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REVIEW

Kinetics of Protein Aggregation. Quantitative Estimation of the Chaperone-Like Activity in Test-Systems Based on Suppression of Protein Aggregation

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Abstract—The experimental data on the kinetics of irreversible aggregation of proteins caused by exposure to elevated temperatures or the action of denaturing agents (guanidine hydrochloride, urea) have been analyzed. It was shown that the terminal phase of aggregation followed, as a rule, first order kinetics. For the kinetic curves registered by an increase in the apparent absorbance (A) in time (t) the methods of estimation of the corresponding kinetic parameters A_{lim} and k_I (A_{lim} is the limiting value of A at $t \to \infty$ and k_I is the rate constant of the first order) have been proposed. Cases are revealed when the reaction rate constant k_I calculated from the kinetic curve of aggregation of the enzymes coincides with the rate constant for enzyme inactivation. Such a situation is interpreted as a case when the rate of aggregation is limited by the stage of denaturation of the enzyme. A conclusion has been made that, in order to establish the mechanism of protein aggregation, the kinetic investigations of aggregation should be carried out over a wide range of protein concentrations. The refolding experiments after denaturation of proteins by guanidine hydrochloride or urea have been also analyzed. It was shown that aggregation accompanying refolding follows first order kinetics at the final phase of the process. The model of protein refolding explaining such a kinetic regularity has been proposed. When aggregation of protein substrate follows first order kinetics, parameters A_{lim} and k_I may be used for the quantitative characterization of the chaperone-like activity in the test-systems based on suppression of protein aggregation.

Key words: aggregation, denaturation, refolding, kinetics, molecular chaperones, guanidine hydrochloride, urea

Molecular chaperones function in a range of protein homeostatic events, including cotranslational protein folding, assembly and disassembly of supramolecular protein structures, and protein transport across membranes [1-18]. Many molecular chaperones are known as heat-shock proteins (Hsp), which are synthesized in cells of all organisms in response to supraoptimal temperatures. Heat-shock proteins are needed to protect cells from heat damage, and they assist in normalization of functions during recovery [19-25].

Molecular chaperones represent a large class of proteins, including Hsp27, Hsp40 (hdj-1), Hsp60, Hsp70, Hsp90, Hsp104, and Hsp110, which are functionally related based on common properties of influencing the conformation of protein substrates. Molecular chaperones interact preferentially with certain intermediates of protein folding, having a favorable effect on the folding kinetics. Whereas some chaperones such as Hsp70 and Hsp60 (GroEL) facilitate refolding to the native state, other chaperones, including α-crystallins, small heat-shock proteins, immunophilins, cdc37, and Hsp90, prevent aggregation but do not fully restore unfolded proteins to their final native state [4, 19, 21, 26-32]. The estimation of the chaperone activity in the test-systems based on suppression of aggregation of protein substrates needs an understanding of the mechanism of protein aggregation.

The goal of the present work was to discuss the shape of the kinetic curves of aggregation of proteins caused by the action of heating or denaturants like guanidine hydrochloride (GuHCl) or urea. It was of interest to elucidate the correctness of a very popular comment that the time-course of protein aggregation follows the kinetics of a reaction of the second or higher order with respect to protein. Besides, we planned to specify the kinetic parameters of aggregation of protein substrate, which may be used for the quantitative estimation of the chaperone-like activity.

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ANALYSIS OF EXPERIMENTAL DATA ON THE KINETICS OF AGGREGATION OF PROTEINS

Order of aggregation with respect to protein. The shape of the kinetic curve describing the time-dependence of the concentration of a substance can be characterized by the order of the reaction with respect to the substance. The protein aggregates possess an enhanced ability to scatter incident light. Therefore the kinetics of protein aggregation can be easily followed by monitoring an increase in the apparent absorbance (of light scattering intensity) of the protein solution in the visible region. Suppose that absorbance 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 Jennings et al. [33] when they studied aggregation of the mutant form of interleukin 1β K971, accompanying refolding of the protein after denaturing action of GuHCl.

One can expect that a strict proportionality between absorbance and the amount of the aggregated protein exists in the time region where growth of aggregates already formed occurs. The results of our investigations of glycogen phosphorylase *b* from rabbit skeletal muscle provide indirect grounding in such a suggestion. It was shown [34] that adsorption of glycogen phosphorylase *b* (a protein with a molecular mass of 195 kD) to the glycogen particles of molecular mass of $5.5 \cdot 10^6$ daltons was accompanied by an increase in the light scattering intensity of the glycogen solution, an increment of the light scattering intensity being a linear function of the number of the enzyme molecules adsorbed to the glycogen particle. Besides, an increment of absorbance of the glycogen solution in the course of the enzymatic reaction catalyzed by glycogen phosphorylase *b* (substrates are glycogen and glucose 1-phosphate) is directly proportional to the number of glucose residues attached to a polysaccharide chain [35, 36].

It should be noted that the assumption of proportionality of absorbance and the amount of the aggregated protein was used in the present work only for analysis of *the terminal phase* of aggregation (i.e., the initial parts of the kinetic curves usually including a lag period were ignored).

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

$$n\mathrm{P} \xrightarrow{k} \mathrm{P}_{agg} \qquad (1)$$

(P_{agg} is the aggregated form of the protein, k is the rate constant of the n-th order). The rate of aggregation (v_{agg}) can be written as the diminishing of the concentration of the non-aggregated protein:

$$v_{agg} = -\mathrm{d}[\mathrm{P}]/\mathrm{d}t = nk[\mathrm{P}]^n, \qquad (2)$$

where t is time and 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:

$$\frac{\mathrm{d}A}{\mathrm{d}t} = \frac{nk[\mathrm{P}]_0^{n-1}}{A_{lim}^{n-1}}(A_{lim} - A)^n. \qquad (3)$$

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

$$\mathrm{d}A/\mathrm{d}t = k_1(A_{lim} - A), \qquad (4)$$

where k_1 is the rate constant of the first order. Integration of Eq. (4) gives the expression describing the dependence of A on t:

$$A = A_{lim}\{1 - \exp[-k_1(t - t_0)]\} \qquad (5)$$

(t_0 is the value of t, at which $A = 0$) or

$$A = A_0 + (A_{lim} - A_0)[1 - \exp(-k_1 t)] \qquad (6)$$

(A_0 is the value of A at $t = 0$).

Combination of Eqs. (4) and (6) gives the relation between $\mathrm{d}A/\mathrm{d}t$ and t:

$$\mathrm{d}A/\mathrm{d}t = k_1(A_{lim} - A_0)\exp(-k_1 t) =$$

$$= (\mathrm{d}A/\mathrm{d}t)_0\exp(-k_1 t), \qquad (7)$$

where $(\mathrm{d}A/\mathrm{d}t)_0$ is the value of $\mathrm{d}A/\mathrm{d}t$ at $t = 0$.

Thus, to prove the first order of aggregation with respect to protein, the following equations can be used: Eq. (3) for analysis of the dependence of A on t with the aid of special programs, which allow the kinetic data to be described by the differential kinetic equations (in the case under discussion the value of n should be equal to unity), Eq. (4) (the dependence of $\mathrm{d}A/\mathrm{d}t$ on A should be the linear function), Eq. (5) (the dependence of A on t should be the exponential function with time retardation; $t_0 > 0$) and Eq. (6) (the dependence of $\mathrm{d}A/\mathrm{d}t$ on t should be the exponential function).

Consider the situation when $n = 2$ (*the second order of aggregation with respect to protein*). Equation (3) is transformed as follows:

$$dA/dt = 2k_{II}[P]_0(A_{lim} - A)^2/A_{lim}, \quad (8)$$

where k_{II} is the rate constant of the second order. Integration of Eq. (8) gives the expression describing the dependence of A on t:

$$A = A_{lim}\frac{2k_{II}[P]_0\,(t-t_0)}{1 + 2k_{II}[P]_0\,(t-t_0)} \quad (9)$$

or

$$A = A_{lim}\frac{A_0 + 2(A_{lim} - A_0)k_{II}[P]_0\,t}{A_{lim} + 2(A_{lim} - A_0)k_{II}[P]_0\,t}. \quad (10)$$

Combination of Eqs. (8) and (10) gives the relation between dA/dt and t:

$$\frac{dA}{dt} = \frac{2A_{lim}\,(A_{lim} - A_0)^2k_{II}[P]_0\,t}{\{\,A_{lim} + 2(A_{lim} - A_0)k_{II}[P]_0\,t\,\}^2}. \quad (11)$$

Equations (3) and (8)-(11) may be used for demonstration of the second order of aggregation with respect to protein.

Kinetics of thermal aggregation of proteins. Thermal denaturation of proteins is often accompanied by aggregation of denatured molecules [37, 38]. This circumstance complicates the application of spectral, hydrodynamic, and calorimetric methods for investigation of denaturation. Unfolding of the protein molecule exposes hydrophobic residues. Hydrophobic forces are the driving-force of interaction of denatured molecules [39-41]. One must discriminate between association and aggregation of the protein molecules. *Association* (or self-assembly) is interaction of the protein molecules resulting in the formation of stoichiometrically determined oligomeric structures, whereas *aggregation* is interaction of the unfolded protein molecules that are responsible for the formation of agglomerates of irregular form as a result of "incorrect" protein–protein contacts [42].

Consider as an example the kinetics of thermal aggregation of malate dehydrogenase from pig heart mitochondria (the data obtained by Nakamoto et al. [43]). Aggregation was registered by the increase in absorbance at 360 nm. The initial part of the kinetic curve includes a lag period (Fig. 1a). The appearance of lag period is due to the following reasons. First, aggregation is preceded by the stage of unfolding of the protein molecule:

$$N \longrightarrow U \quad (12)$$

(N is the native state of the protein molecule and U is the unfolded state, which is prone to aggregation). Second, an increment of absorbance becomes appreciable only after the appearance of sufficiently large aggregates U_m in the solution [44]. Most likely the increase in absorbance for the main part of the kinetic curve is connected with the enlargement of aggregates formed at the initial stage of aggregation.

In the present work attention focuses on the analysis of the terminal phase of aggregation, namely the part of the kinetic curve after passing the inflexion point. For the kinetic curve presented in Fig. 1a we analyzed the part of the curve at $t > 11$ min using the Scientist computer program (MicroMath, USA). This program allows the kinetic curve to be described in the framework of the kinetic scheme presented by a differential equation (or the system of differential equations). The value of n calculated in such a manner is found to be 1.02 ± 0.02 (Fig. 1b). Thus, the terminal phase of aggregation follows first order kinetics.

This conclusion was additionally substantiated using Eqs. (4), (5), and (7). Figure 1a shows the description of the experimental curve by the integral form of the equation corresponding to first order kinetics, namely Eq. (5). The following values of parameters were obtained: $A_{lim} = 0.0473 \pm 0.0001$, $k_I = 0.0940 \pm 0.0003$ min^{-1}, and $t_0 = 3.71 \pm 0.01$ min. As can be seen from Fig. 1a, the range of the values of A/A_{lim} where first order kinetics are applicable comprises about 80% (from $A/A_{lim} \approx 0.2$ and higher).

After differentiation of the dependence of A on t carried out with the aid of the Origin 5.0 computer program (Microcal Software, Inc., USA) we could construct the dA/dt versus A and dA/dt versus t plots. Approximation of the dependence dA/dt on A by a straight line at $A > 0.015$ in accordance with Eq. (4) gives the following values of parameters A_{lim} and k_I: $A_{lim} = 0.0473 \pm 0.0001$ and $k_I = 0.0942 \pm 0.0004$ min^{-1}. Previously we used coordinates $\{dA/dt; A\}$ for demonstration of the first order of aggregation with respect to protein in the case of aggregation of glycogen phosphorylase *b* in the presence of GuHCl [45]. Approximation of the dependence dA/dt on t by the exponential function (7) at $t > 10$ min gives the value of k_I: $k_I = 0.0944 \pm 0.0004$ min^{-1}. Thus, approximation of the experimental data by Eqs. (4), (5), or (7) gives similar values of the rate constant of the first order.

Figure 2 shows the examples of the kinetic curves of thermal aggregation where the terminal phase follows first order kinetics. In the case of aggregation of beef liver catalase [46] the terminal phase of aggregation is described by Eq. (5) at the following values of parameters: $A_{lim} = 0.5312 \pm 0.0003$, $k_I = 0.0795 \pm 0.0002$ min^{-1}, and $t_0 = 7.55 \pm 0.02$ min (Fig. 2a). The range of the values of A/A_{lim} where first order kinetics are applicable comprises about 84% (from $A/A_{lim} \approx 0.16$ and higher). The application of Eq. (5) for description of the terminal phase of aggregation of pig heart citrate synthase [47] gives the following values of parameters: $A_{lim} = 0.855 \pm 0.002$, $k_I =$

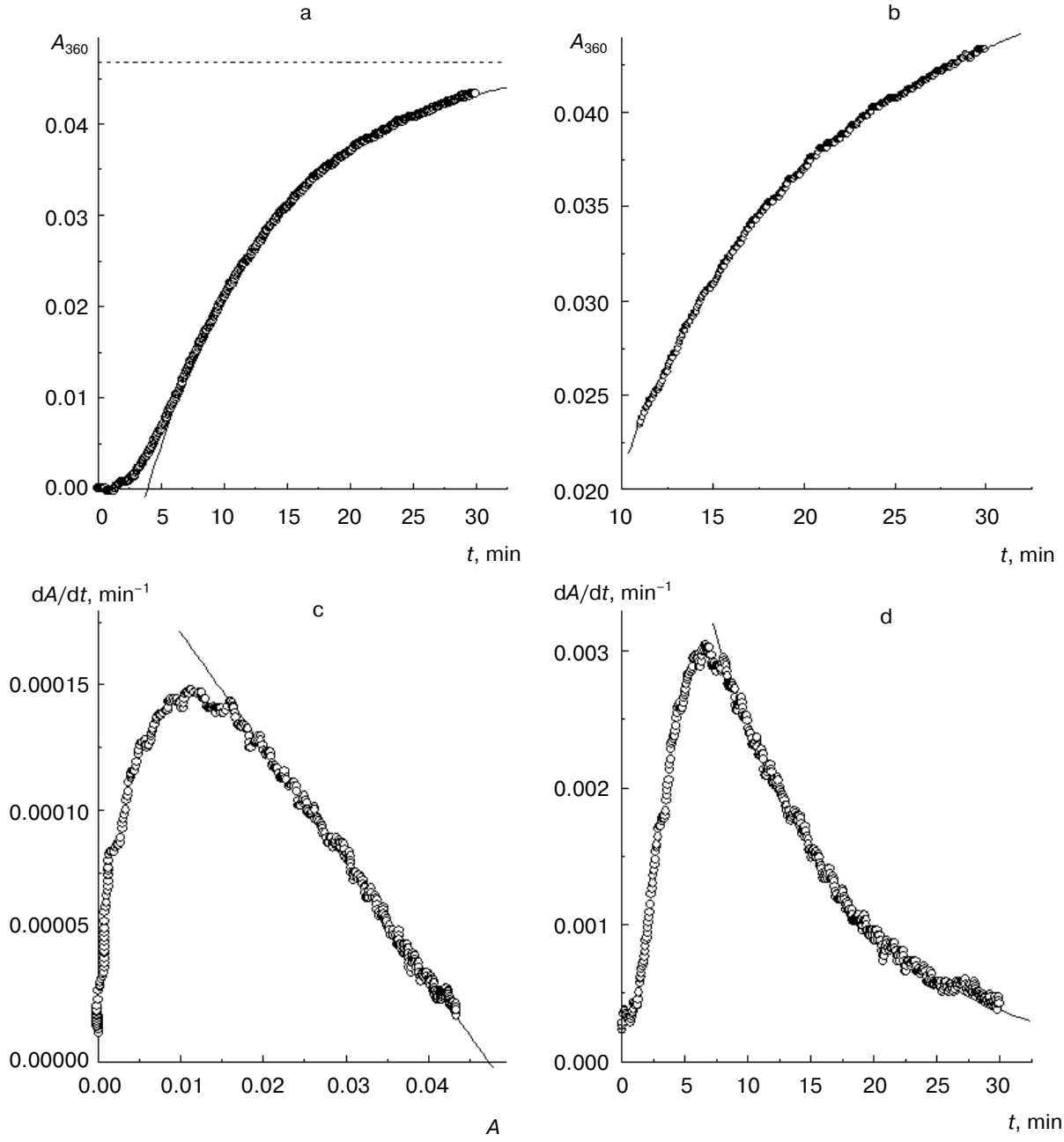


Fig. 1. Analysis of the kinetics of thermal aggregation of malate dehydrogenase from pig heart mitochondria (45°C). Points are the experimental data [43]. a) The dependence of absorbance at 360 nm (A_{360}) on time. The solid curve is calculated from Eq. (5). The horizontal dotted line corresponds to the A_{lim} value. b) Description of the terminal phase of aggregation ($t > 11$ min) by Eq. (3) using the Scientist computer program. c) The dependence of dA/dt on A. The terminal phase is described by the linear function (4). d) The dependence of dA/dt on t. The solid curve is calculated from Eq. (7).

0.0928 ± 0.0005 min^{-1}, and t_0 = 3.63 ± 0.02 min (Fig. 2b). First order kinetics are applicable in the range of the values of A/A_{lim} from ≈0.24 and higher. In the case of aggregation of β_{low}-crystallin [48] the terminal phase of the process is described by Eq. (5) at the following values of parameters: A_{lim} = 0.911 ± 0.001, k_I = 0.124 ± 0.001 min^{-1}, and t_0 = 4.08 ± 0.01 min (Fig. 2c). First order kinetics is applicable in the following range of the values of A/A_{lim}: $0.33 < A/A_{lim} < 0.90$. It should be noted that at high values of time ($t > 25$ min) the experimental points lie below the theoretical curve. The discrepancy between experimental and approximated curves is most probably due to the precipitation of large aggregates of protein.

Kinetics of aggregation of proteins in the presence of GuHCl and urea. The study of denaturation of proteins under the action of GuHCl or urea showed that in a certain interval of the denaturant concentrations the protein under study revealed generally a high propensity for aggregation. The phenomenon may be explained as follows. Low concentrations of denaturant induce denaturation of the protein. Interaction of denatured protein molecules results in their aggregation. However at high concentrations of denaturant the protein–protein interactions are weakened, resulting in the enhancement of protein solubility and breakdown of aggregates [38, 44, 49]. Such a situation is realized, for example, on treatment of bovine liver rhodanese [50], bovine growth hormone [51], creatine kinase from chick muscle [52], G-actin [53], D-glyceraldehyde-3-phosphate dehydrogenase from rabbit skeletal muscle [54], phosphofructokinase-1 from *Saccharomyces cerevisiae* [55], and glycogen phosphorylase *b* from rabbit skeletal muscle [45] with GuHCl and on treatment of tryptophanase from *Escherichia coli* [56], the mutant of a human anti-β-galactosidase single chain antibody fragment scFv13R4c [57], and chicken liver fatty acid synthase with urea [58]. The fact the degree of aggregation at moderate concentrations of denaturant decreases with diminishing temperature is indicative of the important role of hydrophobic interactions in the "sticking" of the unfolded protein molecules [51, 52].

Analysis of the kinetics of aggregation of glycogen phosphorylase *b* in the presence of 1 M GuHCl (0.08 M Hepes-buffer, pH 6.8; 25°C) showed that at relatively low protein concentrations (for example, 0.25 mg/ml) the terminal phase of aggregation registered by the increase in absorbance at 600 nm followed first order kinetics (k_I = 0.082 min^{-1}) [32].

Kendrick at al. [59] studied the kinetics of aggregation of recombinant human interferon γ in the presence of 0.9 M GuHCl (25°C). The degree of aggregation was determined by separation of the aggregated protein in 5 mM sodium succinate (pH 5.0; 5°C). It was shown that the decrease in the concentration of the non-aggregated protein followed first order kinetics (k_I = 0.033 min^{-1}).

Consider as an example the kinetics of aggregation of chicken liver fatty acid synthase in the presence of 3 M urea (1 M Na-phosphate buffer, pH 7.0; 37°C). Aggregation was registered by the increase in absorbance at 400 nm (ΔA_{400}) [58]. As can be seen from Fig. 3a, the terminal phase of aggregation (t > 25 min) is described satisfactorily by Eq. (5), suggesting that the order of aggregation with respect to protein is equal to unity. The following values of parameters were obtained: ΔA_{lim} = 1.197 ± 0.001, k_I = 0.0494 ± 0.0002 min^{-1}, and t_0 = 22.46 ± 0.04 min. The range of the values of $\Delta A/\Delta A_{lim}$ where first order kinetics are applicable comprises approximately 75% (from $\Delta A/\Delta A_{lim} \approx 0.25$ and higher). The first order of aggrega-

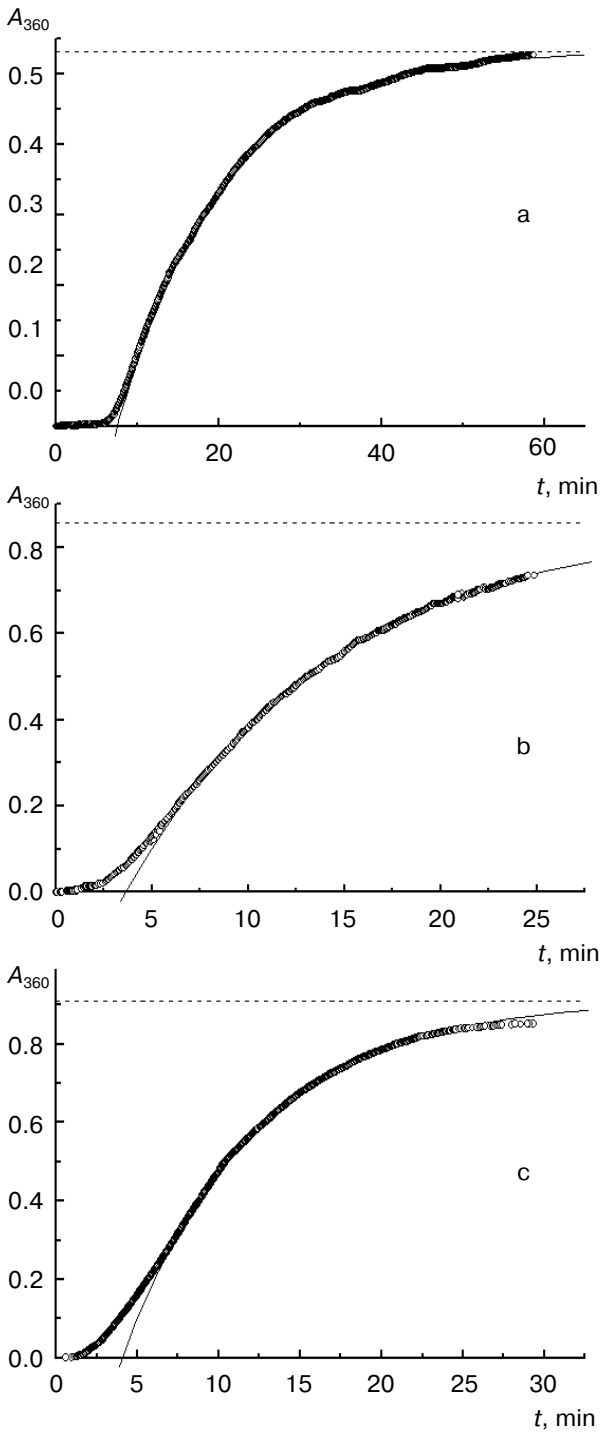


Fig. 2. Examples of thermal aggregation of proteins where the terminal phase of the kinetic curves follows first order kinetics. Kinetics of aggregation were registered by the increase in absorbance at 360 nm. a) Aggregation of beef heart catalase (50 mM Na-phosphate buffer, pH 7.0; 55°C). Points are the experimental data [46]. b) Aggregation of beef heart citrate synthase (50 mM Hepes-buffer, pH 7.0; 39.5°C). Points are the experimental data [47]. c) Aggregation of β_{low}-crystallin (20 mM Na-phosphate buffer, pH 6.9, containing 100 mM Na_2SO_4; 55°C). Points are the experimental data [48]. The solid curves are calculated from Eq. (5).

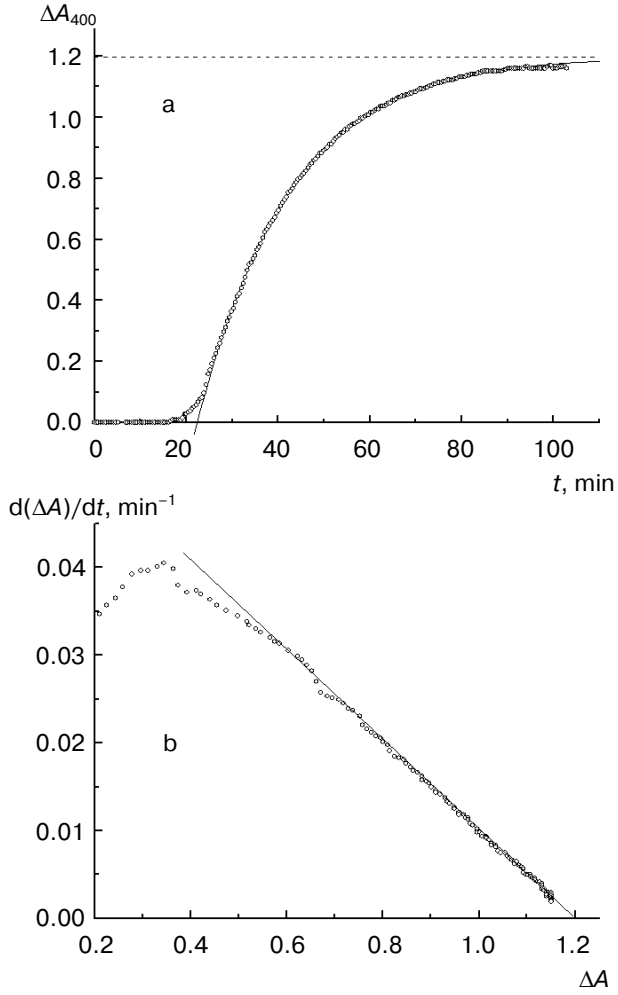


Fig. 3. Analysis of the kinetics of aggregation of chicken liver fatty acid synthase in the presence of 3 M urea (1 M Na-phosphate buffer, pH 7.0; 37°C). The final concentration of the enzyme was 1.3 μM. Points are the experimental data [58]. a) The dependence of the absorbance increment at 400 nm (ΔA_{400}) on time. The solid curve is calculated from Eq. (5). b) The dependence of $d(\Delta A)/dt$ on ΔA.

tion with respect to protein is supported by linearization of the terminal phase of the kinetic curve in coordinates $\{d(\Delta A)/dt; \Delta A\}$ (Fig. 3b).

We analyzed also the data on the kinetics of aggregation of bovine growth hormone (bGH) in the presence of 3.5 M GuHCl (bGH 1.75 mg/ml, 3°C) [51]. Aggregation was registered by the increase in absorbance at 450 nm. The kinetic curve of aggregation is described satisfactorily by Eq. (5) at the following values of parameters: $A_{lim} = 0.29 \pm 0.01$, $k_I = 0.66 \pm 0.07$ min^{-1}, and $t_0 = 0.39 \pm 0.05$ min (first order kinetics are applicable in the range of the values of A/A_{lim} from 0.08 and higher).

Aggregation accompanying refolding of proteins. In certain cases the proteins denatured by high concentrations of GuHCl or urea are capable of restoring the native structure after diminishing the denaturant concentration. Generally refolding is accompanied by aggregation of the protein, and the yield of the native protein decreases with increasing protein concentration [56, 60-65]. The structure of aggregates formed during refolding of lactate dehydrogenase from pig skeletal muscle after the treatment of the enzyme by 6 M GuHCl was studied using electron microscopy and circular dichroism [60]. Aggregates form an irregular network of filaments, the dimensions of the structural elements of the filaments being 10-15 times greater than those of the native tetramer (molecular mass of tetramer is 140 kD). Molecular mass of aggregates is several millions daltons. Aggregates consist of individual monomeric chains possessing partially recovered secondary structure.

Aggregation accompanying refolding of proteins is also used in the test-systems designed for estimation of the chaperone-like activity.

Our calculations show that in certain cases the time course of aggregation accompanying refolding of proteins follows first order kinetics. Figure 4 shows as an example the kinetics of aggregation of the mutant form of maltose-binding protein MalE31. The protein was denatured by 4 M GuHCl. Refolding was started by dilution of the protein by 50 mM Hepes-buffer, pH 7.5. The kinetics of aggregation accompanying the refolding process were registered by the increase in the increment of the light scattering intensity at 320 nm [66] (Fig. 4a). The terminal phase of the kinetic curve of aggregation ($t > 2.2$ min) was approximated by Eq. (3) using Scientist program. Value of n obtained ($n = 1.03 \pm 0.02$) is indicative of the applicability of first order kinetics. Figure 4a shows the results of description of the experimental data by the equation, which was obtained from Eq. (5) through substitution of an increment of light scattering intensity ΔI for absorbance A:

$$\Delta I = \Delta I_{lim}\{1 - \exp[-k_I(t - t_0)]\}, \qquad (13)$$

where ΔI_{lim} is the limiting value of ΔI at $t \to \infty$ and t_0 is the value of t, at which $\Delta I = 0$. Application of Eq. (13) gives the following values of parameters: $\Delta I_{lim} = 4.563 \pm 0.005$, $k_I = 0.572 \pm 0.008$ min^{-1}, and $t_0 = 1.47 \pm 0.03$ min. First order kinetics are applicable in the range of the values of $\Delta I / \Delta I_{lim}$ from 0.33 and higher. The first order of aggregation with respect to protein is supported by linearization of the terminal phase of the kinetic curve in coordinates $\{d(\Delta I)/dt; \Delta I\}$ (Fig. 4b).

Our calculations show that the situation where the terminal phase of aggregation accompanying refolding follows first order kinetics is realized also at refolding of lactate dehydrogenase from pig skeletal muscle (at low concentrations of the enzyme) [60] (Fig. 5a), the mutant of a human anti-β-galactosidase single chain antibody fragment scFv13c [57] (Fig. 5b), and creatine kinase from

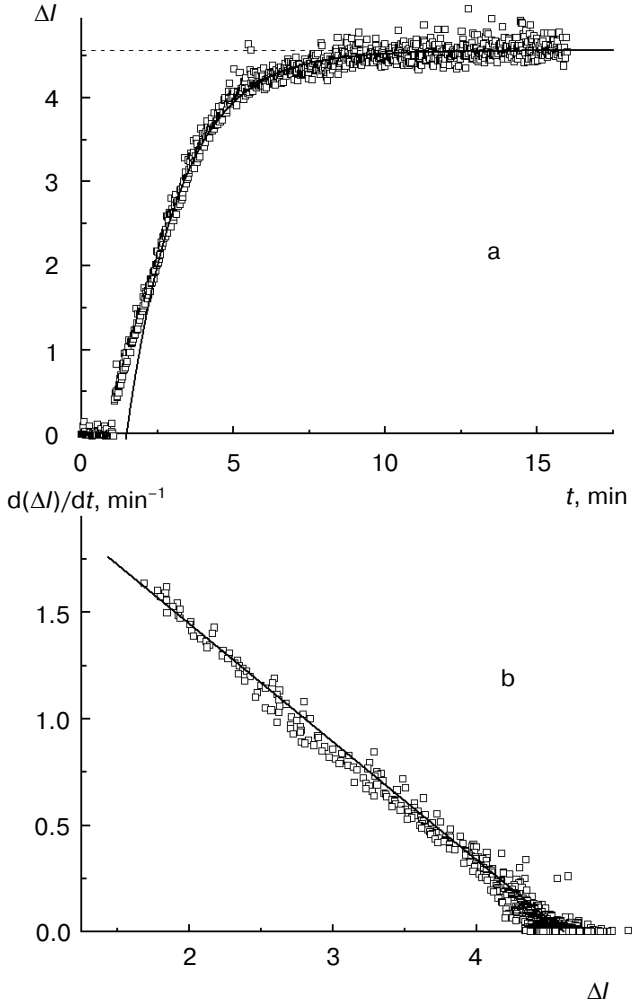


Fig. 4. Analysis of aggregation of the mutant form of the maltose-binding protein, MalE31, denatured by 4 M GuHCl after dilution by 50 mM Hepes-buffer, pH 7.5. The final concentration of the protein was 1.25 μM. Points are the experimental data [66]. a) The dependence of the increment of light scattering intensity at 320 nm (ΔI_{320}) on time. The solid curve was calculated from Eq. (13). b) The dependence of $d(\Delta I)/dt$ on ΔI.

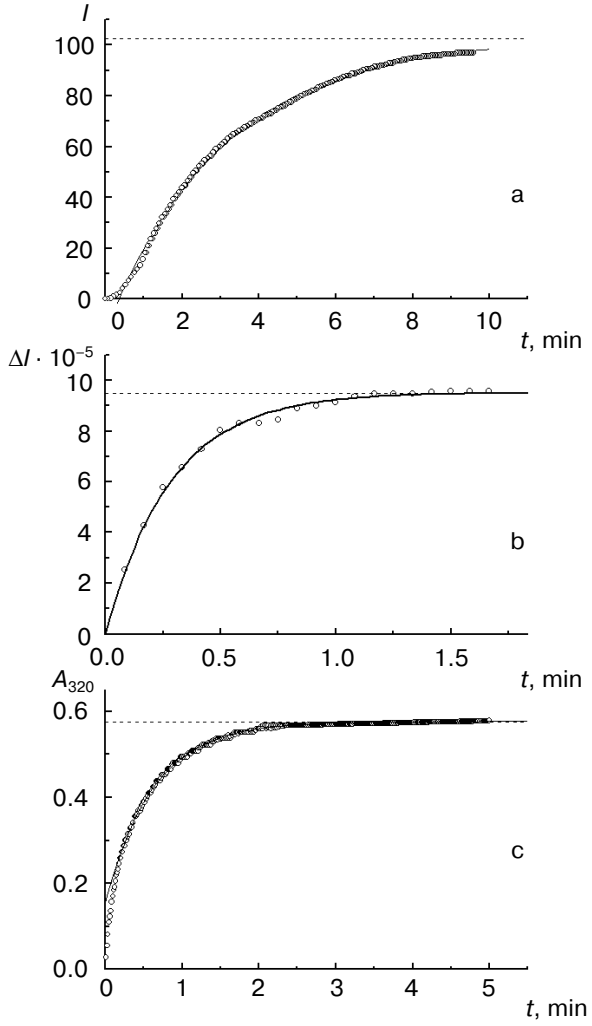


Fig. 5. Aggregation accompanying refolding of proteins. a) Aggregation of lactate dehydrogenase from rabbit skeletal muscle during refolding of the enzyme denatured by 6 M GuHCl (pH 7.0; 20°C; 0.05 μM lactate dehydrogenase). The kinetics of aggregation was registered by the increase in the intensity of light scattering (I is the light scattering intensity in arbitrary units). Points are the experimental data [60]. The solid curve was calculated from Eq. (13) at the following values of the parameters: $I_{lim} = 102.6$, $k_I = 0.326$ min^{-1}, and $t_0 = 0.35$ min. b) Aggregation of the mutant of a human anti-β-galactosidase single chain antibody fragment scFv13c accompanying refolding of the protein denatured by 7.5 M urea (25°C; protein concentration 5 μg/ml). The kinetics of aggregation was registered by the increase in the intensity of light scattering at 350 nm (ΔI is an increment of the light scattering intensity in arbitrary units). Points are the experimental data [57]. The solid curve was calculated from Eq. (13) at the following values of the parameters: $\Delta I_{lim} = 9.50 \cdot 10^5$, $k_I = 3.52$ min^{-1}, and $t_0 = 0$. c) Aggregation of creatine kinase from rabbit skeletal muscle during refolding of the enzyme denatured by 6 M urea (30 mM Tris-HCl-buffer, pH 8.0; 25°C; 2 μM creatine kinase). The kinetics of aggregation was registered by the increase in absorbance at 320 nm (A_{320}). Points are the experimental data [67]. The solid curve was calculated from Eq. (5) at the following values of the parameters: $A_{lim} = 0.575$, $k_I = 1.61$ min^{-1}, and $t_0 = -0.19$ min.

rabbit skeletal muscle [67] (Fig. 5c). First order kinetics are applicable in the range of the values of $\Delta I / \Delta I_{lim}$ from 0.20, 0.26, and 0.44, respectively, and higher (Fig. 5, a-c).

QUANTITATIVE ESTIMATION OF CHAPERONE-LIKE ACTIVITY

If aggregation of protein substrate follows first order kinetics, parameters A_{lim} and k_I may be used for the quantitative estimation of the activity of chaperone in the test-system based on suppression of protein aggregation. Consider as an example the results of the study of the chaperone-like activity of the C-terminal domain

(residues 518-803) of glucose-regulated protein (grp94). grp94 is a glycoprotein with molecular mass of 94 kD possessing multiple Ca^{2+}-binding sites. The molecular mass of the C-terminal domain (grp94-CT) measured in the presence of 1 mM dithiothreitol was found to be 36 kD. In the absence of reducing agents grp94-CT exists as a dimer (however, high-order oligomers are also observed). The chaperone-like activity of grp94-CT was studied by Itarte et al. [68] in the test-system based on suppression of aggregation of the catalytic subunit of protein kinase CK2 (CK2α) at 40°C. Figure 6a shows the kinetic curves of aggregation registered by the increase in the light scattering intensity at 360 nm. The terminal phase of all the kinetic curves obtained in the absence and in the presence of grp94-CT follows first order kinetics. The experimental curve obtained in the absence of grp94-CT was described by Eq. (13) at the following values of the parameters: $I_{lim} = 8.30 \pm 0.06$, $k_I = 0.100 \pm 0.005$ min^{-1}, and $t_0 = 4.0 \pm 0.4$ min (curve *1* in Fig. 6a). First order kinetics are applicable in the range of the values of I/I_{lim} from 0.33 and higher. The values of I_{lim} and k_I for the kinetic curves of aggregation obtained in the presence of grp94-CT are shown in Figs. 6b and 6c as functions of the molar ratio [grp94-CT]/[CK2α].

A decrease in the I_{lim} value in the presence of chaperone is due to binding of denatured protein by chaperone. When bound to chaperone, denatured protein is not being involved in aggregation. If affinity of denatured protein to chaperone is rather high (in other words, the concentrations of chaperone and denatured protein exceed substantially the value of the microscopic dissociation constant for the corresponding complex), the dependence of I_{lim} on the concentration of chaperone allows the stoichiometry of the complexation of chaperone with denatured protein to be determined. The intercept on the abscissa axis may be used for this purpose. For example, for the data presented in Fig. 6a the complete suppression of aggregation is observed at the [grp94-CT]/[CK2α] ratio equal to 4.3 ± 0.2.

It should be noted that this method of determination of the stoichiometry of the complex formed by chaperone and denatured protein can be considered as reliable if the dependences of I_{lim} on the concentration of chaperone obtained at various fixed concentrations of protein substrate give the same value of stoichiometry of the complex.

As for the dependence of the rate constant k_I on the concentration of chaperone, k_I varies insignificantly when the [grp94-CT]/[CK2α] ratio increases to 2 : 1 (Fig. 6c). Further increase in the [grp94-CT]/[CK2α] ratio results in a decrease in the rate constant k_I. In our opinion, analysis of the dependence of k_I on the concentration of chaperone needs understanding of the mechanism of aggregation of protein substrate used.

Thus, the action of chaperone on aggregation of protein substrate is connected with the change in parameters A_{lim} (or I_{lim}) and k_I. It is evident that each of these parameters is not a direct characteristic of the rate of aggregation. It is of interest to discuss how the influence of chaperone on *the rate* of aggregation may be characterized. In our opinion, the $k_I \cdot A_{lim}$ (or $k_I \cdot I_{lim}$) value is especially suitable for this purpose. In order to explain the physical sense of the $k_I \cdot A_{lim}$ product, one must draw on the scheme presented in Fig. 7. This scheme includes an experimental dependence of A on t (curve *1*) and approximation of this dependence by the theoretical equation (5) (curve *2*). The theoretical curve cuts the time axis at $t = t_0$. The straight line *4* is a tangent to the theoretical curve at the point with coordinates $t = t_0$ and $A = 0$. The slope of this tangent is $k_I \cdot A_{lim}$. Thus, the $k_I \cdot A_{lim}$ product is the initial rate of aggregation (expressed in units of absorbance per time unit) on the assumption that aggregation completely follows first order kinetics. Figure 6d shows the dependence of $k_I \cdot I_{lim}$ on the [grp94-CT]/[CK2α] ratio for the above-mentioned suppression of aggregation of CK2α in the presence of grp94-CT.

COMPARISON OF THE KINETICS OF AGGREGATION WITH THE KINETICS OF DENATURATION OF PROTEINS

Some investigators studied simultaneously the kinetics of thermal aggregation of certain enzymes and the kinetics of their inactivation. It is of special interest to compare the kinetic constants calculated for aggregation and inactivation of the enzymes.

Itarte et al. [68] studied the kinetics of inactivation of the catalytic subunit of protein kinase CK2 at 40°C and influence of grp94-CT on the process of inactivation (Fig. 8). As can be seen from the figure, chaperone does not affect the rate of inactivation of CK2α. Our calculations show that the kinetic curve of inactivation is described satisfactorily by the exponential equation of the type:

$$v/v_0 = (v/v_0)_{lim} + [1 - (v/v_0)_{lim}]\exp(-k_I t), \qquad (14)$$

where v is the rate of the enzymatic reaction and v_0 is the value of v at $t = 0$. The following values of parameters were obtained: $(v/v_0)_{lim} = 0.144 \pm 0.014$ and $k_I = 0.080 \pm 0.005$ min^{-1} (when estimating these parameters, we used all the experimental points presented in Fig. 8). The fact that the value of $(v/v_0)_{lim}$ is different from zero may be explained in the belief that the enzyme preparation contains a fraction with high thermostability. The rate constant for the process of inactivation is close to the rate constant of the first order calculated by us for the terminal phase of aggregation of CK2α ($k_I = 0.100$ min^{-1}).

When comparing the kinetics of thermal aggregation and inactivation of creatine kinase from rabbit skeletal muscle, we observed the analogous situation. The termi-

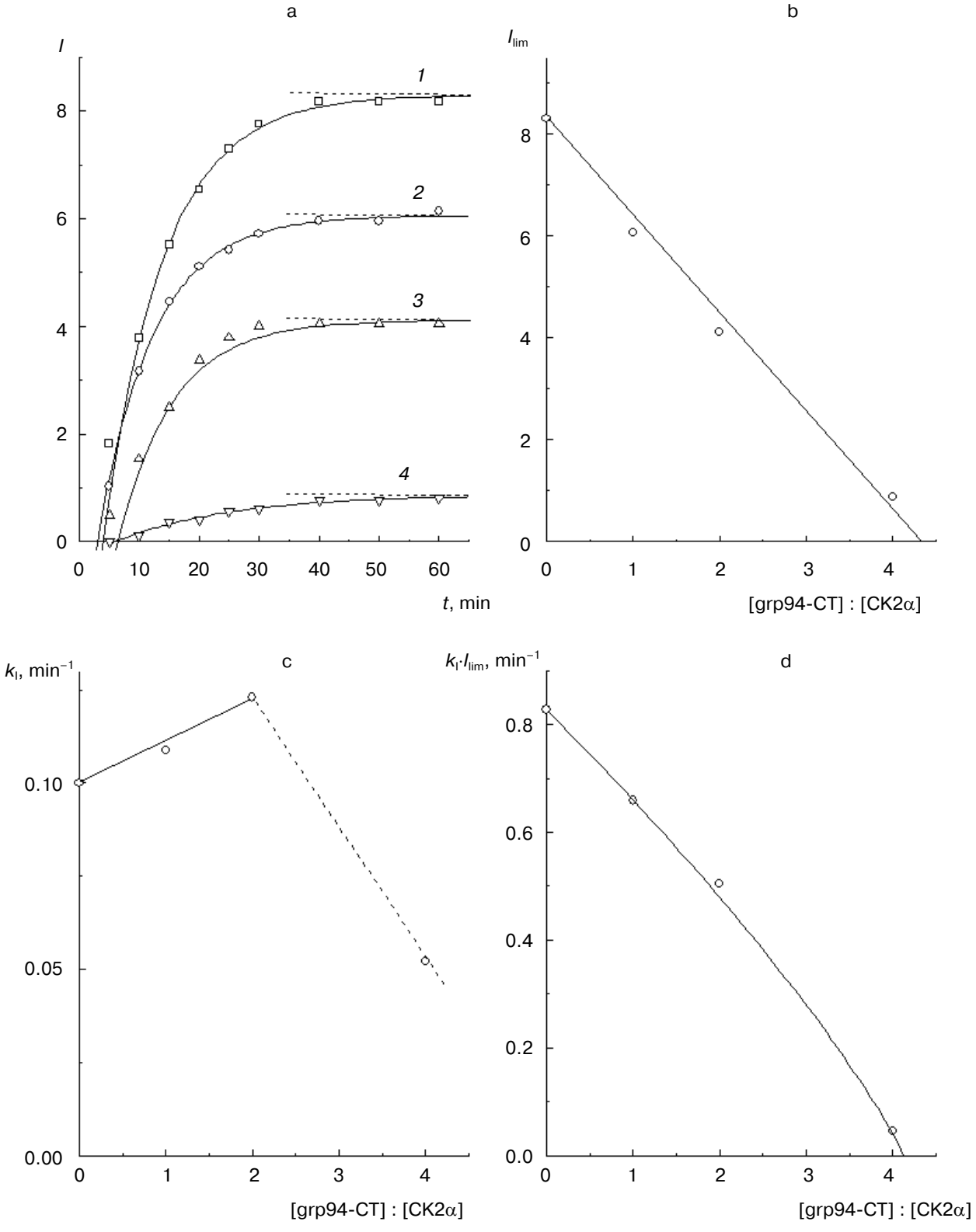


Fig. 6. Analysis of chaperone-like activity using a test-system based on suppression of aggregation of protein substrate. a) The kinetics of thermal aggregation of the catalytic subunit of protein kinase CK2 (CK2α; 40°C) registered by the increase in the intensity of light scattering at 360 nm (I, arbitrary units). Curve *1* is a control (in the absence of grp94-CT). Curves *2-4* were obtained at the molar ratio [grp94-CT]/[CK2α] equal to 1 : 1, 2 : 1, and 4 : 1, respectively. Points are the experimental data [68]. The solid curves were calculated from Eq. (13). b-d) The dependences of I_{lim}, k_I, and $k_I \cdot I_{lim}$ on the ratio [grp94-CT]/[CK2α], respectively.

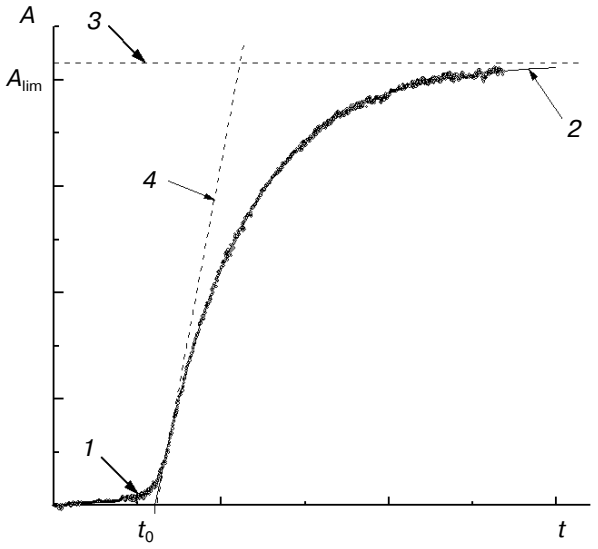


Fig. 7. Illustration of the physical sense of the $k_I \cdot A_{lim}$ magnitude: *1*) the experimental dependence of absorbance (A) on time; *2*) the theoretical dependence corresponding to Eq. (5); *3*) the limiting value of A corresponding to A_{lim}; *4*) a tangent to the curve *2* in the point with coordinates $t = t_0$ and $A = 0$.

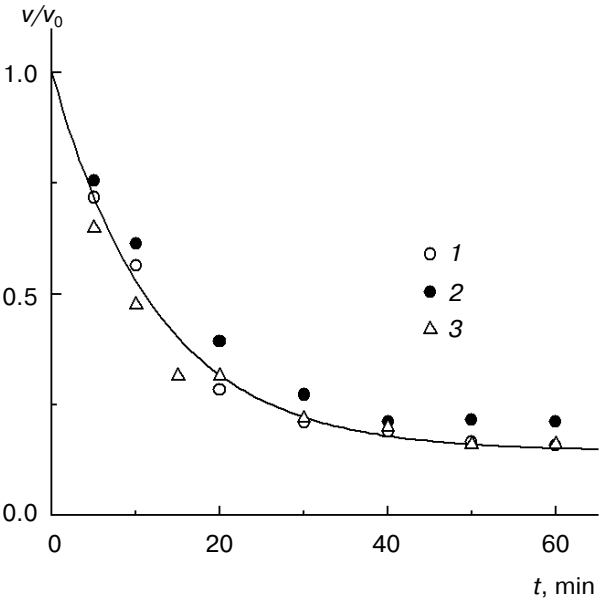


Fig. 8. Kinetics of thermal inactivation of the catalytic subunit of protein kinase CK2 (CK2α) at 40°C. The dependence of the relative enzymatic activity of CK2α, v/v_0 (v is the rate of the enzymatic reaction and v_0 is the value of v at $t = 0$) on time. Points are the experimental data [68]; *1*) inactivation in the absence of grp94-CT; *2*, *3*) inactivation at the values of the [grp94-CT]/[CK2α] ratio equal to 2 : 1 and 4 : 1, respectively. The solid curve is calculated from Eq. (14).

nal phase of aggregation of creatine kinase registered by the increase in absorbance at 400 nm follows first order kinetics. The rate constant of the first order k_I was found to be 0.15 min^{-1} (30 mM Hepes-buffer, pH 8.0; 50.6°C; protein concentration 0.4-0.6 mg/ml). This value of k_I is close to the rate constant of the process of inactivation, which obeys the exponential law [69].

We now turn our attention to the kinetic curve of thermal aggregation of catalase (Fig. 2a, the data obtained by Hook and Harding [46]). The authors of the cited work studied the kinetics of inactivation of catalase at 55°C. Our calculations showed that it is impossible to describe the kinetic curve by the exponential equation. This circumstance hampers the comparison the kinetics of denaturation and inactivation. Nevertheless, it is worthy of note that the initial slope of the kinetic curve in coordinates $\{v/v_0; t\}$, i.e., the specific initial rate of inactivation w_0, is equal to 0.077 ± 0.006 min^{-1}. This value of w_0 is close to the rate constant of the first order calculated by us for the terminal phase of aggregation (k_I = 0.0795 min^{-1}).

DISCUSSION

Aggregation involves interaction of two (or several) protein molecules transformed to the unfolded state. Therefore, many investigators believe that the time course of aggregation follows kinetics of second (or higher) order [42, 60-65, 70-74]. For example, Kiefhaber et al. [70] published a theoretical work where the rate of aggregation is assumed to be controlled by the initial stage of dimerization. This initial stage is followed by fast stages of aggregation, which give rise to aggregate with an aggregation number N. On these assumptions, the rate of aggregation is proportional to the current concentration of the denatured molecules D squared:

$$(d[D]/dt)_{agg} = Nk_{II}[D]^2. \quad (15)$$

However our calculations show that irreversible aggregation of proteins caused by the action of heating or denaturants and aggregation accompanying refolding follows generally first order kinetics at the terminal phase.

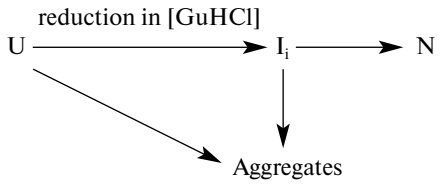
The situation when denaturation of the protein registered by the loss in the enzymatic activity and protein aggregation (at the terminal phase) follows first order kinetics and the corresponding rate constants of the first order are similar implies that the rate-limiting stage of aggregation is the stage of unfolding of the protein molecule. In other words, under conditions studied the rate of the stage of aggregation far exceeds that for the stage of denaturation of the protein molecule. One can expect that, if chaperone does not affect the rate of protein denaturation, the rate constant of the first order calculated from the kinetic curve of aggregation remains unchanged under variation of the concentration of chaperone (in a certain range of chaperon concentration). Such a situation is realized in the studies of the action of chaperone grp94-CT on aggregation of the catalytic sub-

unit of protein kinase CK2 (Fig. 6c). We analyzed also the effect of a protein possessing a chaperone-like activity HspA from *Synechococcus vulcanus* on thermal aggregation of malate dehydrogenase from pig heart mitochondria at 45°C (the experimental data obtained by Nakamoto et al. [43]). Molecular mass of the monomeric form of HspA is 16 kD. However HspA exists in solution as an oligomer with molecular mass of 393 kD. The authors of the cited work showed that HspA did not affect the rate of thermal aggregation of malate dehydrogenase. According to our calculations, the rate constant for enzyme inactivation is equal to 0.14 min^{-1}. This value of k_I is close to the rate constant of the first order calculated from the kinetic curve of aggregation (k_I = 0.094 min^{-1}; Fig. 1). Analysis of the data on the influence of HspA on aggregation of malate dehydrogenase shows that the change in the k_I value in the presence of HspA is insignificant: k_I was found to be 0.115 ± 0.005 and 0.137 ± 0.001 min^{-1} at molar ratio [HspA oligomer]/[malate dehydrogenase] equal to 0.147 and 0.294, respectively.

Since the stage of aggregation is a bi(multi)molecular reaction, one can expect that in the region of sufficiently low concentrations of the denatured protein the rate-limiting stage of aggregation is the stage of aggregation of denatured protein molecules (the situation when the rate of protein molecule unfolding far exceeds that for the stage of aggregation). It is clear that, to understand the mechanism of aggregation, the kinetic investigations of aggregation should be carried out in a wide range of the protein concentrations. In particular, if the conclusion has been drawn that the rate-limiting stage of aggregation is the stage of protein molecule unfolding, this conclusion should be substantiated by the protein concentration-independence of the rate constant of the first order calculated from the kinetic curve of aggregation (strictly speaking, in a certain range of the values of $[P]_0$).

There are no systematic investigations of the kinetics of thermal aggregation of protein in a wide range of the protein concentrations. Therefore, in the present work our attention was focused on the order of aggregation with respect to protein calculated from individual kinetic curves. It should be noted that in the general case the order of the reaction with respect to the substance (n_C) calculated from the dependence of the initial rate of the reaction v_0 on the initial concentration of the substance C_0 differs from that calculated from an individual kinetic curve. For example, previously [75] we studied the kinetics of denaturation of lactate dehydrogenase from rabbit skeletal muscle under the conditions where denaturation on the surface of the vessel was of considerable importance in the overall process of denaturation. It was shown that the time course of the loss in the enzymatic activity follows the exponential law (i.e., n_C = 1), whereas the character of the dependence of the initial rate of inactivation on the initial concentration of the protein testified to the applicability of the zeroth order of inactivation with respect to protein (n_C = 0). Comparison of the values of n_C calculated from the individual kinetic curves and the dependence of v_0 on C_0 is essential for elaboration of the kinetic mechanism of the process under study.

The fact that aggregation accompanying refolding of proteins in certain cases follows first order kinetics is surprising. Actually it is commonly supposed that refolding proceeds through the formation of intermediates (I_i), undergoing further transformation via two pathways. One of the pathways gives rise to the native form (N) and the other is responsible for the formation of protein aggregates [33, 38, 40, 42, 56, 60-65, 71-83]:



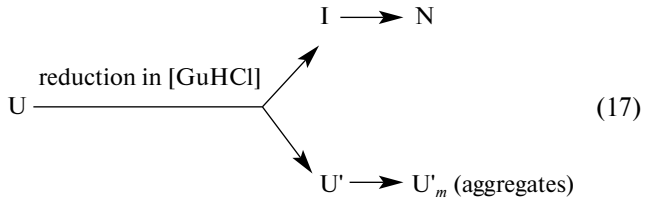
(16)

U is the unfolded state of the protein in the solution of denaturant. When drawing the scheme, we took into account the possibility of aggregation of form U as suggested by some investigators. For example, renaturation of the above-mentioned mutant form of maltose-binding protein includes two stages (renaturation was registered by the change in fluorescence of tryptophan residues of the protein molecule). The fast stage is completed over a period of time comparable with the time taken to mix the enzyme solution containing GuHCl with buffer. The slow stage is characterized by the rate constant of the first order equal to 0.24 min^{-1} [66, 84]. Recall that the value of k_I equal to 0.572 min^{-1} was obtained by us for aggregation accompanying refolding under these conditions. Thus, the times of half-conversion for the slow stage of renaturation and aggregation are the magnitudes of the same order.

There is little likelihood that under the conditions of competence of two processes (transformation of intermediate to the native form and aggregation of intermediate) aggregation will follow first order kinetics. In our opinion, only when the kinetic mechanisms of aggregation is elaborated on the basis of the kinetic investigations in a wide range of the protein concentrations can reasonably justified kinetic models of protein refolding accompanied by aggregation be developed. It is of special interest to analyze the region of the protein concentrations where the rate of the stage of aggregation is comparable with that for the stage of the protein molecule unfolding.

We return to refolding accompanied by protein aggregation. What mechanism can be proposed to explain the first order of aggregation with respect to protein? Let U be

the completely unfolded state of the protein in the presence of high concentration of GuHCl. Suppose that when diluting a part of U is rather rapidly transformed to intermediate I, which is capable of producing the native form N, whereas other part of U remains in the unfolded state, which is incapable of reverting to the native state (let us designate this form as U′). Time course of aggregation is connected with the "sticking" of U′. The general scheme of refolding under discussion has the following form:



(17)

In this scheme the stage of transformation of intermediate I to the native state and the stage of aggregation $mU' \rightarrow U'_m$ proceed independently of one another. In the framework of scheme (17) aggregation is similar to thermal aggregation of the protein under the conditions where over a short period of time the protein is completely transformed to the unfolded state (for example, at high temperatures) and time course of aggregation is due solely to interaction of the unfolded forms. Taking into account this circumstance, one can expect that aggregation will follow first order kinetics at the terminal phase.

Recombinant proteins produced in *Escherichia coli* are prone to aggregation and form inclusion bodies [85-88]. Superexpression results in high local concentrations of the folding intermediates and a significant part of these intermediates becomes included in insoluble precipitate because of competition between the reactions of folding and aggregation. Aggregation is a severe problem in biotechnology, since isolation and purification of recombinant proteins are made difficult by the appearance of protein aggregates. Rinas et al. [89] analyzed the kinetics of the formation of inclusion bodies and showed that the inclusion of monomers in the precipitate follows first order kinetics (provided that the time-dependent decrease in the rate of the production of the initial product of translation obeys the exponential law). The authors of the cited work drew an analogy between the kinetics of the formation of inclusion bodies and the kinetics of inclusion of β-amyloid protein in amyloid fibrillar plaques of Alzheimer's disease [90] (see also [91-96]).

The presence of the stage where monomer disappears with the rate proportional to the first power of the monomer concentration is characteristic of the mechanism of protein association including the stage of nucleation (nucleation-dependent polymerization). This model was first developed by Oosawa and Kasai [97]. The model includes the stage of the formation of a nucleus

$$n\mathrm{M} \rightleftarrows \mathrm{M}_n \qquad (18)$$

(M is a monomer and M_n is a nucleus) and the stage of growth of an associate

$$\mathrm{M}_i + \mathrm{M} \rightleftarrows \mathrm{M}_{i+1}\,. \qquad (19)$$

If the concentration of nuclei remains constant in the course of association and the stage of growth of associate is irreversible, the rate of disappearance of monomer in the reaction of growth of associate is proportional to [M], i.e., the bimolecular reaction $M_i + M \rightarrow M_{i+1}$ proceeds as a reaction of pseudo-first order. From the above reasoning one can assume that the first order of aggregation with respect to protein can be observed under the conditions where growth of aggregate proceeds as an irreversible reaction of attachment of the unfolded form of the protein to nuclei formed in the solution and the concentration of nuclei (or, to be more precise, concentration of the points of growth) remains constant in the course of aggregation. The rate of aggregation expressed as a rate of diminishing the monomer concentration has the following form:

$$v_{agg} = -\mathrm{d[M]}/\mathrm{d}t = k_{II}[\mathrm{R}][\mathrm{M}] = k_I[\mathrm{M}], \qquad (20)$$

where [R] is the concentration of nuclei, k_{II} is the rate constant of the second order, and k_I is the rate constant of pseudo-first order ($k_I = k_{II}[\mathrm{R}]$). It should be noted that the presence of the stage of nucleation might be one of the reasons of appearance of lag period on the kinetic curves of aggregation.

In the present work we showed that irreversible aggregation of protein caused by the action of heating or denaturants (GuHCl, urea) and aggregation accompanying refolding of proteins denatured by GuHCl or urea follow, as a rule, first order kinetics at the terminal phase. For each kinetic curve we can calculate two parameters: the limiting value of absorbance A_{lim} (or the light scattering intensity I_{lim}) and the rate constant of the first order k_I. When estimating the chaperone-like activity in the test-systems based on suppression of aggregation of protein substrates, these parameters may be used for the quantitative characterization of the efficiency of action of chaperones. A similar approach can be applied for the quantitative estimation of the effects of the substances of non-protein nature (osmolytes, derivatives of cyclodextrin, and so on) on the rate of protein aggregation as well as the quantitative description of the change in the aggregation rate under variation of the experimental conditions (temperature, ionic strength, pH).

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