A tentative mechanism of the ternary complex formation between phosphorylase kinase, glycogen phosphorylase *b* and glycogen

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Abstract The kinetics of rabbit skeletal muscle phosphorylase kinase interaction with glycogen has been studied. At pH 6.8 the binding of phosphorylase kinase to glycogen proceeds only in the presence of Mg^{2+}, whereas at pH 8.2 formation of the complex occurs even in the absence of Mg^{2+}. On the other hand, the interaction of phosphorylase kinase with glycogen requires Ca^{2+} at both pH values. The initial rate of the complex formation is proportional to the enzyme and glycogen concentrations, suggesting the formation of the complex with stoichiometry 1:1 at the initial step of phosphorylase kinase binding by glycogen. According to the kinetic and sedimentation data, the substrate of the phosphorylase kinase reaction, glycogen phosphorylase *b*, favors the binding of phosphorylase kinase with glycogen. We suggest a model for the ordered binding of phosphorylase *b* and phosphorylase kinase to the glycogen particle that explains the increase in the tightness of phosphorylase kinase binding with glycogen in the presence of phosphorylase *b*.

Key words: Phosphorylase kinase; Glycogen; Glycogen phosphorylase *b*

1. Introduction

Phosphorylase kinase catalyzing phosphorylation and activation of glycogen phosphorylase *b* plays a key role in the cascade system of regulation of glycogen metabolism [1]. Phosphorylase kinase activity is pH dependent. It is very low at pH 6.8 and increased at pH 8.2. The enzyme molecule is a hexadecamer with the subunit formula $(\alpha\beta\gamma\delta)_4$ and a molecular mass of 1320 kDa [2]. Electron microscopic studies of rabbit skeletal muscle phosphorylase kinase showed that the tight complex of the catalytic γ-subunit (44.7 kDa) with the regulatory δ-subunit (16.7 kDa) identical to the Ca^{2+} binding protein calmodulin forms the nucleus of the structure, whereas the regulatory α-(138.4 kDa) and β-subunits (125.2 kDa) form ridges on the surface of the molecule, giving it the shape of a 'butterfly' or 'chalice' [3–5].

In skeletal muscle, 20–40% of phosphorylase kinase is located with other enzymes of glycogen metabolism on the surface of the glycogen granules [6]. It is known that glycogen increases the affinity of phosphorylase kinase to its protein substrate, phosphorylase *b*, 2–3-fold [7,8]. The ability of phosphorylase kinase to complex with glycogen was studied in the presence of Mg^{2+} [9] and also with Ca^{2+} and Mg^{2+} [10]. The sensitivity of the enzyme for Ca^{2+} is provided by the δ-subunit [2], whereas two Mg^{2+} binding sites are located in the N-terminal domain of the γ-subunit [11,12]. Fisher and Krebs [13] and also Zemskova et al. [8] showed a direct influence of glycogen on the rate of autoactivation of phosphorylase kinase by phosphorylation of its α- and β-subunits. Chen and Graves demonstrated the activation of the αγδ-complex but not the γδ-complex under the influence of glycogen suggesting important role for the α-subunit of phosphorylase kinase in the interaction with glycogen [14]. In 1993 Veronica E. Sanchez showed in her thesis [15] that glycogen specifically protects the α-subunit from carboxymethylation. The activating effect of glycogen on phosphorylase kinase was lost when the α-subunit was destroyed by partial proteolysis, suggesting a role for the α-subunit in the formation of a phosphorylase kinase-glycogen complex [16]. The existence of this complex was also demonstrated by the gel filtration method [8]. Shmelev and Serebrenikova ,using the turbidimetric method, showed the formation of the ternary complex (glycogen-phosphorylase kinase-phosphorylase *b*) under the conditions of a phosphorylase kinase assay [17].

The goal of the present paper is to determine the stoichiometry of the phosphorylase kinase-glycogen complex formation at various relationships between the components and to study the influence of pH, on the sensitivity of the process under study, to Ca^{2+} and Mg^{2+} ions. In order to elucidate the mechanism of the influence of phosphorylase *b* on the interaction of phosphorylase kinase with glycogen, in addition to the kinetic method, the sedimentation analysis giving a direct information about binding of proteins to glycogen has been used.

2. Materials and methods

Phosphorylase kinase was purified from rabbit skeletal muscle according to Cohen [2] using ion exchange chromatography on DEAE Toyopearl at the final step of purification [18]. Rabbit skeletal muscle phosphorylase *b* was prepared by the method of Fisher and Krebs [13] using dithiothreitol instead of cysteine and was recrystallized at least three times. The enzyme was freed of AMP by treatment with Norit A. The preparation of phosphorylase *b* used here did not contain phosphorylase *a*. The concentrations of phosphorylase kinase and phosphorylase *b* were determined spectrophotometrically using the extinction coefficients of 12.4 and 13.2, respectively, for 1% solutions [2,19].

HEPES was purchased from Sigma (USA). Glycogen from pig liver with an average molecular mass of 5.5×10^6 Da was from Biolar (Latvia). Other reagents (of high purity) were from Russian suppliers.

The kinetics of the complex formation between phosphorylase kinase and glycogen were followed by the increase in the absorbance at 360 nm using a spectrophotometer Hitachi-557 (Japan) equipped with a thermostated cell holder (the length of the optical path 1 cm) at 20°C in 40 mM HEPES, containing 1 mM β-mercapthoethanol. The initial rates of the interaction of phosphorylase kinase with glycogen were calculated by the approximation of the kinetic curves by poly-

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nomial of the third degree using the MicroCal Origin 4.0 program for a IBM compatible computer.

The sedimentation of phosphorylase kinase in the presence of glycogen and phosphorylase *b* was studied at 20°C in a Spinco model E analytical ultracentrifuge (Beckman) equipped with a photoelectric scanner, a multiplexer and a monochromator. A titanium rotor An-F-Ti and 12 mm double sector centerpieces were used in the experiments. The rotor speed was 40 000 rpm. The sedimentation was registered by the measurement of protein absorbance at 280 nm.

3. Results

3.1. The kinetics of phosphorylase kinase binding with glycogen

Since the glycogen particle of the preparation used (molecular mass 5.5×10^6 Da) is rather large, it could probably bind several molecules of phosphorylase kinase. On the other hand, the hexadecameric phosphorylase kinase molecule containing specific sites for glycogen binding on the α-subunits [14] should be capable of interacting with several glycogen particles.

To find out the stoichiometry of the complex formation between phosphorylase kinase and glycogen at the initial step of this process, we studied the initial rate of the enzyme interaction with glycogen at various concentrations of the components. The kinetics of binding was followed by the increase in the absorbance at 360 nm. As shown in Fig. 1, the initial rate of phosphorylase kinase interaction with glycogen is directly proportional to the concentration of each component. Thus, we could suppose that the stoichiometry of the complex formation of phosphorylase kinase with glycogen at the initial step of their interaction is 1:1.

3.2. The influence of pH on the complex formation

As was shown previously [10,17], the formation of the complex of phosphorylase kinase with glycogen at pH 6.8 occurs only in the presence of Ca^{2+} and Mg^{2+}. Activity of phosphorylase kinase may be enhanced by alkaline pH [1]. Therefore, it would be of special interest to study the role of Ca^{2+} and Mg^{2+} in the complex formation at pH 8.2.

Fig. 2 shows the time course of the formation of the complex phosphorylase kinase-glycogen at pH 8.2 in the presence of 0.1 mM $CaCl_2$ and 10 mM $MgCl_2$ (upper curve). The dashed line in Fig. 2 corresponds to the kinetic curve of interaction of phosphorylase kinase with glycogen at pH 6.8 in the solution containing 0.1 mM $CaCl_2$ and 10 mM $MgCl_2$.

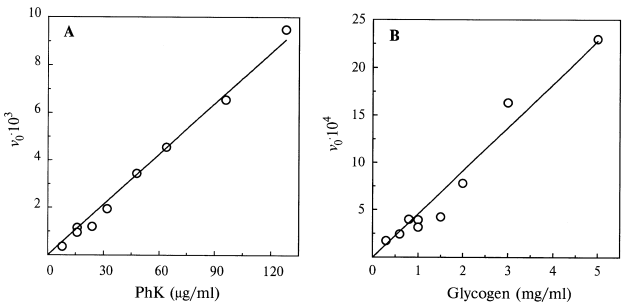

Fig. 1. The dependence of the initial rate of the complex formation v_0 (U absorbance/min) on phosphorylase kinase (PhK: A) and glycogen (B) concentrations. (A) The glycogen concentration was fixed (2 mg/ml). (B) The phosphorylase kinase concentration was fixed (33 μg/ml). The process of complex formation was initiated by the addition of phosphorylase kinase, pre-incubated with Ca^{2+} and Mg^{2+} for 2 min at 20°C, to 40 mM HEPES, pH 6.8, containing glycogen, 0.1 mM $CaCl_2$, and 10 mM $MgCl_2$.

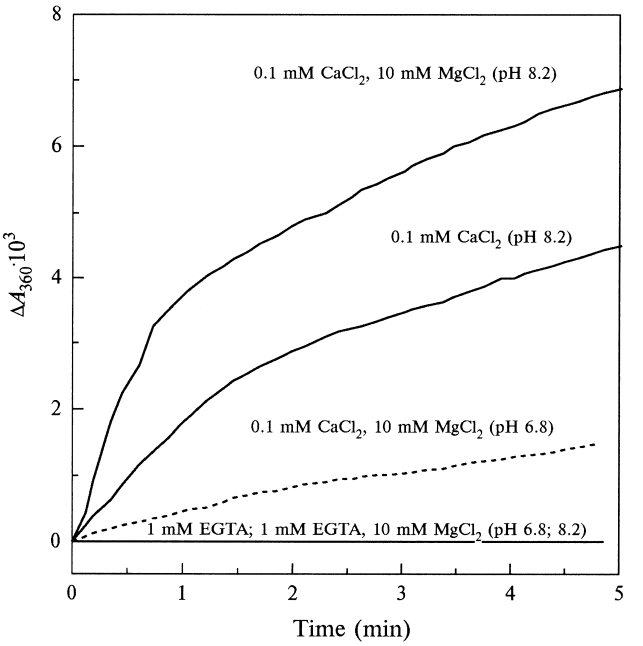

Fig. 2. The interaction of phosphorylase kinase with glycogen in 40 mM HEPES at pH 8.2 (20°C). The kinetic curves of the change in the absorbance at 360 nm (ΔA_{360}) obtained by the addition of phosphorylase kinase to glycogen solution in the presence or absence of Ca^{2+} and Mg^{2+}. The final concentrations: phosphorylase kinase 80 μg/ml, glycogen 0.6 mg/ml.

The comparison of this curve with the kinetic curve obtained at pH 8.2 shows that the change in pH of the solution from 6.8 to 8.2 is accompanied by a 12-fold increase in the initial rate of the complex formation.

We have found that the binding of phosphorylase kinase to glycogen in the absence of Mg^{2+} is pH dependent. If the system at pH 8.2 does not contain Mg^{2+} (the curve obtained in the presence of 0.1 mM $CaCl_2$), only a partial decrease in the rate of the complex formation occurs (the v_0 value decreases 2.8-fold). The addition of 1 mM EGTA (Ca^{2+} binding agent) results in the complete suppression of phosphorylase kinase-glycogen complex formation (lower curve). In addition, we did not observe the complex formation in the solution containing both EGTA (1 mM) and $MgCl_2$ (10 mM).

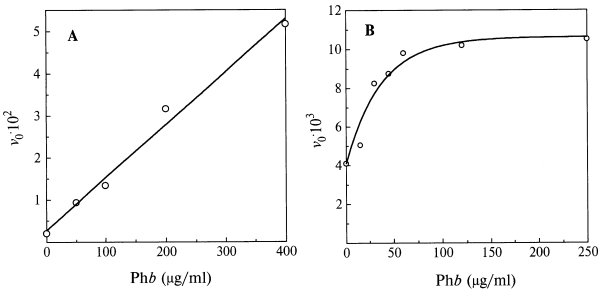

Fig. 3. The dependence of the initial rate of the interaction of phosphorylase kinase with glycogen v_0 (in U absorbance/min) on the concentration of phosphorylase *b* at pH 6.8 (A) and 8.2 (B). The process of the complex formation was initiated by the addition of phosphorylase kinase, pre-incubated with Ca^{2+} and Mg^{2+} for 2 min at 20°C, to the solution containing glycogen (0.6 mg/ml), 0.1 mM $CaCl_2$, 10 mM $MgCl_2$ and various concentrations of phosphorylase *b*. The final concentration of phosphorylase kinase was 35 μg/ml (A) and 80 μg/ml (B).

3.3. The influence of phosphorylase b on the complex formation

The marked increase in the initial rate of phosphorylase kinase binding with glycogen is observed in the presence of the substrate of the kinase reaction, phosphorylase *b*. At pH 6.8, the dependence of v_0 on phosphorylase *b* concentration remains linear up to a phosphorylase *b* concentration of 400 μg/ml (Fig. 3A).

We also studied the influence of phosphorylase *b* on the initial rate of the complex formation at pH 8.2 (Fig. 3B). In this case, at a phosphorylase *b* concentration of approximately 250 μg/ml, the initial rate of the phosphorylase kinase binding with glycogen reaches a limiting value. These data suggest that phosphorylase *b* favors the binding of phosphorylase kinase with glycogen.

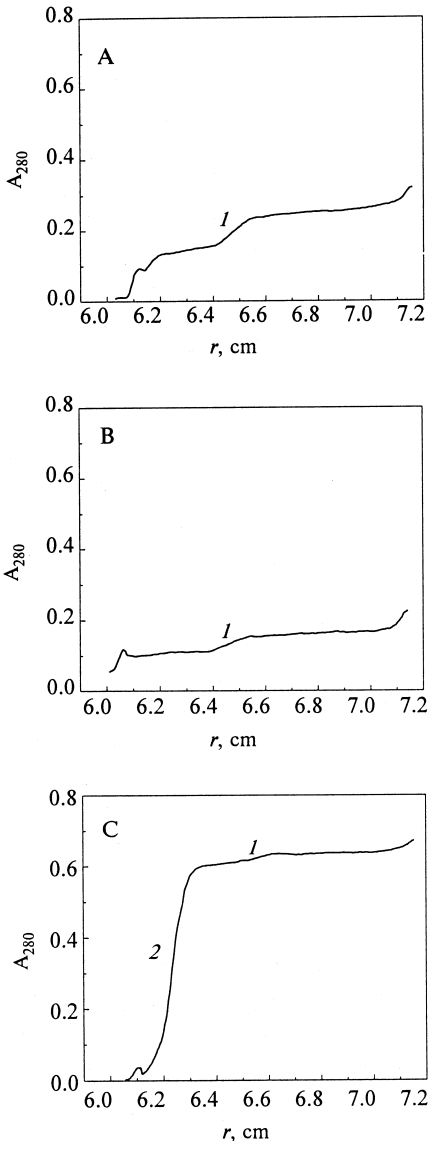

Fig. 4. The effect of phosphorylase *b* on the interaction of phosphorylase kinase with glycogen. (A) Sedimentation of phosphorylase kinase (0.26 mg/ml). (B) Sedimentation of phosphorylase kinase (0.26 mg/ml) in the presence of glycogen (0.6 mg/ml). (C) Sedimentation of phosphorylase kinase (0.26 mg/ml) in the presence of glycogen (0.6 mg/ml) and phosphorylase *b* (0.2 mg/ml). Times of sedimentation (min): 28 (A), 29.5 (B) and 31 (C). The direction of sedimentation is from left to right.

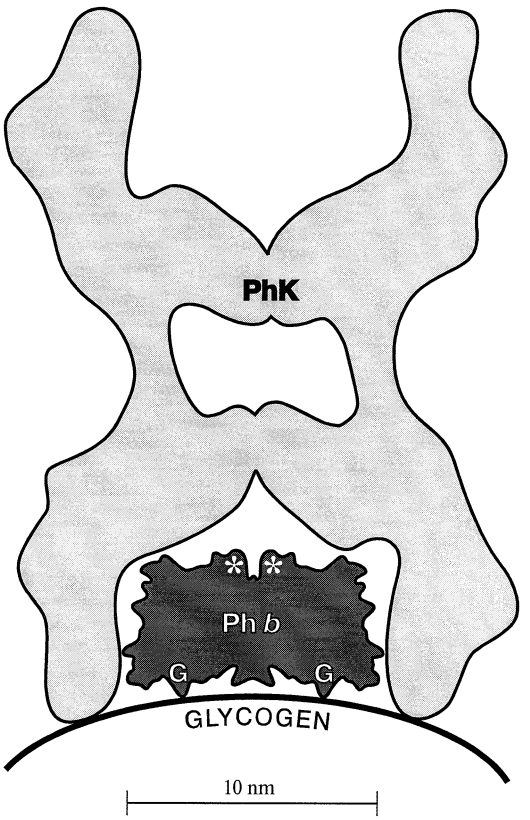

Fig. 5. The model of the binding of phosphorylase kinase and phosphorylase *b* on the glycogen particle. The dimensions and shape of the phosphorylase kinase and phosphorylase *b* molecules are taken from [5] and [24], respectively. G are the glycogen storage sites in the dimeric molecule of phosphorylase *b*. The location of the residues Ser-14 which could be phosphorylated is indicated by asterisks.

The increase in the strength of phosphorylase kinase binding to glycogen in the presence of phosphorylase *b* at pH 6.8 was supported by the sedimentation method. Fig. 4 shows the sedimentation patterns of phosphorylase kinase (A), phosphorylase kinase in the presence of glycogen (B) and phosphorylase kinase in the presence of glycogen and phosphorylase *b* (C). The sedimentation coefficient of phosphorylase kinase (0.26 mg/ml) was found to be 21 S (the boundary *1*, Fig. 4A). When sedimentation of phosphorylase kinase was carried out in the presence of glycogen (0.6 mg/ml), a two-fold decrease in the height of the boundary *1* was observed (Fig. 4B), as compared to sedimentation of the free enzyme. Such a decrease in the height of the boundary corresponds to the decrease in the concentration of free phosphorylase kinase caused by its binding with glycogen. When sedimentation of phosphorylase kinase was carried out in the presence of both glycogen and phosphorylase *b* (0.2 mg/ml), a further two-fold decrease in the height of the boundary corresponding to sedimentation of phosphorylase kinase (boundary *1*) was observed (Fig. 4C). The boundary *2* in this Fig. 4C corresponds to the sedimentation of free phosphorylase *b* (the sedimentation coefficient is equal to 7.3 S). Thus, the presence of phosphorylase *b* causes a tight binding of phosphorylase kinase to glycogen. This result may be due to the interaction between phosphorylase kinase and phosphorylase *b* on the surface of the glycogen particle.

4. Discussion

In order to propose the mechanism of the ternary complex formation between phosphorylase kinase, glycogen phosphorylase *b* and glycogen, we should take into account the following experimental data: (1) glycogen enhances the affinity of phosphorylase kinase to phosphorylase *b* [20]; (2) phosphorylase *b* favors the formation of the complex between phosphorylase kinase and glycogen ([17], the present work); (3) phosphorylase kinase α-subunits participate in the formation of the complex between the enzyme and glycogen [14,15,21]; (4) in the presence of glycogen the activation of the phosphorylase kinase reaction occurs [22,23]. Thus, the ternary complex between phosphorylase kinase, glycogen phosphorylase *b* and glycogen should be a productive one. In other words, a correct orientation of phosphorylase kinase and glycogen phosphorylase *b* in this complex could allow the phosphorylation of Ser-14 in phosphorylase *b*.

According to the results of the X-ray studies carried out by Barford and Johnson [24], the molecular dimensions of the phosphorylase *b* dimer are $6.5\times6.5\times11$ nm [24]. It is known that glycogen storage sites and phosphorylated Ser-14 residues in phosphorylase *b* are situated on the opposite sides of the protein molecule. The electron microscopic studies show that the phosphorylase kinase molecule more often has the 'butterfly' form with dimensions of 22.2×29 nm [5]. The 'butterfly' wings are formed by the α- and β-subunits and are connected by bridges consisting of the γ- and δ-subunits.

According to our data and the geometric characteristics of both enzymes, we propose a model of the complex between phosphorylase kinase, glycogen phosphorylase *b* and glycogen (Fig. 5). In the ternary complex, the phosphorylase *b* molecule is situated between the 'butterfly' wings of phosphorylase kinase in such a way that the Ser-14 residues of phosphorylase *b* are oriented towards the catalytic γ-subunits of phosphorylase kinase. Thus, phosphorylase *b* molecule appears to be 'closed' in the cavity formed by phosphorylase kinase and the surface of the glycogen particle. The structure of the ternary complex proposed by us suggests the ordered mechanism of the formation of this complex. First, on the surface of the glycogen particle the phosphorylase *b* dimer is bound (this process is rather fast, with half-conversion times falling in the ms interval [25]) and then the adsorbed phosphorylase *b* dimer is 'covered' by the phosphorylase kinase molecule. In the complex of such a kind, phosphorylase kinase is held on the surface of the glycogen particle not only by the direct contacts with glycogen via α-subunits, but also by the contacts with the adsorbed phosphorylase *b*. This pattern explains a tighter binding of phosphorylase kinase with glycogen in the presence of phosphorylase *b* and also suggests that the effect of glycogen on the rate of the phosphorylase kinase reaction is both substrate- and enzyme-directed.

The results obtained provide new insights into the role of second messengers in the cell. Ca^{2+}, apart from the regulatory function (the stimulation of the phosphorylase kinase activity), acts as an assembly forming factor enhancing the efficiency of phosphorylase kinase catalysis through the formation of the productive enzyme-substrate complex on the surface of the glycogen particle.

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