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Change in Kinetic Regime of Protein Aggregation with Temperature Increase. Thermal Aggregation of Rabbit Muscle Creatine Kinase

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Abstract—Creatine kinase thermal aggregation kinetics has been studied in 30 mM Hepes-NaOH buffer, pH 8.0, at two temperatures: 50.6 and 60°C. Aggregation kinetics was analyzed by measuring the growth of apparent absorption (A) at 400 nm. It was found that the limiting value of apparent absorption (A_{lim}) is proportional to protein concentration at both temperatures. The first order rate constant (k_1) does not depend on protein concentration in the range 0.05-0.2 mg/ml at temperature 50.6°C, but at temperature 60°C it increases with the growth of protein concentration in the range 0.1-0.4 mg/ml. Kinetic curves, shown in coordinates $\{A/A_{lim}; t\}$, in experiments at 50.6°C fuse to a common curve, which coincides with the theoretical curve of creatine kinase denaturation calculated using the denaturation rate constant determined from differential scanning calorimetry. At temperature 60°C, half-transformation time $t_{1/2} = \ln 2/k_1$ decreases when protein concentration grows. We conclude that when temperature increased from 50.6 to 60°C, change in the kinetic regime of thermal creatine kinase aggregation took place: at 50.6°C aggregation rate is limited by the stage of protein molecule denaturation, but at 60°C it is limited by the stage of protein aggregate growth, which proceeds as a reaction of pseudo-first order. Small heat shock protein Hsp 16.3 *Mycobacterium tuberculosis* suppresses the creatine kinase aggregation.

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Key words: creatine kinase, thermal aggregation, kinetics, small heat shock protein Hsp 16.3

Protein aggregates are formed as a result of interaction of partially or fully unfolded protein molecules leading to generation of agglomerates of arbitrary form [1-9]. In normal cell physiological conditions, there is no accumulation of protein aggregates because of the existence of intra-cell mechanisms "controlling" protein quality. This mechanism involves the combined action of chaperones and proteinases [4, 5, 8, 9]. When generation of irregularly folded proteins exceeds the ability of the cell to disintegrate them, insoluble intra-cell complexes and inclusion bodies are formed. This process underlies the pathogenesis of many degenerative and neurodegenerative human diseases [3-7]. Thus, study of macroscopic aggregation of proteins is one of the important directions in contemporary biology [3-5].

To study thermal aggregation, the following proteins are often used in the experiments: citrate synthase, malic dehydrogenase from mitochondria, β-crystallin, alcohol dehydrogenase, and a number of other proteins [3, 4]. Creatine kinase (CK) as an object for investigation was used only in a few cases [10-15]. Kinetics of CK thermal aggregation has not been studied before at different temperatures and in a wide range of protein concentration. The enzyme is widely distributed in cells; it can be found in large quantities in cytoplasm, in mitochondria, and in sarcoplasmic reticulum of muscle, cerebral, heart, and other tissues. Creatine kinase plays a role of "energy

Abbreviation: CK) creatine kinase.

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buffer", catalyzing the reaction of reversible transfer of phosphoryl group between ATP and creatine, which leads to formation of ADP and creatine phosphate. Creatine phosphate is stored in the cell and is used later by means of ATP regeneration at moments of high cell activity [16, 17]. The enzyme has been found in cells in the form of cytoplasmic isoenzymes, which are binary combinations of either identical subunits (MM, BB) or different subunits (MB) [16, 18], and two mitochondrial isoenzymes [19]. Creatine kinase was first isolated in homogeneous state from rabbit skeleton muscle [20, 21], and its physicochemical properties have been intensively studied [16, 17]. X-Ray analysis of CK from rabbit muscle [22], as well as of mitochondrial CK [23], has shown that each subunit of CK consists of two domains: small (1-112) and large (113-380). An active site is located in the cleft between the two domains. Subunits of the dimer are bound in the region of two contact sites by eight hydrogen bonds and one salt bridge [22]. Despite such a small number of bonds, the interaction between subunits is rather strong. Dissociation of dimers to monomers takes place in hard conditions (8 M urea) and is accompanied by partial unfolding of the monomer [22, 24, 25]. It is worth noting that on warming the protein globule of CK unfolds without preliminary dissociation of dimer to monomers [10, 11]. Thermal denaturation of CK turns out to be a one phase process: M_2 (natural) $\rightarrow$ 2M (dissociated) [11].

Already in early papers, it was shown that CK is rather stable in the region of physiological temperatures (0-40°C). In this region, its activity remains practically unchanged [16]. Even when the enzyme is kept for 30 min at 45°C, it retains 90% of its activity. But further increase in the temperature leads to a quick loss of the enzyme activity. In the region of 53-55°C, the enzyme becomes totally inactivated [10, 16]. Using differential scanning calorimetry, it was shown that when the enzyme was heated with the rate 1 K/min excessive heat capacity reached a maximum at 56°C [11].

The present paper is devoted to the study of thermal aggregation kinetics of rabbit muscle CK in a wide range of protein concentration at two temperatures, 50.6 and 60°C, which correspond to the initial part of protein molecule unfolding and the temperature when the molecule is fully unfolded. The influence on this process of the small heat shock protein Hsp 16.3 *Mycobacterium tuberculosis*, properties of which are well studied [26-31], was also investigated. Earlier it has been shown that this chaperone is an oligomer consisting of nine subunits, which have the property of rapid dynamic dissociation and reassociation [27]. It is highly interesting that warming chaperone from 25 to 65°C leads to dissociation of the nanomer. The higher was the temperature, the higher the degree of chaperone dissociation to oligomers of smaller molecular mass was observed. It is believed that dissociation of Hsp 16.3 to subunits is a necessary condition of opening substrate-binding centers and thus revealing its chaperone activity [29, 30].

MATERIALS AND METHODS

Creatine kinase was isolated from rabbit muscle according to the procedure described by Kuby [20, 21] followed by DEAE cellulose chromatography [32]. The enzyme was lyophilized and kept at −20°C. Homogeneity of the enzyme was checked according to criteria of gradient SDS-polyacrylamide gel electrophoresis. Protein concentration was determined by measuring the absorbance at 280 nm with $A_{280}^{1\,cm} = 0.88$ (mg/ml)$^{-1}$ [33]. The molar concentration of the enzyme was calculated using the molecular mass of the dimer of 85,964 daltons, determined by mass spectrometry [34].

Kinetics of thermal aggregation of the enzyme was studied using a Hitachi-557 spectrophotometer (Japan) interfaced with a computer. The spectrophotometer measured apparent absorption of light traversing the thermo-stabilized cuvette of 1 cm path length containing the enzyme solution. Measurements were carried out at wavelength 400 nm for which the native enzyme did not absorb. The process of protein aggregation was studied in 30 mM Hepes-NaOH buffer, pH 8.0, which was preliminarily warmed to the necessary temperature. Then aliquots of the enzyme solution (5-50 μl), dialyzed against the used buffer, were put into the cuvette with buffer. The cuvette was covered with a lid during experiments. The temperature of the solution in the cuvette was constantly controlled by a thermocouple.

Small heat shock protein *M. tuberculosis* Hsp 16.3 was prepared by the method described by Z. Chang [26] in the form of lyophilized powder and kept at −20°C. In experiments, we used protein portions dissolved in 30 mM Hepes-NaOH buffer, pH 8.0, dialyzed against the same buffer. Protein concentration was determined using the spectrophotometer at 280 nm assuming extinction coefficient $A_{280}^{1\,cm} = 0.2$ (mg/ml)$^{-1}$. Molar concentration of chaperone was calculated using molecular mass of monomer of 16,100 daltons, determined by mass spectrometry [26].

Mathematical analysis of data on aggregation kinetics was performed using the programs Origin 7.0 (Microcal Software, Inc., USA) and Scientist (MicroMath, USA).

RESULTS AND DISCUSSION

Kinetics of CK aggregation at 50.6°C was studied in 30 mM Hepes-NaOH buffer, pH 8.0, in the protein concentration range 0.05-0.2 mg/ml. Figure 1 shows the behavior of typical kinetic curves and increase with time of apparent optical absorbance at 400 nm (A_{400}). Each

curve is the result of averaging of 2-4 parallel measurements. Analysis of kinetic curves was done using the program Scientist for the final stage of aggregation process under the assumption that the apparent optical absorbance is proportional to the amount of aggregated protein. To determine kinetic parameters describing curves, we used the equation derived for examination of the protein aggregation process considered as a reaction of n-th order:

$$dA/dt = k_n(A_{lim} - A)^n, \quad (1)$$

where t is time, A is A_{400} at moment t, A_{lim} is the limiting value of A at $t \to \infty$, k_n is the rate constant of n-th order reaction, and n is the reaction order with respect to protein [3, 4]. Within the range of protein concentrations used in the experiment, the determined value of n was close to one (1.00 ± 0.05) (Fig. 2a). On these grounds, we may conclude that the aggregation reaction is 1-st order with respect to protein.

In cases when the final stage of the reaction is 1-st order, kinetic curves can be described by the equation suggested in our papers [3, 4]:

$$A = A_{lim}\{1 - \exp[-k_I(t - t_0)]\}, \quad (2)$$

where k_I is the rate constant of 1-st order and t_0 is the t value at which $A = 0$. Applicability of this equation was demonstrated earlier in studies of aggregation of CK [35], coat protein of tobacco mosaic virus [35-37], luciferase [38], and a number of other proteins [3, 4].

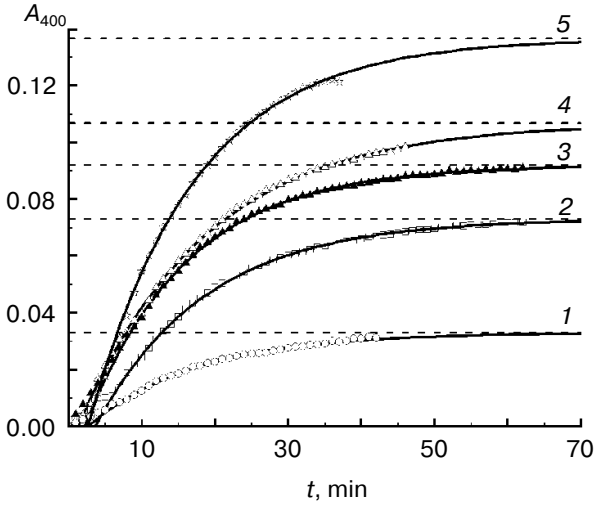

Fig. 1. Kinetics of creatine kinase thermal aggregation at 50.6°C. Aggregation kinetics were registered at protein concentrations: 0.05 (*1*), 0.1 (*2*), 0.125 (*3*), 0.15 (*4*), and 0.2 mg/ml (*5*). Points, experimental data; solid lines, theoretical curves plotted by using Eq. (2); dashed horizontal lines, limiting values of A_{400} (A_{lim}) calculated on the basis of Eq. (2).

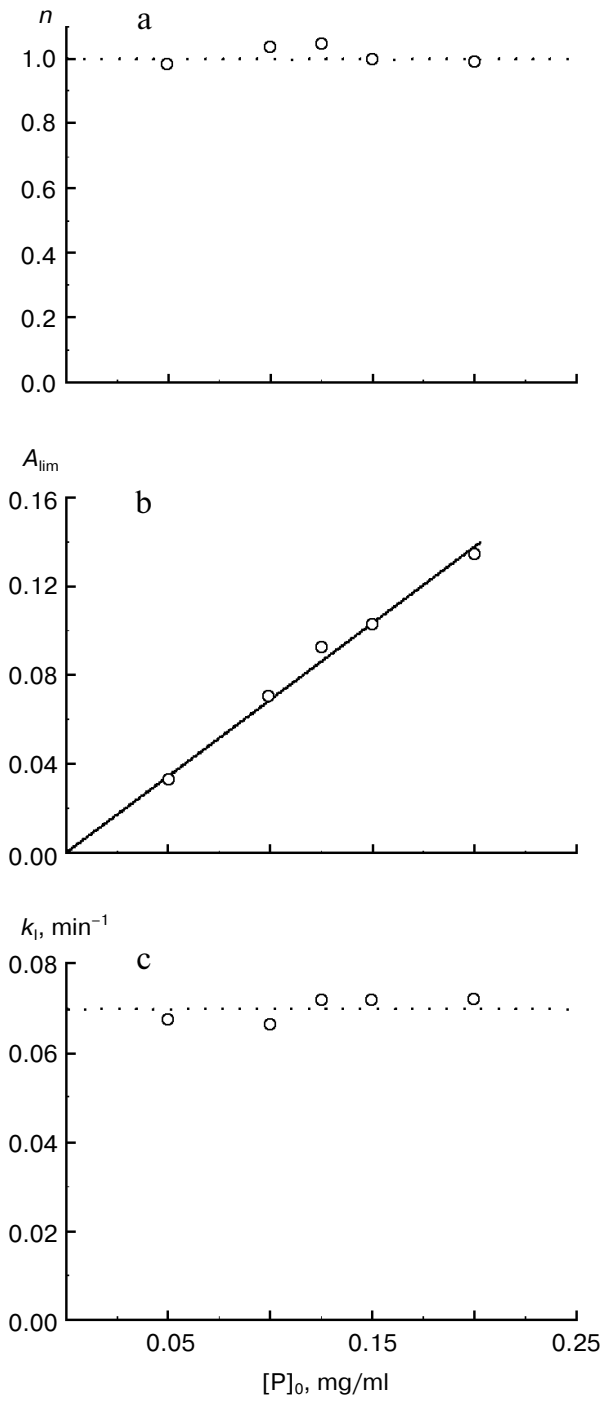

Fig. 2. Analysis of thermal aggregation of creatine kinase at 50.6°C. The dependence of the parameters n (a), A_{lim} (b), and k_I (c) on protein concentration $[P_0]$.

As can be seen from Fig. 1, experimental aggregation curves, shown in coordinates $\{A; t\}$, are satisfactorily described by Eq. (2) (solid lines in Fig. 1). For example, we give equation parameters for two limiting protein concentrations, 0.05 and 0.2 mg/ml, calculated using the

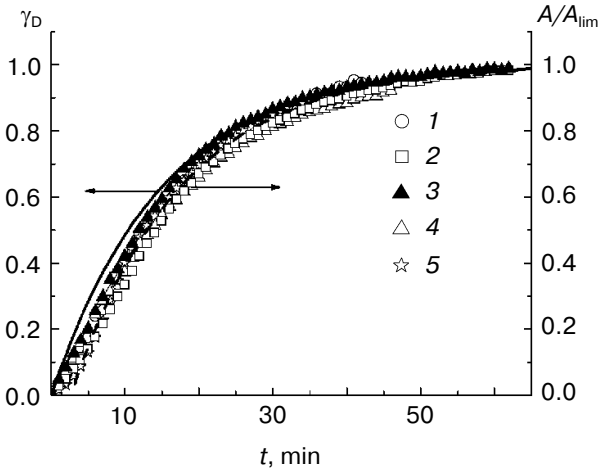

Fig. 3. Kinetic curves of creatine kinase aggregation at 50.6°C shown in coordinates $\{A/A_{lim}; t\}$. Protein concentration are 0.05 (*1*), 0.1 (*2*), 0.125 (*3*), 0.15 (*4*), and 0.2 (*5*) mg/ml. Dashed line, aggregation curve resulting from averaging points relating to different concentration; solid line, theoretical curve of denaturation kinetics plotted according formula (3); γ_D, fraction of denatured protein.

Origin 7.0 program. For CK concentration 0.05 mg/ml the parameters are: $A_{lim} = 0.033 \pm 0.001$; $k_I = 0.068 \pm 0.001$ min^{-1}; $t_0 = 2.69 \pm 0.07$ min. For CK concentration 0.2 mg/ml the parameters are: $A_{lim} = 0.137 \pm 0.001$, $k_I = 0.069 \pm 0.001$ min^{-1}; $t_0 = 2.66 \pm 0.03$ min. The value of A_{lim} increases proportionally to the increase in protein concentration (Fig. 2b). This bears out the assumption that optical absorbance value is proportional to the amount of aggregated protein. It is of interest that the rate constant k_I of the 1-st order reaction turned out to be the same for all investigated protein concentrations (Fig. 2c).

In Fig. 3, kinetic curves of CK aggregation are shown in coordinates $\{A/A_{lim}; t\}$. For enzyme concentrations in the range from 0.05 to 0.2 mg/ml aggregation curves fuse into one curve with averaged rate constant (k_I), equal to 0.066 ± 0.001 min^{-1} and $t_0 = 2.64 \pm 0.01$ min.

We compared CK aggregation kinetics at 50.6°C with its denaturation kinetics at the same temperature. While enzyme is being warmed up, the fraction of the denatured protein (γ_D) is increasing with the time. This increase can be described by the equation:

$$\gamma_D = 1 - \exp(-kt), \quad (3)$$

where k is the denaturation rate constant. The denaturation rate constant was calculated from the data on CK thermal denaturation obtained by the method of differential scanning calorimetry [11] using the Arrhenius equation:

$$k = \exp[E_a(1/T^* - 1/T)/R], \quad (4)$$

where E_a is activation energy, R is the gas constant, T is absolute temperature, and T^* is the temperature at which the rate constant equals 1 min^{-1}. For creatine kinase $E_a = 461.0 \pm 0.7$ kJ/mol and $T^* = 329.01 \pm 0.01$ K [11]. The value of k calculated according to Eq. (4) at 50.6°C is 0.067 ± 0.001 min^{-1} and coincides with the 1-st order aggregation rate constant obtained from the analysis of experimental kinetic curves (0.066 ± 0.001 min^{-1}).

The closeness of the experimental aggregation curves and the denaturation theoretical curve indicates that in the described conditions velocity of CK aggregation is limited by the velocity of CK denaturation (Fig. 3). One should especially discuss difference between kinetic curves of denaturation and aggregation observed at early times. The reason for this difference is connected with the fact that a thermodynamically unfavorable stage of nucleation precedes the phase of protein aggregate growth [4, 39, 40]. Thus, strictly speaking, for the presented experimental data only after completion of the nucleation stage (that is, after the end of the lag period) the stage of protein molecule denaturation becomes the stage that limits the velocity of the general aggregation process. In other words, the stage of aggregate growth is proceeding with much higher velocity than the stage of protein molecule unfolding.

Influence of chaperone Hsp 16.3 *M. tuberculosis* on CK aggregation was studied at 50.6°C in 30 mM Hepes-NaOH buffer, pH 8.0. From Fig. 4 one can see that the enzyme aggregation is suppressed in the presence of chaperone, the suppression increasing as chaperone concentration is increased. The chaperone action efficiency was quantitatively estimated using a method described earlier in treating experimental data relating to chaperone impact on aggregation of catalytic subunits of proteinase

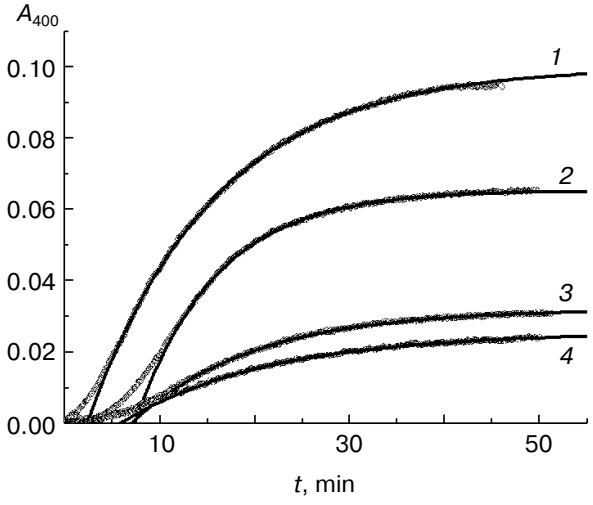

Fig. 4. Creatine kinase (0.15 mg/ml) aggregation kinetics at 50.6°C in the absence (*1*) and presence of chaperone Hsp 16.3 at concentration 0.09 (*2*), 0.18 (*3*), and 0.27 mg/ml (*4*).

CK2 and malic dehydrogenase [4]. As in the referred to cases, the general form of kinetic curve describing CK aggregation in the presence Hsp16.3 is preserved. The final stage of the aggregation process follows the kinetics of 1-st order reaction for all kinetic curves obtained both in absence (Fig. 4, curve *1*) and in presence of chaperone (Fig. 4, curves *2*-*4*). The experimental curves are satisfactorily described by Eq. (2) (see solid lines in Fig. 4). For all curves parameters A_{lim}, k_I, and t_0 were calculated.

Decrease in A_{lim} value in presence of chaperone (Fig. 5a) is due to binding of the denatured protein with chaperone. Bound denatured protein is removed from the aggregation process and A_{lim} is proportional to the protein amount actually taking part in aggregation.

From dependence of A_{lim} on chaperone concentration estimates were made relating to the stoichiometry of complex formed by chaperone with denatured protein. With this purpose, we used the distance cut on the abscissa axis by the straight line in Fig. 5a. One can see that the straight line, describing dependence of A_{lim} on the ratio [Hsp 16.3-monomer]/[CK-dimer], crosses the abscissa axis at point 9. This means that total suppression of CK aggregation will take place when nine monomers of chaperone will bind with one dimer of denatured CK. It is worth noting that for the large value of ratio [chaperone]/[protein-substrate] (≥9.6) there appear deviation from linear dependence. These deviations are caused by the formation of conglomerates consisting of chaperone and denatured protein-substrate. These conglomerates may make additional contribution to apparent optical absorption.

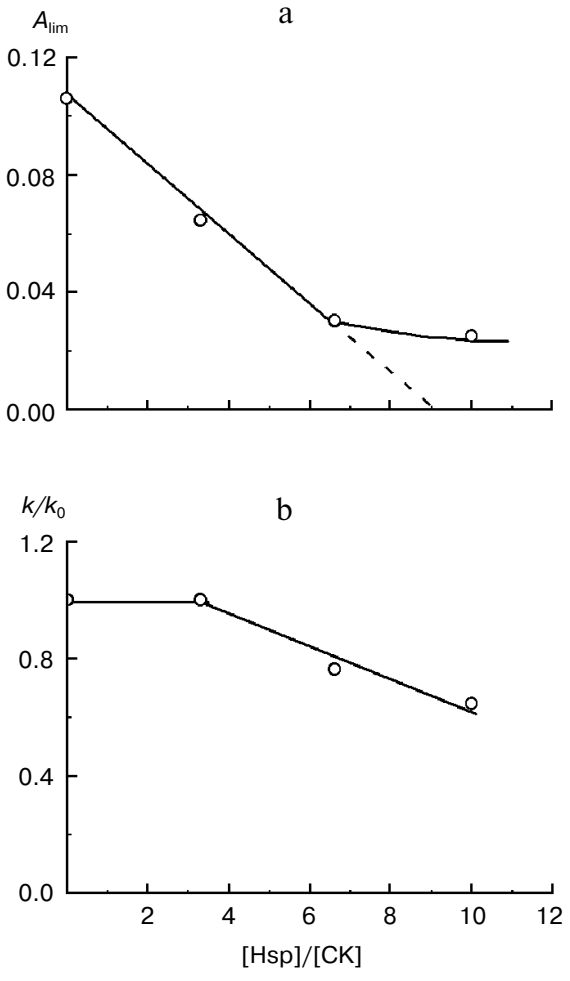

Fig. 5. Dependence of A_{lim} (a) and k_I (b) on molar ratio [Hsp 16.3-monomer]/[CK-dimer].

Another parameter, which can be useful in discussing quantitative aspects of chaperone influence on aggregation process, is the value of aggregation rate constant k_I. As it was shown, the rate constant of 1-st order k_I, obtained from the analysis of kinetic curves of CK aggregation at 50.6°C, did not depend on protein concentration in the region of concentrations 0.05-0.2 mg/ml and coincided with the denaturation rate constant (Fig. 2a). At low chaperone concentration, when the amount of denatured CK bound to chaperone was small, constant k_I did not change (Fig. 5b). When chaperone concentration was increased, one observed decrease in k_I value, which could be connected with the change in aggregation regime, that is appearance of a kinetic regime at which stage of aggregate growth becomes the stage that limits velocity of the general aggregation process. In this case, k_I value decreases in parallel with the decrease in protein-substrate concentration.

Kinetics of CK aggregation at 60°C was studied in 30 mM Hepes-NaOH buffer, pH 8.0, in the range 0.075-0.4 mg/ml of protein concentration. Temperature increase led to acceleration of CK aggregation. Because of this, the initial moment of the reaction could be fixed only at the lowest concentration, 0.075 mg/ml. At higher concentrations A_{400} measured at $t = 0$ is different from 0. Experimental curves of CK aggregation at all investigated protein concentration could be satisfactorily described by the equation:

$$A = A_0 + (A_{lim} - A_0)[1 - \exp(-k_I t)], \qquad (5)$$

which is a modified form of Eq. (2).

For example, we give equation parameters for two limiting protein concentration, calculated using the Origin program. For enzyme concentration 0.075 mg/ml parameters values were: $A_{lim} = 0.007 \pm 0.001$; $k_I = 0.139 \pm 0.006$ min^{-1}; $A_0 = -0.00010 \pm 0.00001$. For protein concentration 0.4 mg/ml these parameters were: $A_{lim} = 0.301 \pm 0.001$; $k_I = 0.80 \pm 0.03$ min^{-1}; $A_0 = 0.16 \pm 0.01$. Value A_{lim} is directly proportional to protein concentration (Fig. 6b). The aggregation rate constant (k_I) in the enzyme concentration range 0.1-0.4 mg/ml increases directly proportionally to protein concentration (Fig. 6c). This means that k_I should be considered as a rate constant of pseudo-first order. In Fig. 6a, experimental curves are

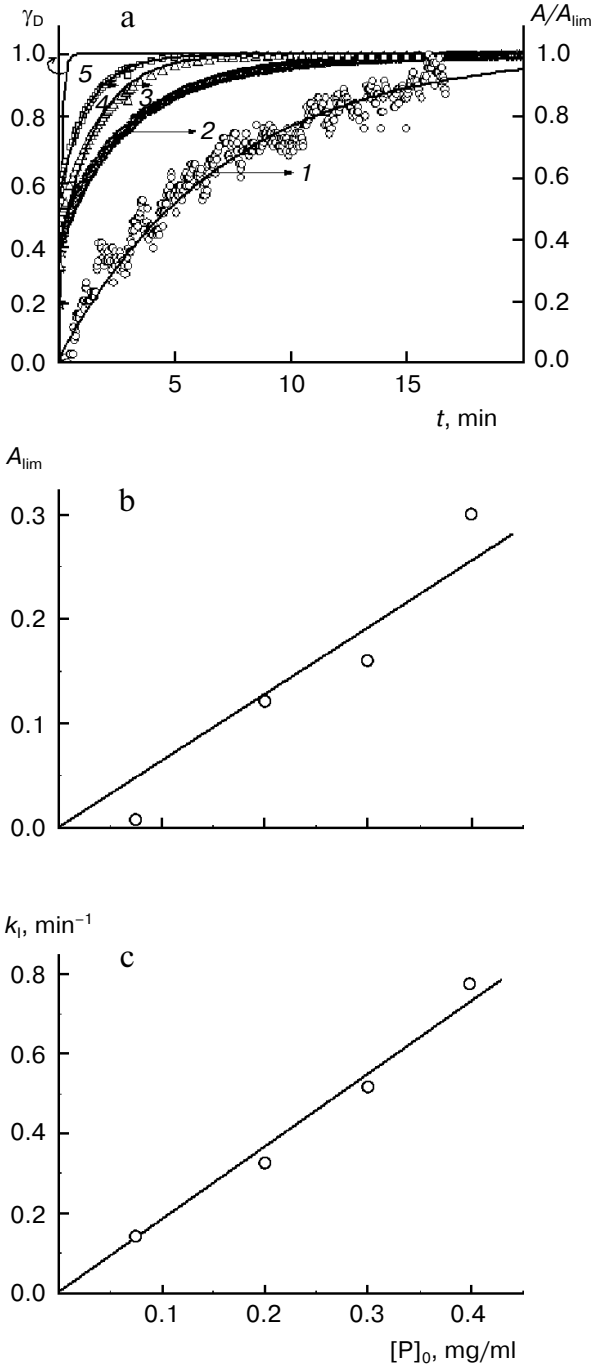

Fig. 6. Kinetics of thermal aggregation and dependence of the fraction of denatured creatine kinase (γ_D) on time at 60°C. a) Kinetic curves in coordinates $\{A/A_{lim}; t\}$; protein concentration: 0.075 (*1*), 0.2 (*2*), 0.3 (*3*), and 0.4 (*4*) mg/ml; b and c) dependences of A_{lim} and k_I on protein concentration, correspondingly.

shown in coordinates $\{A/A_{lim}; t\}$. It is clear that the curves do not fuse into one curve, contrary to what was observed for 50.6°C (see Fig. 3), and when protein concentration increases, they approach the theoretical curve describing the process of CK denaturation. The theoretical CK denaturation curve was plotted on the basis of formula (3) using the denaturation rate constant for 60°C ($k = 9.35 \pm 0.01$ min^{-1}). This constant was calculated from the data of differential scanning calorimetry [11], as explained before. The denaturation rate constant at 60°C is one order of magnitude higher than aggregation rate constant k_I ($k_I = 0.800 \pm 0.031$ min^{-1}) for maximal protein concentration 0.4 mg/ml. Since under these conditions CK denaturation proceeds very quickly (95% of the protein amount denatures during 19 sec), the kinetic curves obtained at 60°C describe solely the CK aggregation process.

Linear dependence of the first order rate constant (k_I) on initial protein concentration, calculated from kinetic aggregation curves, was observed earlier for the thermal denaturation of the coat protein of tobacco mosaic virus (52°C; 0.05 M Na-phosphate buffer, pH 8.0) [36] and firefly luciferase (42°C; 0.025 M Tricine buffer, pH 7.5) [38]. For explanation of the observed kinetics regularities in protein aggregation in papers [3, 4, 36, 38] protein aggregation was considered as a process including a nucleation stage

$$n\mathrm{D} \rightarrow \mathrm{R} \qquad (6)$$

(R is a nucleus consisting of n denatured protein molecules D) and a stage of aggregate growth

$$\mathrm{R} + \mathrm{D} \xrightarrow{k_{II}^{macro}} \mathrm{R}' \qquad (7)$$

(R′ is a nucleus with attached to it additional molecule of denatured protein). The stage of aggregate growth is a bimolecular reaction. Its velocity is given by

$$v_{agg} = -\mathrm{d[D]}/\mathrm{d}t = k_{II}^{macro}[\mathrm{R}][\mathrm{D}], \qquad (8)$$

where k_{II}^{macro} is a macroscopic rate constant of the second order reaction. If one assumes that concentration of nuclei [R] remains constant in the process of protein aggregation, the stage of aggregate growth can be considered as a reaction of first order where the rate constant of pseudo-first order k_I equals to the product k_{II}^{macro} [R]. The value of k_I in this case changes when protein concentration is varied. Linear dependence of rate constant of pseudo-first order reaction on protein concentration indicates that the concentration of nuclei, on which aggregate growth takes place, is directly proportional to the initial protein concentration.

Thus, the increase in temperature leads to the change in the kinetic regime in aggregation process. When temperatures are relatively low, the kinetic regime is realized at which velocity is limited by the stage of unfolding of the protein molecule (rigorously speaking after completion of the nucleation stage). When temperatures are relatively high, the kinetic regime is realized in

which the velocity-limiting stage of the general aggregation process is the stage of protein aggregate growth.

Decrease in the initial protein concentration influences the kinetics of the aggregation similarly to the increase in temperature. For instance, in studying thermal aggregation of the coat protein of tobacco mosaic virus (42°C; 0.1 M Na-phosphate buffer, pH 8.0) half-transformation time ($t_{1/2}$) for aggregation kinetic curves remained constant at protein concentration from 0.1 to 0.4 mg/ml. That means that under these conditions the velocity-limiting stage of the aggregation process is the stage of protein denaturation. Lowering initial protein concentration leads to the increase in $t_{1/2}$, that is the velocity of aggregate growth becomes comparable with the velocity of the protein molecule unfolding.

In the present paper in studying CK thermal aggregation it has been demonstrated that at the same conditions (30 mM Hepes-NaOH buffer, pH 8.0) and within the same region of protein concentration (0.05-0.4 mg/ml) only increase in temperature from 50.6 to 60°C causes the change in aggregation regime. At 50.6°C, aggregation velocity is limited by the velocity of protein molecule denaturation, and at 60°C the aggregation process is wholly determined by the velocity of protein aggregate growth.

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