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Effect of molecular crowding on self-association of phosphorylase kinase and its interaction with phosphorylase *b* and glycogen

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Self-association of phosphorylase kinase (PhK) and its interaction with glycogen ($M = 5500$ kDa) and phosphorylase *b* (Ph*b*) has been studied using analytical ultracentrifugation and turbidimetry under the conditions of molecular crowding arising from the presence of high concentrations of osmolytes. In accordance with the predictions of the molecular crowding theory, trimethylamine *N*-oxide (TMAO) and betaine greatly favor self-association of PhK induced by Mg^{2+} and Ca^{2+} and PhK interaction with glycogen. In contrast, proline suppresses these processes, probably, due to its specific interaction with PhK. All osmolytes tested prevented the complex formation between PhK and its physiological substrate, Ph*b*. The specific interactions of PhK and Ph*b* with glycogen, in the living cell, presumably is a factor allowing the negative effect of crowding on the recognition of Ph*b* by PhK to be overcome. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords: **phosphorylase kinase; phosphorylase *b*; association; glycogen; molecular crowding; osmolyte; sedimentation; turbidimetry**

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INTRODUCTION

Phosphorylase kinase (PhK; EC 2.7.1.38) catalyzing phosphorylation and activation of glycogen phosphorylase *b* (Ph*b*) play a key role in the cascade system of regulation of glycogen metabolism (Krebs *et al.*, 1959; Brushia and Walsh, 1999; Livanova, 1993). The PhK molecule is a hexadecamer with subunit composition $(\alpha\beta\gamma\delta)_4$ and molecular mass of 1320 kDa (Cohen, 1973). The oligomeric state of the native enzyme is dependent on the protein, Ca^{2+} and Mg^{2+} concentrations. Ca^{2+} and Mg^{2+} ions stimulate the PhK activity by inducing tertiary and subsequent quaternary structural changes in the enzyme molecule (Nadeaw *et al.*, 1999; Wilkinson *et al.*, 1999). In the absence of Ca^{2+} and Mg^{2+}, the enzyme exists in the monomeric and dimeric forms with $s_{20,w} = 23$ S (that corresponds to a molecular mass of 1320 kDa) and $s_{20,w} = 36.5$ S, respectively (Cohen, 1973; Chebotareva *et al.*, 2002). However, the addition of 0.1 mM Ca^{2+} and 10 mM Mg^{2+} results in the appearance of higher-order associates (Carlson and King, 1982; Chebotareva *et al.*, 2002). In skeletal muscle about 40% of phosphorylase kinase is localized, together with Ph*b* and other enzymes of glycogen metabolism, on the surface of glycogen granules (Meyer *et al.*, 1970), and is really functioning as a part of the protein–glycogen complex. Thus, to understand the mechanisms of action of the enzymes of glycogen metabolism, it is very important to study the enzyme–enzyme interactions and interaction of these enzymes with glycogen under conditions which imitate those in the cell.

Molecular crowding has a pronounced effect on macromolecular interactions, the rate and the reaction equilibrium of the biochemical processes in living systems (Ellis, 2001; Ellis and Minton, 2003; Minton, 1997, 1998, 2001; Ralston, 1990; Zimmerman and Minton, 1993). Crowding influences the conformation and degree of association of macromolecules. It is well known that osmolytes, small organic molecules, stabilize proteins and therefore protect organisms against different stresses (Bolen and Baskakov, 2001; Yancey *et al.*, 1982). Under stress conditions cells accumulate high concentrations of osmolytes. Using high concentrations of osmolytes *in vitro* permits the simulation of molecular crowding conditions. Previously we showed that self-association of PhK was greatly stimulated in the presence of high concentrations of the natural osmolyte trimethylamine *N*-oxide (TMAO) and large associates with $s_{20,w} = 189$ and 385 S were registered (Chebotareva *et al.*, 2002).

The main goal of this paper was to examine the effect of molecular crowding arising from the high concentrations of osmolytes on self-association of PhK, binding of PhK with phosphorylase *b* (Ph*b*) and interaction of PhK with glycogen.

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Abbreviations used: Ph*b*, phosphorylase *b*; PhK, phosphorylase kinase; TMAO, trimethylamine *N*-oxide.

MATERIALS AND METHODS

The influence of the high concentrations of osmolytes (TMAO, betaine, and proline) on self-association of PhK from rabbit skeletal muscle and its interaction with Ph*b* and glycogen has been studied in 40 mM Hepes, pH 6.8, at 20 °C using analytical ultracentrifugation and the turbidimetric method. Sedimentation velocity experiments were carried out in a Model E analytical ultracentrifuge (Beckman), equipped with a photoelectric scanner and monochromator; 12 mm double sector cells were used. Sedimentation profiles were recorded by measuring the absorbance of the enzyme at 280 nm. The sedimentation coefficients were calculated according to the formula:

$$s = \ln\,(r/r_m)w^2t \qquad (1)$$

where r and r_m are radial positions of the boundary and meniscus, respectively, w is the angular velocity, and t is time of sedimentation. The sedimentation coefficients were corrected to solvent density and viscosity (20 °C, water) in the standard way.

PhK was isolated from rabbit skeletal muscle according to Cohen (1973), except using ion-exchange chromatography on DEAE–Toyopearl instead of DEAE–cellulose at the final step of purification (Morozov *et al.*, 1989). The concentration of PhK was determined spectrophotometrically at 280 nm using an extinction coefficient $\varepsilon^{1\%}_{1\,cm} = 12.4$.

Ph*b* was isolated from rabbit skeletal muscle by the method of Fischer and Krebs (1962), except using dithiothreitol instead of cysteine. After four crystallizations the AMP-free Ph*b* preparations were stored at −20 °C in β-phosphoglycerate–NaOH (pH 6.8) supplemented with 50% glycerol (v/v). The concentration of Ph*b* was determined spectrophotometrically at 280 nm using an extinction coefficient $\varepsilon^{1\%}_{1\,cm} = 13.2$ (Kastenschmidt *et al.*, 1968). Prior to sedimentation experiments, Ph*b* was dialyzed against 40 mM Hepes buffer, pH 6.8, at 4 °C for 2 h.

TMAO, betaine (minimum 99%), proline (minimum 99%), and Hepes were purchased from Sigma Chemical Co. (USA). The reagents were used without additional purification. When preparing the solutions containing Phb or PhK and osmolyte, the initial pH value of osmolyte solution in 40 mM Hepes buffer was adjusted so that the final pH value was 6.8. Glycogen was from Oline (Latvia). Pig liver glycogen was precipitated with ethanol. The sedimentation coefficient (s) determined at 20 °C for 1% glycogen solution in water was 106 S, which corresponded to an average molecular mass of 5.5×10^6 (Chebotareva *et al.*, 1979; Klinov *et al.*, 1982) according to the empirical equation proposed by Brammer *et al.* (1972): $M = 5 \times 10^3 s^{3/2}$.

The kinetics of self-association of PhK and its interaction with glycogen were followed by an increase in the optical absorbance at 360 nm using a Hitachi 557 spectrophotometer (Japan) equipped with a thermostated cell holder. The kinetic experiments were performed at 20 °C in 40 mM Hepes buffer, pH 6.8, containing 1 mM β-mercaptoethanol. Prior to sedimentation and kinetic experiments, PhK was dialyzed against 40 mM Hepes buffer, pH 6.8, at 4 °C for 2 h. There were no changes in turbidity of the glycogen solutions in the presence of osmolytes.

The initial rates of the binding of PhK to glycogen were calculated by setting the kinetic curves using a second degree polynomial with a Microcal ORIGIN 5.0 program.

RESULTS

Effect of osmolytes on self-association of PhK

Figure 1 shows the time course of PhK self-association registered by the turbidimetric method at various concentrations of TMAO. The concentration of TMAO was varied in the range from 0.5 to 1.4 M. TMAO was found to promote the rate of association. The higher the TMAO concentration, the higher the initial rate of PhK association became.

Additional quantitative information on the enhanced association of PhK due to TMAO and other osmolytes was obtained by sedimentation analysis. In previous work, we showed that in the presence of 0.6 M TMAO, 0.1 mM Ca^{2+}, and 2 mM Mg^{2+}, apart from the PhK associates consisting of a rather low number (n) of PhK molecules ($n = 2$–6), two distinct rapidly moving boundaries with $s_{20,w} = 189$ and 365 S were registered on the sedimentation profiles (Chebotareva *et al.*, 2002). These boundaries correspond to 24- and 70-mers. In the present work we have compared the effect of TMAO, betaine and proline on self-association of PhK.

Sedimentation velocity studies of self-association of PhK in 80 mM Hepes, pH 6.8, containing 0.1 mM Ca^{2+} and 10 mM Mg^{2+} show that the formation of very large associates takes place in the presence of high concentrations of TMAO and betaine. The species with the sedimentation coefficients of 23 700 and 21 800 S were registered at rotor speed 2000 rpm for PhK in the presence 1 M TMAO or 1 M betaine, respectively. In contrast, proline does not stimulate the formation of large aggregates of PhK. The data obtained by the turbidimetric method show that the addition of proline (0.6–1.0 M) to the PhK solution containing 0.1 mM Ca^{2+} and 10 mM Mg^{2+} does not result in an increase in

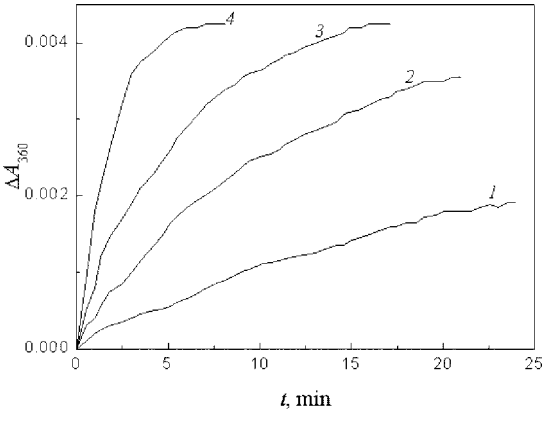

Figure 1. The effect of TMAO on the kinetics of PhK self-association (40 mM Hepes, pH 6.8; 0.1 mM Ca^{2+} and 10 mM Mg^{2+}; 20 °C). The concentration of PhK was 53 μg/ml. Association was initiated by the addition of the PhK solution preincubated with Ca^{2+} and Mg^{2+} at 20 °C for 2 min to the solution containing TMAO, Ca^{2+} and Mg^{2+}. The time dependence of the change in the absorbance at 360 nm (ΔA_{360}) was obtained at various concentrations of TMAO: 0.5 M (*1*), 0.75 M (*2*), 1.0 M (*3*) and 1.4 M (*4*).

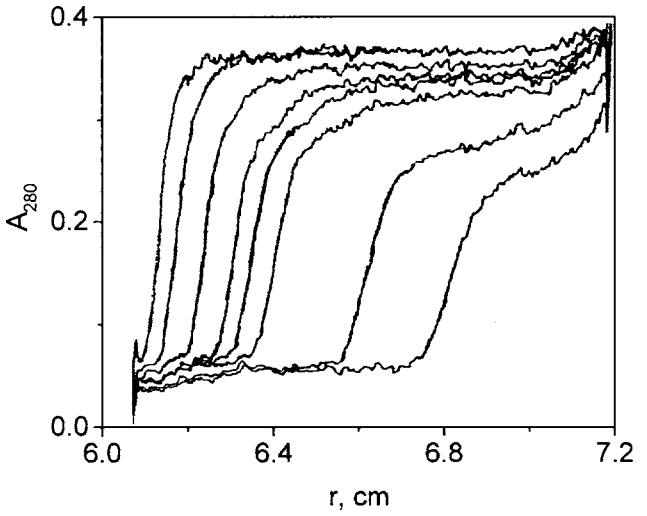

Figure 2. The sedimentation velocity patterns of PhK (0.3 mg/ml) in the presence of 0.6 M proline (40 mM Hepes, pH 6.8; 0.1 mM Ca^{2+}, and 10 mM Mg^{2+}; 20 °C). Rotor speed was 40 000 rpm. The sedimentation direction was from left to right. Times of sedimentation after the attainment of the maximum speed were the following: 1, 6, 12, 16, 20, 37 and 52 min. The PhK solution was preincubated with osmolyte, Ca^{2+} and Mg^{2+} for 90 min at 20 °C.

absorbance at 360 nm. This result suggests that there are no large aggregates in the presence of proline.

We also studied the sedimentation of PhK in the presence of osmolytes at a higher speed (40 000 rpm). In such experiments the enzyme was pre-mixed with osmolytes in the presence of Ca^{2+} and Mg^{2+} for 90 min. In the presence of 1 M TMAO or 1 M betaine, large aggregates of PhK precipitated prior to the attainment of the maximum rotor speed. Figure 2 shows the sedimentation behavior of PhK at 40 000 rpm in the presence of 0.6 M proline. There are only two boundaries ($s_{20,w} = 22.6$ and 30.5 S) corresponding to the monomeric and dimeric forms, respectively. Thus, trimethylamines, TMAO and betaine stimulate the self-association of PhK, whereas proline prevents the formation of large aggregates of the enzyme.

Effect of osmolytes on the interaction of PhK with glycogen

Figure 3 shows the effect of proline on the kinetics of PhK interaction with glycogen (in 40 mM Hepes, pH 6.8, containing 0.1 mM Ca^{2+} and 2 mM Mg^{2+}; 20 °C). The absorbance change at 360 nm [Fig. 3(A), control curve *1*] is interpreted to mean that the interaction of PhK with glycogen takes place under these conditions. When the binding of PhK to glycogen is studied in the presence of proline, the rate and extent of the change in absorbance appear to decrease [Fig. 3(A), curves *2–6*], suggesting that proline interferes with the PhK–glycogen complex formation. Figure 3(B) demonstrates the diminution of the relative initial rate of interaction of PhK with glycogen when proline concentration increases. Direct evidence of the propensity of proline to suppress PhK–glycogen complex formation was obtained by sedimentation velocity. Since glycogen, and its complex with the enzyme, sediment significantly faster than the free

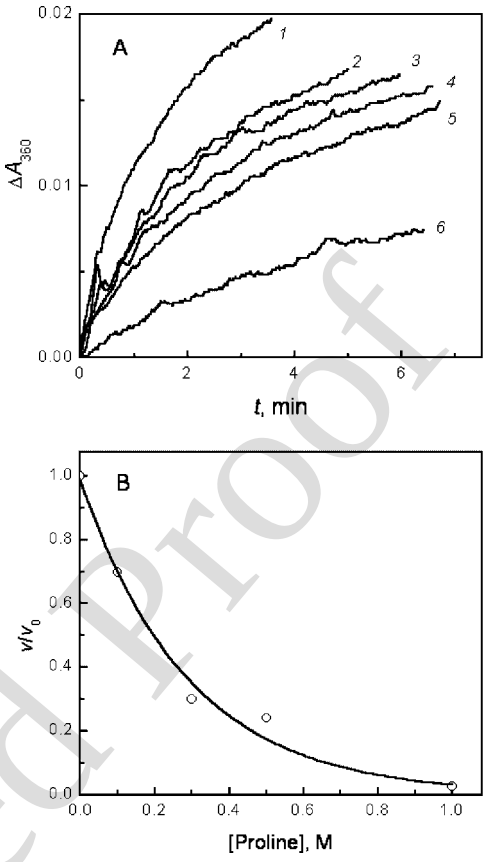

Figure 3. The effect of proline on the kinetics of PhK interaction with glycogen (40 mM Hepes, pH 6.8; 0.1 mM Ca^{2+} and 10 mM Mg^{2+}; 20 °C). The concentration of PhK was 25 μg/ml, glycogen 0.7 mg/ml. The process of complex formation was initiated by the addition of Ca^{2+} and Mg^{2+} to the mixture of PhK with glycogen. (A) The time dependence of the change in the absorbance at 360 nm (ΔA_{360}) was obtained at various concentrations of proline: 0 M (*1*), 0.1 M (*2*), 0.3 M (*3*), 0.5 M (*4*), 0.7 M (*5*) and 1.0 M (*6*). (B) The dependence of the relative initial rate of the interaction of PhK with glycogen (v/v_0) on the proline concentration.

enzyme, it is possible to measure the concentration of free enzyme after the precipitation of glycogen and the PhK–glycogen complex. Figure 4 shows the sedimentation behavior of PhK (0.33 mg/ml) pre-mixed with glycogen (0.9 mg/ml) in the absence of proline (A) and in the presence of 0.3 and 0.6 M proline (B and C, respectively) (40 mM Hepes, pH 6.8, containing 0.1 mM Ca^{2+} and 4 mM Mg^{2+}; 20 °C). From the data presented in Fig. 4 we can conclude that the fraction of free PhK increases with increasing proline concentration. It is equal to 45% without proline, 67% in 0.3 M proline, and 100% in 0.6 M proline. Thus, sedimentation velocity data support the capability of proline to interfere with the binding of PhK to glycogen.

We also investigated the effect of betaine on the initial rate and extent of the interaction of PhK with glycogen using the turbidimetric method. In contrast to proline, betaine enhances the rate and extent of PhK complexation with glycogen [Fig. 5(A)]. Figure 5(B) demonstrates the increase of the relative initial rate of the binding of PhK to glycogen with increasing betaine concentration.

The sedimentation velocity data is additional evidence that betaine favors the complexation of PhK with glycogen. In the presence of betaine, the fraction of free PhK

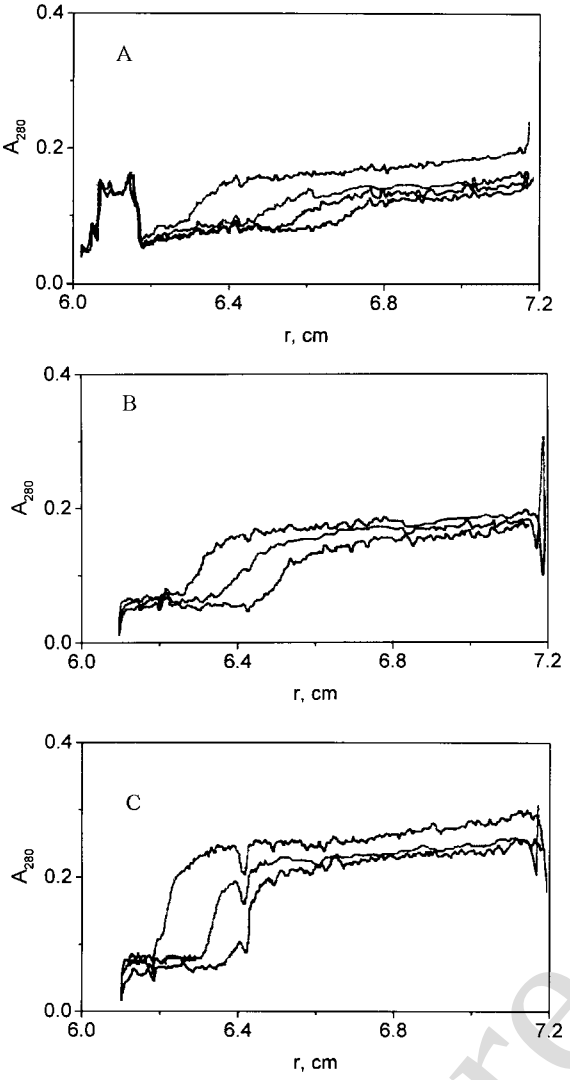

Figure 4. The sedimentation patterns of the system PhK (0.33 mg/ml) and glycogen (0.9 mg/ml) obtained at various concentrations of proline: 0 M (A), 0.3 M (B) and 0.6 M (C) (40 mM Hepes, pH 6.8, containing 0.1 mM Ca^{2+} and 4 mM Mg^{2+}; 20 °C). Rotor speed was 48 000 rpm. Direction of the sedimentation was from left to right. Times of sedimentation were the following: 3, 11, 15, 20 min (A); 8, 13, 18 min (B); 4, 12, 17 min (C).

registered after the precipitation of glycogen and the PhK–glycogen complex decreases with increasing betaine concentration (Table 1). The effect of TMAO on the formation of PhK–glycogen complex is similar to that of betaine (Table 1).

Effect of osmolytes on the interaction of PhK with its protein substrate Ph*b*

It is of interest to study the interaction of PhK with its substrate Ph*b* under the molecular crowding conditions arising from the presence of high concentrations of TMAO, betaine or proline. The experiments were performed in the presence of 0.1 mM Ca^{2+} and 2 mM Mg^{2+}. Sedimentation velocity was used to measure the concentration of free Ph*b*. Since the sedimentation coefficient of PhK is much larger than that of Ph*b*, both PhK and its complex with Ph*b*

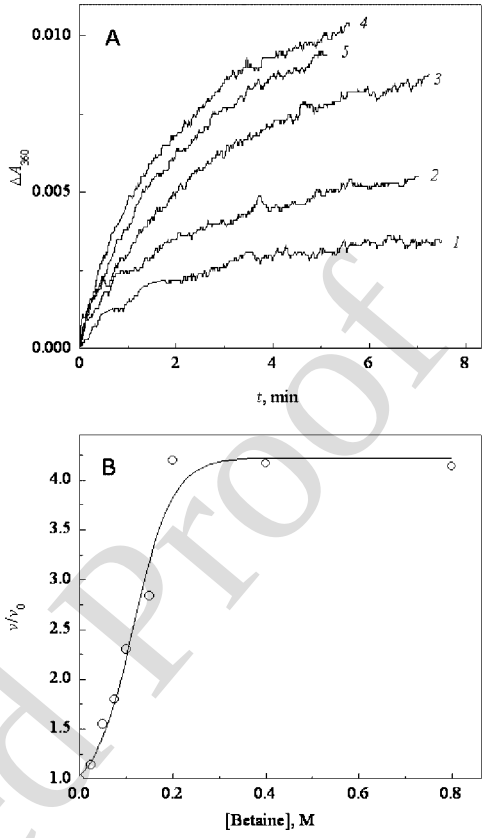

Figure 5. The effect of betaine on the kinetics of PhK interaction with glycogen (40 mM Hepes, pH 6.8; 0.1 mM Ca^{2+} and 10 mM Mg^{2+}; 20 °C). The PhK concentration was 22 μg/ml, glycogen 0.7 mg/ml. The complex formation was initiated by the addition of Ca^{2+} and Mg^{2+} to the mixture of PhK with glycogen. (A) The time dependence of the change in the absorbance at 360 nm (ΔA_{360}) was obtained at various concentrations of betaine: 0 M (*1*), 0.05 M (*2*), 0.1 M (*3*), 0.2 M (*4*) and 0.8 M (*5*). (B) The dependence of the relative initial rate of the PhK interaction with glycogen (v/v_0) on the betaine concentration.

Table 1. The effect of betaine and TMAO on the PhK binding with glycogen (40 mM Hepes, pH 6.8, 0.1 mM $CaCl_2$, 10 mM $MgCl_2$; 20 °C)

PhK, mg/ml	Glycogen, mg/ml	Osmolyte, M	Fraction of free enzyme[a]
		Betaine	
0.25	0.9	0	50%
0.25	0.9	0.2	50%
0.25	0.9	0.6	35%
0.25	0.9	1.2	20%
		TMAO	
0.18	0.08	0	70%
0.18	0.08	1	30%

[a] Fraction of free enzyme was estimated from the sedimentation data.

sediment significantly faster than Ph*b* at high speed. Thus, the sedimentation plateau formed near the meniscus after the precipitation of PhK and PhK–Ph*b* complex corresponds to the concentration of free Ph*b*. Figure 6 demonstrates the sedimentation behavior of the PhK and Ph*b* mixture in the absence of osmolytes (A), in the presence of 0.6 M proline (B), in the presence of 0.7 M betaine (C), and in

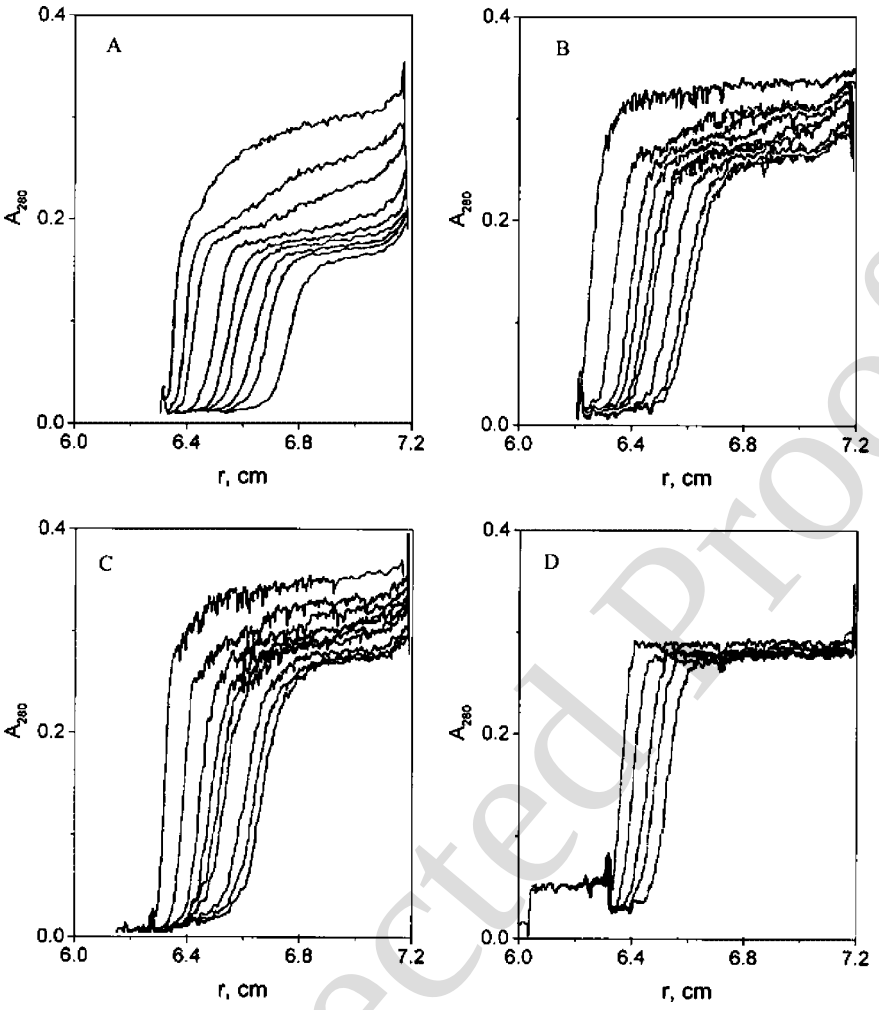

Figure 6. The sedimentation velocity analysis of the interaction of PhK (0.18 mg/ml) with Ph*b* (0.39 mg/ml) (40 mM Hepes, pH 6.8, containing 0.1 mM Ca^{2+}, 2 mM Mg^{2+}; 20 °C). (A) Sedimentation profiles in the absence of osmolytes, (B) in the presence of 0.6 M proline, (C) in the presence of 0.7 M betaine, (D) in the presence of 0.3 M TMAO. The run was carried out at 48 000 rpm. Times of sedimentation were the following: 11, 16, 25, 32, 37, 44, 49, 58 min (A); 6, 19, 28, 33, 38, 41, 50, 57, 61 min (B); 13, 20, 29, 35, 39, 42, 51, 56, 59 (C); 4, 10, 16, 20, 27 min (D).

the presence of 0.3 M TMAO (D). The slowly sedimenting boundary corresponds to Ph*b* ($s_{20,w} = 8.79$ S). The fraction of free Ph*b* calculated from the plateau of the sedimentation patterns increases from 65% in the absence of osmolytes to 100% in the presence of 0.6 M proline, or 0.7 M betaine, or 0.3 M TMAO. Thus, proline, betaine, and TMAO at these concentrations completely prevents the formation of PhK–Ph*b* complex.

DISCUSSION

The effects of osmolytes on the self-association of PhK and interaction of PhK with glycogen or Ph*b* are summarized in Table 2. The effect of trymethylamines (TMAO and betaine) on self-association of PhK and its interaction with glycogen is consistent with the general notion about the molecular crowding as a factor favoring protein oligomerization and the formation of supramolecular structures (Shearwin and Winzor, 1988; Cann *et al.*, 1994; Shtilerman *et al.*, 2002; Zimmerman and Minton, 1993; Patel *et al.*, 2002). A rigorous interpretation of the effect of TMAO and betaine on the interaction of PhK with glycogen is made difficult by the fact that these osmolytes provoke the formation of large associates of PhK. It is not clear how such a self-association of PhK affects the binding of the enzyme to glycogen.

Table 2. Effect of high concentrations of osmolytes (0.3–1.0 M) on self-association of PhK and its interaction with glycogen and Ph*b* (0.1 mM Ca^{2+}, 2–10 mM Mg^{2+}, 40 mM Hepes, pH 6.8, 20 °C)

Reaction type	Osmolyte		
	TMAO	Betaine	Proline
Self-association of PhK	+	+	−
Binding of PhK to glycogen	+	+	−
Interaction of PhK with Ph*b*	−	−	−

(+) means that osmolyte favors the reaction under study.
(−) means that osmolyte interferes with the process under study.

However it is evident that the overall effect of molecular crowding is the favoring of the interaction of PhK with glycogen. The suppression of the interaction of PhK with glycogen by proline is unexpected and is probably due to the specific interaction of proline with PhK.

The investigation of the effect of crowding on PhK is of special interest because of several reasons. Firstly, the physiological substrate of PhK is a large protein Ph*b* ($M = 195$ kDa); thus, the enzyme–substrate interaction is really a protein–protein interaction. Recently, it was shown that the interaction of PhK with Ph*b* was realized not only with the participation of the catalytic γ subunit of PhK but also with some fragment of the regulatory α subunit of PhK, close to the phosphorylation site (Andreeva *et al.*, 2001, 2002). Secondly, both these proteins are localized on the high-molecular-weight matrix–glycogen particles (Meyer *et al.*, 1970). It is known that both PhK and Ph*b* directly interact with glycogen (Steiner and Marshall, 1982; Chebotareva *et al.*, 1979; Klinov *et al.*, 1982; Zemskova *et al.*, 1989; Andreeva *et al.*, 1999a,b). Each subunit of the dimeric Ph*b* molecule contains a specific glycogen storage site (Oikonomakos *et al.*, 1992). As for PhK, the participation of the α subunit in the binding with glycogen has been demonstrated (Chan and Graves, 1982). The presence of Ph*b* favors the binding of PhK to glycogen (Shmelev and Serebrenikova, 1997; Andreeva *et. al.*, 1999a,b).

When interpreting the effect of osmolytes on the PhK–Ph*b* interaction, one should take into account that the protein conformation may be affected by the molecular crowding conditions. For example, Chebotareva *et al.* (2001) studied the effect of molecular crowding on self-association of Phb in the presence of 1 mM AMP. It was found that the enhancement of the association constant for the equilibrium of dimer–tetramer was significantly less than the theoretical value calculated on the basis of the estimates of the second virial coefficients of dimer and tetramer. To explain the experimental dependence of the association constant on TMAO concentration, an additional stage of dimer isomerization was introduced. The conclusion was made that TMAO displaced the isomerization equilibrium for Ph*b* dimer towards the more compact inactive T-state (Chebotareva *et al.*, 2001). An isomerization transition of Ph*b* of this kind may prevent its interaction with PhK. However, it cannot be excluded that the crowding conditions also affect the conformational state of PhK.

The fact that high concentrations of osmolytes simulating the crowding conditions in the cell prevent the complex formation between PhK and its substrate, Ph*b*, appears to be physiologically unreasonable. However it is known that the interaction PhK with Ph*b* is strongly affected by the presence of glycogen (Zemskova *et al.*, 1989; Morange and Buc, 1979). As has been shown in the present work, the crowding conditions arising from the presence of trymethylamines favor the binding of PhK to glycogen. Therefore, one can assume that the specific interactions of PhK and Ph*b* with glycogen overcome the negative effect of molecular crowding on the recognition of Ph*b* by PhK.

Overall, the data presented here indicate that the effect of crowding cannot be ignored when studying biochemical processes *in vitro*. In this work, we simulated the solute concentrations found in cell using high concentrations of osmolyte and showed that the crowding conditions strongly affect the protein–glycogen binding and protein–protein interactions.

Acknowledgments

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