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Influence of Osmolytes on Inactivation and Aggregation of Muscle Glycogen Phosphorylase *b* by Guanidine Hydrochloride. Stimulation of Protein Aggregation under Crowding Conditions

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Abstract—The effects of the osmolytes trimethylamine-N-oxide (TMAO), betaine, proline, and glycine on the kinetics of inactivation and aggregation of rabbit skeletal muscle glycogen phosphorylase *b* by guanidine hydrochloride (GuHCl) have been studied. It is shown that the osmolytes TMAO and betaine exhibit the highest protective efficacy against phosphorylase *b* inactivation. A test system for studying the effects of macromolecular crowding induced by osmolytes on aggregation of proteins is proposed. TMAO and glycine increase the rate of phosphorylase *b* aggregation induced by GuHCl.

Key words: muscle glycogen phosphorylase *b*, guanidine hydrochloride, inactivation, aggregation, osmolyte

Many living organisms accumulate large amounts of osmolytes in response to stress (osmolytic, chemical, or thermal). Osmolytes are low molecular weight organic compounds. These include polyols, some amino acids, and methylamines. Under conditions of stress, osmolytes protect proteins by stabilizing protein structure. Osmolytes have been found in plant and animal cells and also in microorganisms. They stabilize native protein structure at increased temperatures, pH changes, and at high pressure in deep-water fishes [1] and at high salt concentrations in plants [2]. Osmolytes also promote protein refolding into a native state and suppress aggregation that accompanies protein refolding [2, 3]. Denaturing agents compete with water molecules for interaction with protein and decrease water–protein interaction; this results in protein molecule unfolding [4, 5]. According to Bolen et al. [6], osmolyte capacity for protein stabilization under denaturing treatments may be attributed to unfavorable interaction between an osmolyte and peptide backbone (the "osmophobic effect"). This results in transition of the protein into a more compact state characterized by lower free energy.

Biochemical processes occur in media containing high concentrations of macromolecules (50-400 mg/ml), which occupy a significant part of the medium volume (up to 40%) and so volume available to intracellular components in the cell decreases [7-9]. This is a characteristic feature of living systems. In the literature, such cell conditions have been defined as macromolecular crowding. Folding of most proteins involves formation of unstable intermediate states that tend to aggregate [10]. Molecule crowding (the effect of volume exclusion) promotes the aggregation process [10, 11]. Protein aggregates form inclusions, so-called "aggresomes", which reflect general cell response to the presence of incorrectly folded proteins [12]. The study of protein aggregation is important for understanding of the mechanisms of cell protection against protein aggregates inducing "protein conformational diseases".

Good experimental evidence exists that osmolytes exert a protective effect during protein denaturation under stress conditions [6, 13-21], but molecular mechanisms underlying osmolyte action remain poorly understood. Data on the osmolyte effect on protein aggregation are contradictory. For example, trimethylamine-N-oxide (TMAO) inhibited formation of fibrillar structures from β-amyloid peptides [22], whereas betaine and proline protected *Escherichia coli* proteins against aggregation at high NaCl concentration (0.5 M) [2]. Proline protected chicken liver fatty acid synthase against aggregation in the presence of urea [23] and chicken egg lysozyme aggregation in the presence of triacetic acid [24]. Proline also prevented aggregation during refolding of chicken egg lysozyme [3] and rabbit skeletal muscle creatine phosphokinase [18] denatured by guanidine hydrochloride. However, other authors reported that osmolytes either

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potentiate or have no effect on protein aggregation. For example, TMAO promoted aggregation during refolding of RNase fragments at pH 7.0, and the whole enzyme at pH 8.0 [25]. Glycine even at high concentrations (1.8 M) caused minor influence on chicken liver fatty acid synthase aggregation in the presence of urea [23]; it did not inhibit protein aggregation during refolding of chicken egg lysozyme [3].

In the present study, we have investigated the effects of TMAO, betaine, proline, and glycine on inactivation and aggregation of rabbit muscle phosphorylase *b* by guanidine hydrochloride (GuHCl).

MATERIALS AND METHODS

The following reagents were used in the study: guanidine hydrochloride, betaine, and AMP from ICN (USA); Hepes, glucose-1-phosphate, and TMAO from Sigma (USA); glycogen from Olaine (Latvia); glycine from Reanal (Hungary). Glycine was crystallized twice before use.

Glycogen phosphorylase *b* was isolated from rabbit skeletal muscles by the method of Fisher and Krebs [26] using dithiothreitol instead of cysteine; the enzyme was recrystallized three times. Phosphorylase *b* concentration was determined spectrophotometrically at 280 nm using absorbance coefficient of 13.2 for 1% protein solution [27]. Enzyme activity was determined by the turbidimetric method [28] based on registration of the increase of glycogen solution absorbance at 360 nm using 1 cm cuvettes and Hitachi-557 spectrophotometer (Japan) equipped with a thermostatted cell. Spectrophotometric data were recorded to an IBM-compatible computer.

The effect of osmolytes (TMAO, betaine, proline, glycine) on phosphorylase *b* inactivation by 0.7 M GuHCl was evaluated by enzyme activity assayed after preincubation with GuHCl in the absence or in the presence of the osmolytes during various time intervals. Phosphorylase *b* (0.33 mg/ml) was preincubated with 0.7 M GuHCl in 0.08 M Hepes-NaOH buffer, pH 6.8, containing 0.2 mM EDTA at 25°C. During enzyme activity assay the sample was diluted by 125 times and kinetics of enzymatic reaction was registered at 25°C in 0.08 M Hepes-NaOH buffer, pH 6.8, containing 0.2 mM EDTA. The reaction mixture also contained 0.25 mg/ml glycogen, 1 mM AMP, and 6 mM glucose-1-phosphate. The rate constant for phosphorylase *b* inactivation by GuHCl was calculated using the following equation:

$$v/v_0 = \exp(-kt), \quad (1)$$

where v_0 is the rate of reaction catalyzed by phosphorylase *b* in the absence of GuHCl, v is the rate of enzymatic reaction in the presence of GuHCl, k is inactivation rate constant, and t is incubation time.

Phosphorylase *b* aggregation in the presence of 1 M GuHCl was carried out in 0.08 M Hepes-NaOH buffer, pH 6.8, containing 0.2 mM EDTA at 25°C in the presence or absence of osmolytes. Since protein aggregate formation is accompanied by increased turbidity of protein solution, kinetics of aggregation was monitored by increase in apparent absorbance at 360 nm. Aggregation kinetics was studied using a DU-650 spectrophotometer (Beckman, USA) in 1 cm cuvettes.

Experimental data were treated using Origin 7.0 (Microcal Software, Inc., USA) and Scientist (MicroMath, USA) software.

RESULTS AND DISCUSSION

Phosphorylase *b* inactivation in the presence of osmolytes. Figure 1 (a and b) shows kinetic curves of phosphorylase *b* inactivation by 0.7 M GuHCl in the presence of various concentrations of proline. Experimental data treated using Eq. (1) demonstrate a protective effect of proline. Similar kinetic curves of phosphorylase *b* inactivation by GuHCl were obtained in the presence of various concentrations of TMAO, betaine, and glycine.

To compare the protective effect of the osmolytes studied (TMAO, betaine, proline, and glycine), results were plotted as dependences of $\ln(k/k_0)$–osmolyte concentration (Fig. 2) where k_0 and k are the rate constants for phosphorylase *b* inactivation by 0.7 M GuHCl in the presence and absence of the osmolyte, respectively.

Within the theory of activated complex [29] enzyme stability is characterized by free activation energy ($\Delta G_0^{\#}$) of the denaturation process and the stabilizing effect is characterized by $\Delta\Delta G_0^{\#}$ value:

$$\Delta\Delta G_0^{\#} = \Delta G_{0,\mathrm{osm}}^{\#} - \Delta G_0^{\#}, \quad (2)$$

where $\Delta G_0^{\#}$ and $\Delta G_{0,\mathrm{osm}}^{\#}$ are free activation energy in the absence and in the presence of osmolyte, respectively. According to Tams et al. [30]:

$$\Delta\Delta G_0^{\#} = -\mathrm{RT}\ln(k/k_0). \quad (3)$$

The tangent of the slope on the plot of dependence of $\ln(k/k_0)$ on osmolyte concentration is a measure of protein stabilization. Figure 2 shows that TMAO and betaine exhibit the highest protective effect, whereas glycine has the lowest protective effect against GuHCl inactivation (table). It should be noted that osmolytes exhibit different effects on various proteins. As in the case of phosphorylase *b*, proline was more effective than glycine in protection of chicken liver fatty acid synthase against inactivation by 1.5 M urea [23]. Analysis of experimental data on rabbit skeletal muscle creatine phosphokinase inactivation by 0.8 M GuHCl [31] as dependence

of $\ln(k/k_0)$ versus osmolyte concentration demonstrates more effective protection by glycine than proline.

For description of the interaction between osmolytes and proteins, two types of models are used in the literature. One type of model is based on effects of exclusion volume [32-44], and the other type is based on protein binding to osmolytes [3, 18, 45, 46]. A recent review [47] highlighted in detail the protective effect of polyols on equilibration of isomerization between folded (F) and unfolded (U) states of proteins. For explanation of the osmolyte effect, models based on concepts of thermodynamic non-ideality (effect of exclusion volume and preferential hydration) and models based on preferential protein interaction with osmolyte are considered. However, none of these models comprehensively explains all experimental data available, but the authors of [47] believe that molecular crowding makes a significant contribution to protein stabilization.

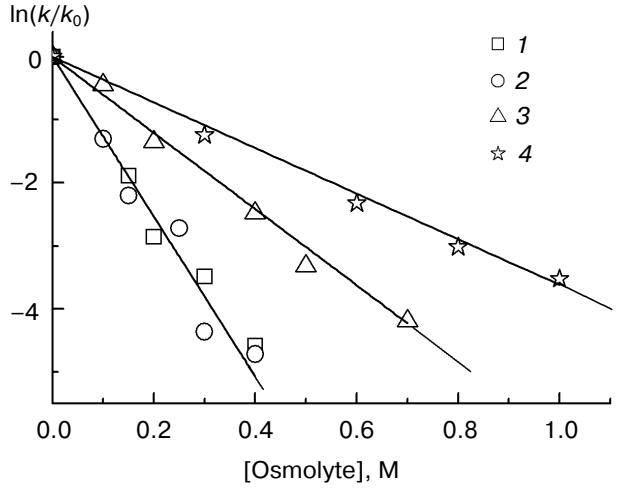

Fig. 2. Protective effect of osmolytes during phosphorylase *b* inactivation by 0.7 M GuHCl at 25°C (0.08 M Hepes, pH 6.8, 0.2 mM EDTA): *1*) TMAO; *2*) betaine; *3*) proline; *4*) glycine.

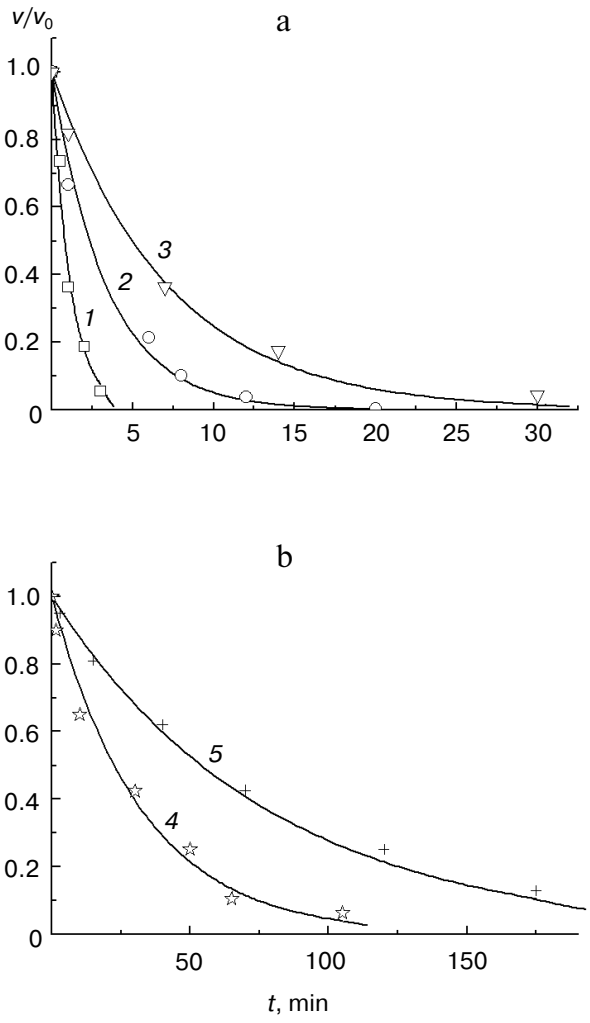

Fig. 1. Effect of proline on phosphorylase *b* inactivation by 0.7 M GuHCl at 25°C (0.08 M Hepes, pH 6.8, 0.2 mM EDTA) (v_0 and v are rates of phosphorylase reaction in the absence and in the presence of GuHCl, respectively). Proline concentrations were: 0 (*1*), 0.1 (*2*), 0.4 (*3*), 0.5 (*4*), and 0.7 M (*5*).

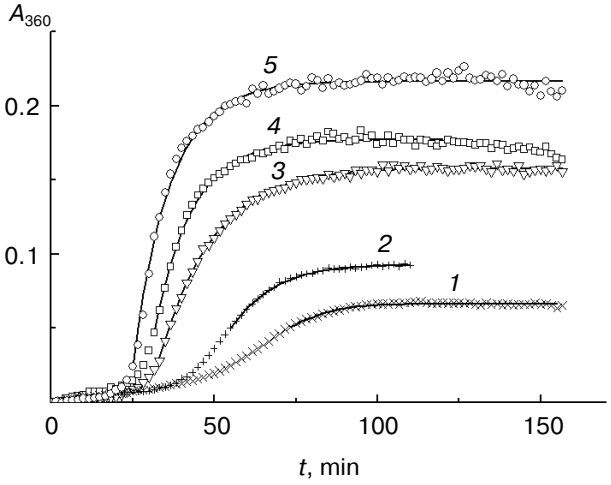

Fig. 3. Kinetic curves of phosphorylase *b* aggregation by 1 M GuHCl at 25°C (0.08 M Hepes, pH 6.8, 0.2 mM EDTA) based on the increase in absorbance at 360 nm. Points represent experimental data. Solid curves were drawn in accordance with Eq. (6). Protein concentrations were: 40 (*1*), 50 (*2*), 75 (*3*), 90 (*4*), and 110 μg/ml (*5*).

Phosphorylase *b* aggregation in the presence of osmolytes. Figure 3 shows kinetic curves of phosphorylase *b* aggregation in the presence of 1 M GuHCl at 25°C (0.08 M Hepes, pH 6.8, 0.2 mM EDTA). Phosphorylase *b* concentration was varied within the range from 40 to 110 μg/ml. We have analyzed regions of kinetic curves above 25 min using the equation previously employed by Eronina et al. [48]:

Stabilizing effect of osmolytes during phosphorylase *b* inactivation by 0.7 M GuHCl (25°C, 0.08 M Hepes, pH 6.8, 0.2 mM EDTA)

Osmolyte	$\Delta\Delta G_0^{\#}$, kJ/mol
TMAO	26.7
Betaine	24.9
Proline	12.9
Glycine	6.6

$$dA/dt = (nk/\alpha^{n-1})(A_{lim} - A)^n, \quad (4)$$

where $\alpha = A_{lim}/[P]_0$ ($[P]_0$ is initial protein concentration), A_{lim} is limiting value of optical absorbance A reached at $t \to \infty$ (t is time), k is the rate constant of n order, and n is reaction order.

Using the Scientist software, we determined the parameter n for each of the kinetic curves of Fig. 3 (see Fig. 4a). The mean value of n was 0.96 ± 0.03. Consequently, in the terminating phase the aggregation process follows kinetics of first-order reaction. At $n = 1$ the derivative dA/dt in the Eq. (4) is a linear function of optical absorbance A:

$$dA/dt = k_I(A_{lim} - A), \quad (5)$$

where k_I is the rate constant of the first-order reaction. The integral form of the Eq. (5) for the kinetics of the first-order reaction can be presented as:

$$A = A_{lim} - \{1 - \exp[-k_I(t - t_0)]\}, \quad (6)$$

where t_0 is the mean of t at which $A = 0$. The parameter t_0 is the duration of a lag period on the kinetic curves. The linear behavior of the dependence A_{lim} on phosphorylase *b* concentration (Fig. 4b) is consistent with the suggestion that optical absorbance is proportional to the amount of aggregated protein. Figure 4c shows that k_I value increases linearly with the increase in phosphorylase *b* concentration. This suggests that the stage of protein aggregate growth [49-52] is the rate-limiting stage of the aggregation process. So subsequent experiments on the effect of osmolytes on phosphorylase *b* aggregation were carried out at the enzyme concentration of 80 μg/ml (corresponding to this region).

The lag period of the kinetic curves of phosphorylase *b* aggregation (t_0) corresponds to the stage of protein molecule unfolding N → U (where N is native state of the protein molecule, U is unfolded state of this molecule tending to aggregation) and the stage of nucleation ($mU \leftrightarrow U_m$; U_m is a nucleus) [48]. The increase in turbidity of solution is related to the stage of aggregate growth (i.e., it is directly related to the aggregation process).

For evaluation of the osmolyte effect on the general process of enzyme aggregation, the osmolytes were added simultaneously with GuHCl at the initial moment of the reaction. Figure 5 (a and b) shows typical curves of aggregation of phosphorylase *b* (80 μg/ml) by 1 M GuHCl in the absence and in the presence of TMAO and glycine,

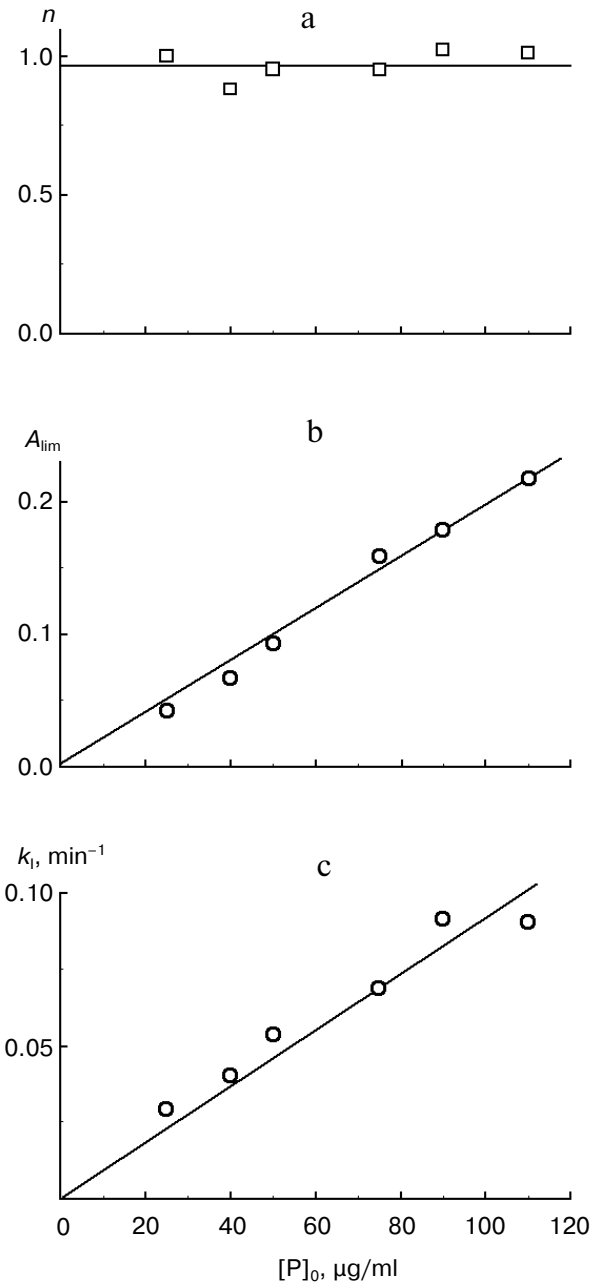

Fig. 4. Dependence of parameters n (a), A_{lim} (b), and k_I (c) on protein concentration, $[P]_0$, during phosphorylase *b* aggregation induced by 1 M GuHCl at 25°C (0.08 M Hepes, pH 6.8, 0.2 mM EDTA).

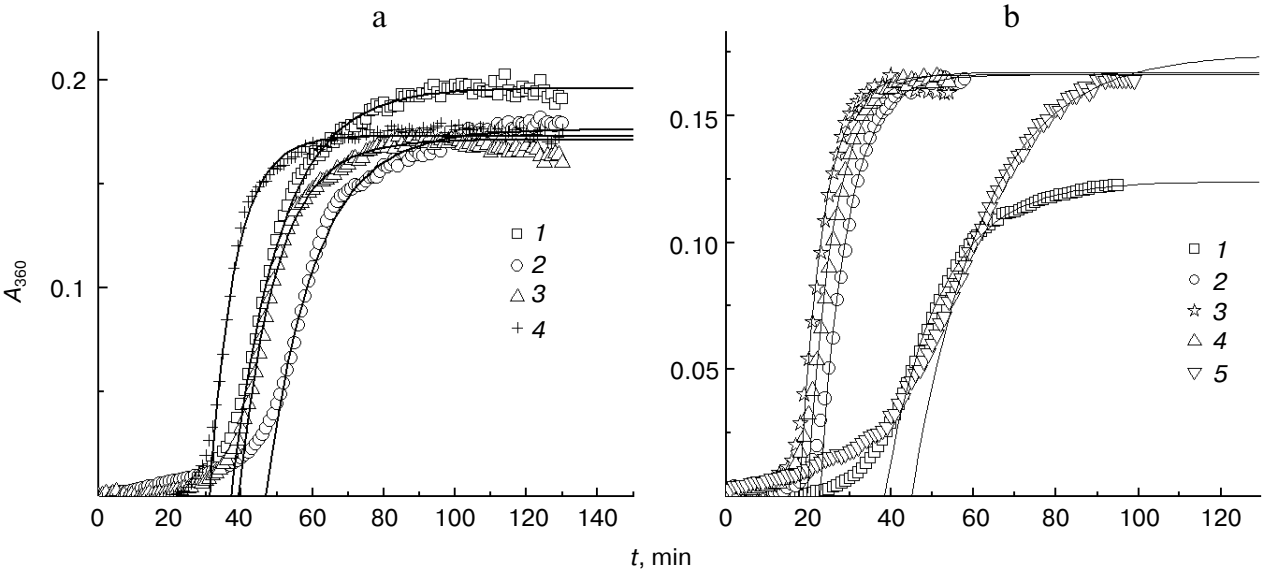

Fig. 5. Kinetic curves of phosphorylase *b* (80 μg/ml) aggregation induced by 1 M GuHCl at 25°C (0.08 M Hepes, pH 6.8, 0.2 mM EDTA). Points represent experimental data. Solid curves were drawn in accordance with Eq. (6). a) TMAO concentration: 0 (*1*), 0.1 (*2*), 0.2 (*3*), and 0.4 M (*4*). b) Glycine concentrations: 0 (*1*), 0.3 (*2*), 0.6 (*3*), 0.8 (*4*), and 1.5 M (*5*).

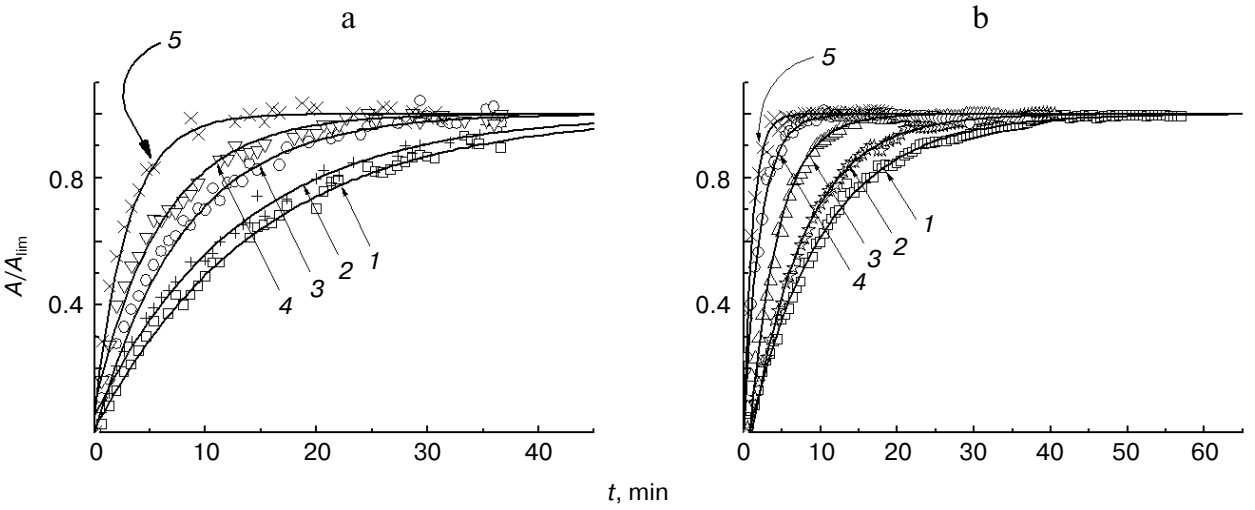

Fig. 6. Kinetics of phosphorylase *b* aggregation by 1 M GuHCl at 25°C (0.08 M Hepes, pH 6.8, 0.2 mM EDTA) plotted as the dependence of A/A_{lim} versus *t*. The osmolyte was added into the reaction mixture after the time interval equal to the duration of the lag period (38 min). a) TMAO concentration: 0 (*1*), 0.05 (*2*), 0.1 (*3*), 0.2 (*4*), and 0.3 M (*5*). b) Glycine concentration: 0 (*1*), 0.15 (*2*), 0.3 (*3*), 0.7 (*4*), and 0.94 M (*5*).

respectively. In the presence of 0.1 M TMAO the duration of lag period of the kinetic curve increased; however, subsequent increase in the osmolyte concentration reduced this parameter compared with control. At 0.4 M TMAO, the k_I value calculated by Eq. (6) increased by 1.88-fold.

Increase in glycine concentration up to 0.6 M resulted in decrease in the lag period, but subsequent increase in glycine concentration to 1.5 M increased this parameter (Fig. 5b). Increase in lag period duration suggests that in the presence of an osmolyte the enzyme retains compact structure for a longer period. Decrease in t_0 value may be attributed to the fact that duration of the lag period is partially determined by the nucleation stage, and its duration decreases under conditions of crowding.

For investigation of the effect of osmolytes on the particular stage of protein aggregation, we added osmolytes after the stages of protein unfolding and nucle-

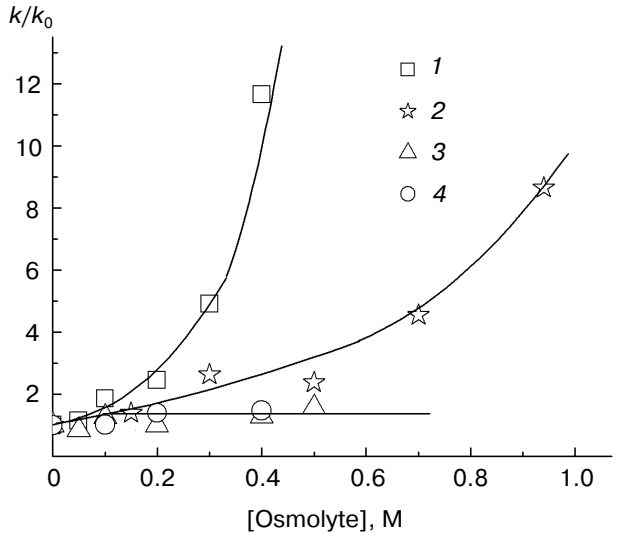

Fig. 7. Dependence of k/k_0 ratio on osmolyte concentration during phosphorylase *b* aggregation by 1 M GuHCl at 25°C (0.08 M Hepes, pH 6.8, 0.2 mM EDTA). k_0 and k are rate constants of the first-order reaction calculated by Eq. (6) in the absence and in the presence of osmolyte, respectively. Osmolyte was added into the reaction mixture after the time equal to the lag period (38 min). The osmolytes: glycine (*1*), proline (*2*), TMAO (*3*), betaine (*4*).

ation, i.e., after termination of the lag period. We used the following plan of the experiment: phosphorylase *b* was initially incubated with 1 M GuHCl for the time interval equal to duration of the lag period on the curve of phosphorylase *b* aggregation in the absence of osmolyte (38 min) and then osmolyte solution containing 1 M GuHCl was added. Figure 6 (a and b) shows the kinetic curves of phosphorylase *b* aggregation in the presence of various concentrations of TMAO and glycine, respectively. Analysis of kinetic curves revealed that the kinetics of aggregation can be described by Eq. (6) at $t_0 = 0$. The rate constants of the first-order reaction (as k/k_0 ratio, where k_0 and k are reaction rate constants in the absence and presence of osmolyte, respectively) calculated using this equation are shown in Fig. 7 as a function of osmolyte concentration. Under our experimental conditions, there was clear increase in the rate of phosphorylase *b* aggregation in the presence of 1 M GuHCl at increasing concentrations of the osmolytes. Figure 7 also shows results of calculation of k/k_0 ratio for proline and betaine. These osmolytes did not influence phosphorylase *b* aggregation induced by 1 M GuHCl.

The proposed test system allows investigating the effect of molecular crowding created by high concentrations of osmolytes on the process of protein aggregation. Using this test system, we found accelerated aggregation of phosphorylase *b* induced by 1 M GuHCl in the presence of TMAO and glycine.

In conclusion, we should stress that crowding influences all biochemical processes in the cell, which are accompanied by changes in exclusion volume. These processes include folding of newly synthesized polypeptide chains into compact functional proteins, protein unfolding induced by chemical or thermal stress, formation of oligomeric structures and multienzyme complexes in metabolic pathways, aggregation of proteins into nonfunctional aggregates such as bacterial inclusion bodies and plaques found in human amyloid diseases (e.g., Parkinson's disease, Alzheimer's disease). Crowding agents are employed in *in vitro* biochemical studies for modeling of reaction conditions to *in vivo* conditions [9, 43]. Such experiments can underline the putative role of crowding on biochemical processes. Considering the putative importance of osmolytes in the cell we can indicate that they play a dual role: they protect the proteins against denaturation but promote aggregation of unfolded molecules.

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