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Kinetics of Denaturation of Rabbit Skeletal Muscle Glycogen Phosphorylase *b* by Guanidine Hydrochloride

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Abstract—The kinetics of denaturation and aggregation of rabbit muscle glycogen phosphorylase *b* in the presence of guanidine hydrochloride (GuHCl) have been studied. The curve of inactivation of phosphorylase *b* in time includes a region of the fast decline in the enzymatic activity, an intermediate plateau, and a part with subsequent decrease in the enzymatic activity. The fact that the shape of the inactivation curves is dependent on the enzyme concentration testifies to the dissociative mechanism of inactivation. The dissociation of phosphorylase *b* dimers into monomers in the presence of GuHCl is supported by sedimentation data. The rate of phosphorylase *b* aggregation in the presence of GuHCl rises as the denaturant concentration increases to 1.12 M; at higher concentration of GuHCl, suppression of aggregation occurs. At rather low concentration of the protein (0.25 mg/ml), the terminal phase of aggregation follows the kinetics of a monomolecular reaction (the reaction rate constant is equal to 0.082 min^{-1}; 1 M GuHCl, 25°C). At higher concentration of phosphorylase *b* (0.75 mg/ml), aggregation proceeds as a trimolecular reaction.

Key words: muscle glycogen phosphorylase *b,* denaturation, dissociation, guanidine hydrochloride

Glycogen phosphorylase (1,4-α-D-glucan:orthophosphate-α-D-glucosyltransferase, EC 2.4.1.1) is the key enzyme of glycogen metabolism in skeletal muscles. The structure and regulatory properties of the enzyme have been studied in detail [1-5]. In resting muscle phosphorylase exists in dephosphorylated form (form *b*) inactive in the absence of AMP. Covalent modification (phosphorylation) of phosphorylase *b* yields form *a* that does not require AMP for revealing the enzymatic activity. In solution phosphorylase *b* exists in the dimeric form consisting of two identical monomers with molecular mass of 97,432 daltons each. Only the dimeric form of the enzyme is catalytically active. The monomer [6] and tetramer [7] do not reveal the enzymatic activity. The active site of phosphorylase includes pyridoxal-5′-phosphate, a cofactor essential for the catalytic function. The aldehyde group of pyridoxal-5′-phosphate forms a Schiff base with Lys680 [8].

Price and Stevens [9] were the first who studied denaturation of phosphorylase *b* by guanidine hydrochloride (GuHCl). When the enzyme was treated by relatively low concentrations of GuHCl (<0.5 M), the enzymatic activity could be recovered by dilution of the enzyme solution. Even short-term treatment by higher concentrations of GuHCl resulted in irreversible changes in the enzyme structure. Phosphorylase *b* is found to be more sensitive to the denaturing agent under discussion than other oligomeric enzymes such as lactate dehydrogenase [9], creatine kinase [10], pyruvate kinase [11], glutamate dehydrogenase [12], and uridine phosphorylase from *Escherichia coli* K-12 [13].

In the present work, the kinetics of the denaturation and aggregation of muscle phosphorylase *b* in the presence of GuHCl at various concentrations of the enzyme and denaturant is studied.

MATERIALS AND METHODS

Phosphorylase *b* was isolated from rabbit skeletal muscle by the method by Fisher and Krebs [14] using dithiothreitol instead of cysteine. The enzyme was thrice crystallized. The enzyme concentration was determined spectrophotometrically with the extinction coefficient of 13.2 for 1% solution of the enzyme [15].

The enzymatic activity of phosphorylase *b* was measured by the turbidimetric method [16, 17] based on the registration of the increase in the optical absorbance of the glycogen solution. The measurements were carried out in 1-cm cells at 360 nm using a Hitachi-557 spectrophotometer (Japan) equipped with a thermostatted cell. To study the denaturing effect of GuHCl, phosphorylase *b* was incubated with different concentrations of

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the denaturing agent at 25°C in 0.08 M Hepes-HCl buffer, pH 6.8, containing 1 mM EDTA. When measuring the enzymatic activity, the sample was diluted so that GuHCl concentration in the reaction mixture did not exceed 0.01 M. The kinetics of the enzymatic reaction catalyzed by phosphorylase *b* were registered at 25°C in 0.08 M Hepes-HCl buffer, pH 6.8, containing glycogen (0.25 mg/ml), 6 mM glucose 1-phosphate, 1 mM AMP, and 1 mM EDTA.

Aggregation of phosphorylase *b* was studied by the turbidimetric method at 600 nm using the Hitachi-557 spectrophotometer (25°C). Aggregation was registered after the addition of the enzyme solution to 0.08 M Hepes-HCl buffer, pH 6.8, containing 1 mM EDTA and GuHCl at a certain concentration. The spectrophotometric data were recorded using an IBM-compatible computer.

Sedimentation analysis of phosphorylase *b* in the presence of GuHCl was carried out using an analytical Spinco ultracentrifuge, model E (Beckman, USA), equipped with an absorption optical system and photoelectric scanner. The sedimentation coefficient was transformed to the standard conditions ($s_{20,w}$) using the formula [18]:

$$s_{20,w} = s_t \left(\frac{\eta_t}{\eta_{20}}\right)\left(\frac{\eta_{sol}}{\eta_w}\right)\frac{(1-\bar{v}\rho_{20,w})}{(1-\bar{v}\rho_{t,sol})}, \quad (1)$$

where η_t is viscosity of water at temperature t, η_{20} is viscosity of water at 20°C, η_{sol}/η_w is the ratio of viscosity of the solution to viscosity of water at given temperature, $\rho_{20,w}$ is density of water at 20°C, $\rho_{t,sol}$ is density of the solution at temperature of the experiment, and $\bar{v}$ is specific partial volume of the enzyme. Density of the solutions of GuHCl at 25°C was calculated as follows [19]:

$$\rho/\rho_0 = 1 + 0.271W + 0.033W^2, \quad (2)$$

where W is weight portion of GuHCl.

Hepes, glucose 1-phosphate, and AMP were purchased from Sigma (USA), pig liver glycogen was from Olaine (Latvia), and guanidine hydrochloride was from Kodak (USA).

All calculations were carried out using the computer program Origin 5.0 (MicroCal Software, Inc.).

RESULTS AND DISCUSSION

Inactivation of phosphorylase *b* in the presence of GuHCl. The kinetics of inactivation of phosphorylase *b* by GuHCl have an unusual character. Over the first minute of incubation a sharp decrease in the enzymatic activity occurred (Fig. 1; 0.5 M GuHCl). Then over tens of minutes the enzymatic activity remained unchanged. The duration of the plateau was about 60 and 80 min at phosphorylase *b* concentration equal to 0.3 and 1.0 mg/ml, respectively (curves *1* and *2* in Fig. 1). After a lapse of time, acceleration of inactivation takes place. As can be seen from Fig. 1, the shape of the inactivation curves varies on variation of the enzyme concentration. This fact indicates that denaturation of phosphorylase *b* by GuHCl follows the dissociative mechanism. A feature of the mechanism is that denaturation involves a stage of reversible dissociation of active dimer into inactive monomers. The monomeric form is more labile than dimer. This is the reason why the overall rate of inactivation increases with decreasing enzyme concentration.

For the inactivation curve obtained at phosphorylase *b* concentration equal to 1 mg/ml (curve *2* in Fig. 1) we studied the possibility of the enzyme inactivated by incubation with GuHCl over a certain period of time for reactivation. As can be seen from Fig. 1 (curve *3*), phosphorylase *b* preincubated with GuHCl for ~80 min or over a shorter interval of time (i.e., in the region of the intermediate plateau) is completely reactivated by 200-fold dilu-

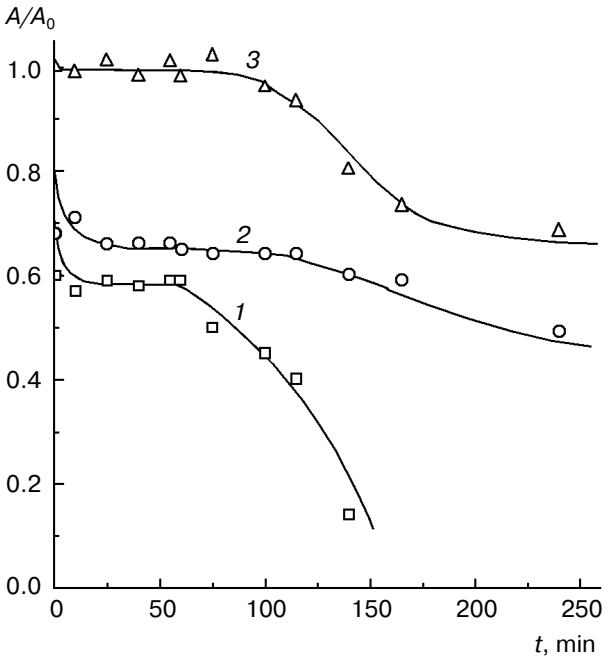

Fig. 1. Kinetic curves of inactivation of phosphorylase *b* in the presence of 0.5 M GuHCl obtained at the following enzyme concentrations (mg/ml): 0.3 (*1*) and 1.0 (*2*) (0.08 M Hepes-HCl buffer, pH 6.8; 25°C). Curve *3* demonstrates the capability of the enzyme for reactivation. When obtaining this curve, the enzyme solution (1 mg/ml) after the incubation with GuHCl over a certain period of time was diluted 200-fold by the reaction mixture containing glycogen (0.25 mg/ml) and 1 mM AMP. After 10-min incubation, the enzymatic reaction was stopped by the addition of glucose 1-phosphate (the final concentration is 6 mM).

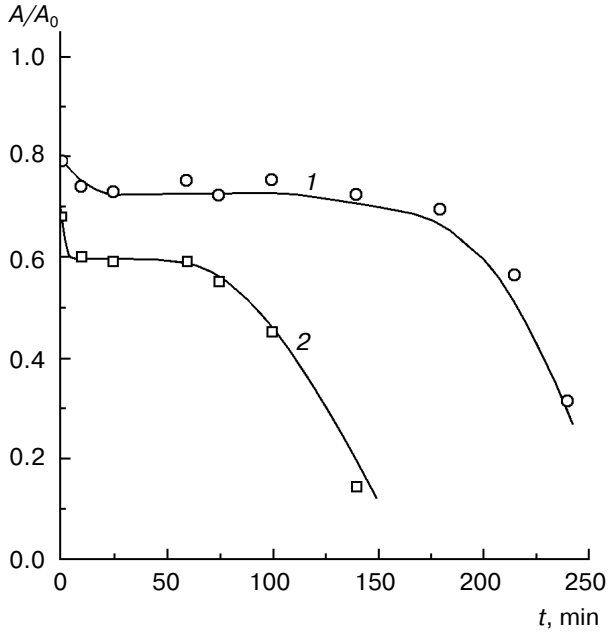

Fig. 2. Kinetic curves of inactivation of phosphorylase *b* (0.3 mg/ml) in the presence of 0.3 M GuHCl (*1*) and 0.5 M GuHCl (*2*).

tion by the reaction mixture and 10-min incubation before the determination of the enzymatic activity. If the enzyme was incubated with GuHCl over a time interval longer than 80 min, the lower value of the degree of reactivation was obtained.

The following changes in the shape of the inactivation curves are observed on variation of GuHCl concentration: the decrease in GuHCl concentration is accompanied by the enhancement of the level of the enzymatic activity corresponding to the intermediate plateau. Simultaneously, the increase in the duration of the intermediate plateau is observed (Fig. 2; cf. curves *1* and *2* obtained with 0.3 and 0.5 M GuHCl, respectively).

The dissociative mechanism of denaturation was demonstrated by us previously for thermal denaturation of phosphorylase *b* [20, 21]. A lag period was observed on the kinetic curves of thermal inactivation and the overall rate of inactivation increased with decreasing enzyme concentration. The kinetic mechanism of thermal denaturation of phosphorylase *b* proposed in [20, 21] involved a stage of reversible dissociation of active dimer into inactive monomers, a stage of irreversible denaturation of monomer, and a stage of aggregation of denatured monomers. Such a kinetic scheme of protein denaturation may be applied to denaturation of phosphorylase *b* by GuHCl. However, to explain the fast initial decline in the enzymatic activity of phosphorylase *b* in the presence of GuHCl, the kinetic scheme of denaturation should be supplemented by a stage of fast conformational changes in the enzyme molecule:

$$\underbrace{\overset{\text{fast}}{D \leftrightarrow D^* \leftrightarrow D^{**}}}_{\text{active dimer}} \leftrightarrow \underbrace{2M \rightarrow 2M_{den}}_{\text{inactive monomer}} \rightarrow \text{aggregation.} \qquad (3)$$

In this scheme D is the original dimeric form of phosphorylase *b*, D* and D** are conformationally changed states of dimer, M is monomer, and M_{den} is the denatured form of monomer.

The stage D* ↔ D** preceding the dissociation of dimer into monomers may be connected with step-by-step disruption of the contact between monomers in a dimer. Such a mode of disruption of the contact is possible when the latter consists of discrete subcontacts. Transformation of D* to D** is accompanied by disruption of a certain number of subcontacts. Splitting the last subcontact means the dissociation of dimer into monomers (D** → 2M). Strictly speaking, if the number of subcontacts is more than two, the transition D* → D** may include not only one, but several stages. The appearance of a lag period (or intermediate plateau) on the kinetic curves of the enzyme inactivation indicates that the D* → D** transition occurs without alterations of the catalytic properties of the enzyme. Such an explanation of the appearance of a plateau on the inactivation curves for oligomeric enzymes under denaturing conditions was given by Poltorak and Chukhrai [22, 23].

The region of the contact between subunits in the dimeric molecule of phosphorylase *b* consists of two subcontacts, which are formed with the participation of caps and towers, respectively [24]. It can be assumed that disruption of these subcontacts proceeds in steps. Splitting one of the subcontacts does not result in the change in the catalytic activity of the dimer. Disruption of the second subcontact means the dissociation of the dimer into monomers. Such a mechanism of denaturation explains the appearance of an intermediate plateau on the kinetic curves and acceleration of inactivation after passing the plateau.

The dissociative mechanism of denaturation by GuHCl characterized by the change in the shape of the kinetic curves of inactivation on variation of the enzyme concentration was demonstrated for uridine phosphorylase from *Escherichia coli* K-12 [13]. The possibility of realization of the dissociative mechanism for denaturation of uridine phosphorylase is predetermined by the existence of the oligomeric structure of the enzyme: uridine phosphorylase from *E. coli* K-12 is a hexamer consisting of identical subunits with a molecular mass of 27.5 kD each.

Sedimentation of phosphorylase *b* in the presence of GuHCl. Sedimentation studies of denaturation of phosphorylase *b* (0.27 mg/ml) were carried out in the range of

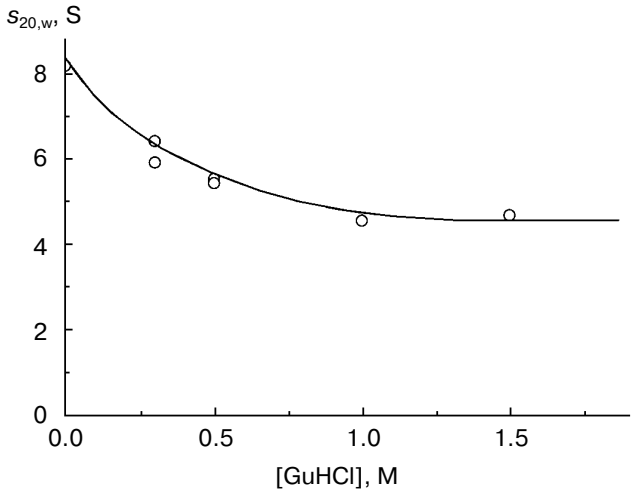

Fig. 3. Dependence of the sedimentation coefficient (s) of phosphorylase b on GuHCl concentration. Before studying sedimentation, the enzyme was incubated with GuHCl for 2 h.

GuHCl concentrations from 0.3 to 1.5 M. Before studying sedimentation, phosphorylase b was incubated in the GuHCl solution for 2 h. Reversible dissociation of the dimeric form of the enzyme into monomers was observed at low concentrations of GuHCl. The sedimentation coefficient ($s_{20,w}$) decreases from 8.2 S in the absence of GuHCl to 5.9 S at 0.3 M GuHCl and 5.4 S at 0.5 M GuHCl (Fig. 3). The sedimentation coefficients $s_{20,w}$ of the dimeric and monomeric forms of phosphorylase b are equal to 8.2 and 5.1-5.4 S, respectively [25]. At GuHCl concentrations equal to 1.0 and 1.5 M, a substantial portion of the enzyme undergoes aggregation and precipitates in the sedimentation experiment. The remaining protein sediments with the sedimentation coefficient of 4.5 S.

Experiments with 0.3 M GuHCl showed that dissociation depended on the concentration of phosphorylase b. When the concentration of the enzyme was varied from 0.05 to 0.5 mg/ml, the sedimentation coefficients fell in the interval from 5.1 S (monomer) to 8.2 S (dimer). Thus, the sedimentation experiments support the dissociative mechanism of denaturation of muscle phosphorylase b by GuHCl.

Aggregation of phosphorylase *b* in the presence of GuHCl. The addition of GuHCl to phosphorylase b results in aggregation of the enzyme revealed as an increase in the optical absorbance of the enzyme solution at 600 nm. Figure 4a shows the kinetic curves obtained at various concentrations of GuHCl (concentration of phosphorylase b is 0.2 mg/ml). First of all, it should be noted that the kinetics of aggregation are characterized by an increase in the rate in the course of the process. Such a picture is typical of the kinetics of protein aggregation caused by different denaturing agents. Thermal aggregation of phosphorylase b is an example [20]. As can be seen from Fig. 4a, increasing GuHCl concentration in the range from 0.56 to 1.12 M results in fast aggregation (curves *1-4* in Fig. 4a). A driving force for aggregation is sticking together of partially unfolded states of the protein molecule. The higher concentrations of GuHCl suppress aggregation of phosphorylase b (curves *5* and *6* in Fig. 4a). The reason is that fully unfolded states of the protein molecule do not interact with each other in the presence of the denaturing agent. It should be noted that similar character of action of GuHCl on protein aggregation was demonstrated for muscle creatine kinase [26]: aggregation of the enzyme was observed in 0.625 M GuHCl but was suppressed when the concentration of GuHCl was raised to 1.5 M.

Since aggregation involves the interaction of several molecules of denatured protein (or interaction of denatured

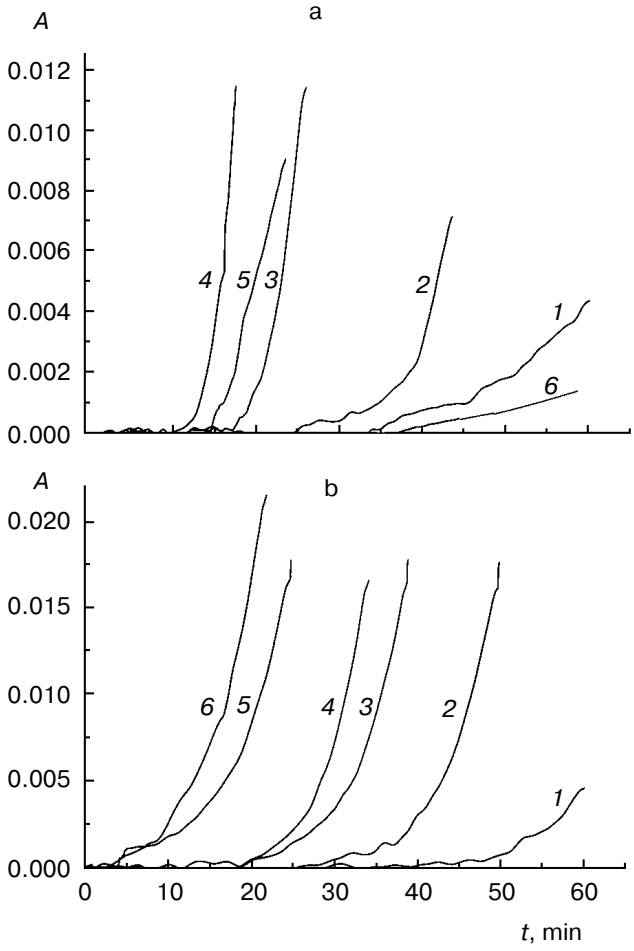

Fig. 4. Aggregation of phosphorylase b in the presence of GuHCl (25°C). a) Kinetic curves of aggregation obtained at various concentrations of GuHCl (M): 0.56 (*1*), 0.7 (*2*), 0.98 (*3*), 1.12 (*4*), 1.28 (*5*), and 1.4 (*6*). A is the optical absorbance at 600 nm. Phosphorylase b, 0.2 mg/ml. b) Kinetic curves of aggregation obtained at various concentrations of phosphorylase b (mg/ml): 0.26 (*1*), 0.38 (*2*), 0.76 (*3*), 1.5 (*4*), 2.3 (*5*), and 3.0 (*6*). 0.7 M GuHCl.

protein with aggregate formed), increasing the protein concentration should result in enhancement of the rate of aggregation. The kinetics of aggregation of phosphorylase *b* studied at various concentrations of the enzyme (Fig. 4b) agree with this prediction. It should be noted that the similar character of the dependence of the rate of aggregation in the presence of urea on the protein concentration was observed for horse heart myoglobin [27].

Figure 5 shows the kinetic curves of aggregation of phosphorylase *b* in the presence of GuHCl over a wider interval of time. At rather high values of time, the optical absorbance approaches the limiting value, which corresponds to the complete transition of the protein to the aggregated state. The kinetic curves were obtained in 1 M GuHCl at two concentrations of phosphorylase *b*: 0.25 mg/ml (Fig. 5, a and b) and 0.75 mg/ml (Fig. 5, c and d).

Suppose that the optical absorbance (A) is proportional to the amount of protein in the aggregated state. With this assumption the kinetic curves of the increase in the optical absorbance correspond to accumulation of the aggregated protein in time. The derivative dA/dt (t is time) characterizes the rate of aggregation of phosphorylase *b*. The limiting value of the optical absorbance (A_{lim}) at $t \to \infty$ is the optical absorbance of the fully aggregated protein. Thus, the portions of the aggregated and non-aggregated protein can be calculated as A/A_{lim} and $(1 - A/A_{lim})$, respectively.

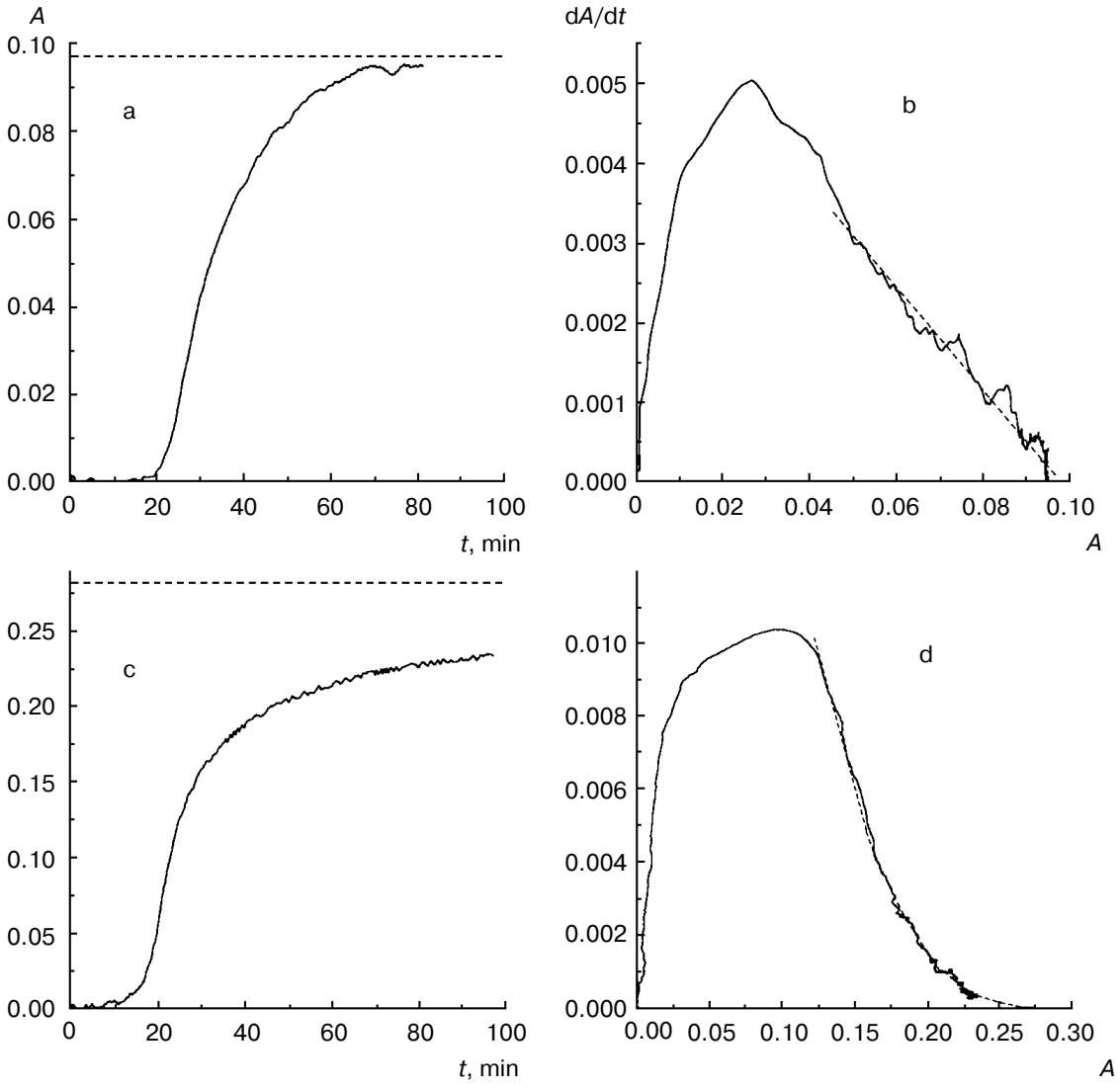

Fig. 5. Analysis of the kinetic curves of aggregation of phosphorylase *b* obtained in the presence of 1 M GuHCl and the following concentrations of the enzyme (mg/ml): 0.25 (a and b) and 0.75 (c and d). a, c) Kinetic curves in coordinates {A; t}. A is the optical absorbance at 600 nm. The dotted lines in panels (a) and (c) correspond to the limiting values of A at $t \to \infty$. b, d) Dependences of dA/dt on A. The dotted curves in panels (b) and (d) are drawn using Eq. (6).

Aggregation is considered as an irreversible association of n molecules of the non-aggregated protein:

$$nP \rightarrow P_{agg}. \quad (4)$$

The rate of aggregation is expressed as follows:

$$v_{agg} = -d[P]/dt = nk[P]^n , \quad (5)$$

where k is the reaction rate constant of the n-th order. The molar concentration of the non-aggregated protein [P] is equal to $(1 - A/A_{lim})[P]_0$ ($[P]_0$ is the value of [P] at $t = 0$).

Substitution of the relationship $[P] = (1 - A/A_{lim})[P]_0$ in Eq. (5) gives the following expression for the rate of the change in the optical absorbance in time:

$$\frac{dA}{dt} = \frac{nk}{\alpha^{n-1}}(A_{lim} - A)^n, \quad (6)$$

where $\alpha \equiv A_{lim}/[P]_0$. We used this equation for analysis of the terminal phase of aggregation of phosphorylase b at enzyme concentrations equal to 0.25 and 0.75 mg/ml (Fig. 5). As can be seen from Figs. 5b and 5d, the dependence of dA/dt on A for the terminal phase of aggregation (the degree of conversion is more than 50%) is described satisfactorily by Eq. (6). The dotted lines in Figs. 5b and 5d are calculated with Eq. (6). Parameters of Eq. (6), namely n, A_{lim}, and the effective parameter nk/α^{n-1}, obtained at two concentrations of phosphorylase b are given in the table. At $[P]_0 = 0.25$ mg/ml, parameter n is close to unity ($n = 0.97 \pm 0.02$). One can conclude that the terminal phase of aggregation proceeds as a monomolecular reaction:

$$dA/dt = k_1(A_{lim} - A) , \quad (7)$$

where k_1 is the reaction rate constant of the first order. The value of k_1 for the curve presented in Fig. 5a is equal to 0.082 ± 0.001 min^{-1}.

Parameters of Eq. (6) used for description of the terminal phase of aggregation of phosphorylase b (0.08 M Hepes-HCl buffer, pH 6.8, containing 1 M GuHCl; 25°C)

$[P]_0$, mg/ml	n	A_{lim}	nk/α^{n-1}
0.25	0.97 ± 0.02	0.097 ± 0.001	0.082 ± 0.001
0.75	3.03 ± 0.09	0.283 ± 0.003	$(2.06 \pm 0.02) \cdot 10^{-8}$

The kinetic regularity observed can be explained by a kinetic scheme including a monomolecular stage of unfolding of the protein molecule followed by a fast stage of growth of aggregates with the participation of the nuclei formed in the solution (U_m):

$$N \xrightarrow{k_1} U \quad \text{protein unfolding;} \quad (8)$$

$$mU \rightarrow U_m \text{ nucleation stage;} \quad (9)$$

$$U_m + U \xrightarrow{k_{agg}} U_{m+1} \text{ aggregate growth,} \quad (10)$$

where N and U are the native and unfolded states of the protein molecule and k_{agg} is the reaction rate constant of the second order for the stage of aggregate growth.

Consider the time interval where the thermodynamically unfavorable stage of the formation of aggregate nuclei (9) is completed and the main reactions proceeding in the system are reactions (8) and (10). The concentration of nuclei is assumed to remain constant ($\sum_{i=0}^{\infty}[U_{m+i}] = \text{const}$; the growth of nuclei proceeds without a change in their number) and the rate of the stage of aggregate growth exceeds substantially the rate of protein unfolding ($k_{agg}[U_m] >> k_1$). With this assumption the reaction rate constant of the first order calculated from the terminal phase of the kinetic curve of aggregation corresponds to the reaction rate constant k_1 for the stage of protein unfolding.

When studying the kinetics of aggregation of human interferon γ in the presence of 0.9 M GuHCl, Kendrick et al. [28] showed that the decrease in the concentration of the native protein follows the exponential law. To determine the concentration of the native protein, aggregation was stopped by the addition of an aliquot withdrawn from the reaction mixture to ice-cold 5 mM solution of sodium succinate (pH 5.0). Aggregated protein was removed by centrifugation. Protein determined in supernatant corresponded to the content of the native protein. To explain the fact that the decrease in the content of the native protein in the course of aggregation follows the kinetics of the first order reaction, the authors used the kinetic scheme where the rate-limiting stage of aggregation is an irreversible monomolecular reaction of protein unfolding.

At higher concentration of phosphorylase b ($[P]_0 = 0.75$ mg/ml) the linear character of the dependence of dA/dt on A for the terminal phase of aggregation is violated, and using Eq. (6) gives the value of n equal to 3.03 ± 0.09. Thus, at this concentration of the protein aggregation proceeds as a trimolecular reaction. It is believed that in this case nuclei of relatively small size are formed in the initial stages of aggregation (the region of acceleration on the kinetic curves); in the terminal stages of aggregation association of such nuclei is registered as a reaction of the third order. The

dependence of the rate of a trimolecular reaction (v) on the concentration of a substance (c) follows the law: $v = kc^3$; that is, threefold decrease in the concentration of a substance should cause a decrease in the rate of aggregation by a factor of 27. This is probably the reason why the rate of association of nuclei is rather low at concentration of the protein equal to 0.25 mg/ml. At this concentration of phosphorylase *b* the main pathway of growth of aggregate is binding of the denatured protein molecules to the preexisting nuclei.

It should be noted that increasing phosphorylase *b* concentration from 0.25 to 0.75 mg/ml results in the increase in the A_{lim} value calculated with Eq. (6) by a factor of 2.92 ± 0.03. The increment of the A_{lim} value is close the expected one, namely 3. This fact may be considered as supporting evidence for the above assumption of proportionality of the optical absorbance and the amount of the protein in the aggregated state.

Thus, the results show that denaturation of muscle phosphorylase *b* by GuHCl proceeds through the dissociative mechanism, i.e., includes a stage of dissociation of active dimer into inactive monomers. It was shown also that at relatively low concentration of the protein (0.25 mg/ml) the terminal phase of aggregation follows the kinetics of a monomolecular reaction. At higher concentration of phosphorylase *b* (0.75 mg/ml) aggregation proceeds as a reaction of the third order.

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