the first-order kinetics, the dependence of the product $k_I \cdot A_{lim}$ on the concentration of chaperone may be constructed. This dependence characterizes the effect of chaperone on the rate of aggregation of the protein substrate. Propose that the concentration of the protein substrate is selected in the region of the relatively low values where the rate-limiting stage of aggregation is the stage of growth of aggregate. In this case one can expect that the k_I value will linearly decrease with chaperone concentration. According to calculations carried out in [61], such a situation is realized for suppression of thermal aggregation of pig muscle malate dehydrogenase by Hsp18.1 from *Pisum sativum* (pea) [62].

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