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Interaction of Phosphorylase Kinase from Rabbit Skeletal Muscle with Flavin Adenine Dinucleotide

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Abstract—The interaction of flavin adenine dinucleotide (FAD) with rabbit skeletal muscle phosphorylase kinase has been studied. Direct evidence of binding of phosphorylase kinase with FAD has been obtained using analytical ultracentrifugation. It has been shown that FAD prevents the formation of the enzyme–glycogen complex, but exerts practically no effect on the phosphorylase kinase activity. The dependence of the relative rate of phosphorylase kinase–glycogen complex formation on the concentration of FAD has cooperative character (the Hill coefficient is 1.3). Under crowding conditions in the presence of 1 M trimethylamine-N-oxide (TMAO), FAD has an inhibitory effect on self-association of phosphorylase kinase. The data suggest that the complex of glycogen metabolism enzymes in protein–glycogen particles may function as a flavin depot in skeletal muscle.

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Phosphorylase kinase (EC 2.7.1.38) plays a key role in the neuronal and hormonal regulation of glycogenolysis in skeletal muscle. The enzyme has a complex molecular organization and forms a hexadecamer consisting of four different subunits with stoichiometry $(\alpha\beta\gamma\delta)_4$ and molecular mass 1320 kD [1-3]. It was shown that the γ-subunit (44.7 kD) has a catalytic function [4], whereas the α- (138.4 kD), β- (125.2 kD), and δ-subunits (16.7 kD) are regulatory ones [5, 6], with the δ-subunit being identical to the Ca^{2+}-binding protein calmodulin [7].

The oligomeric state of phosphorylase kinase depends on the concentration of Ca^{2+} and Mg^{2+}. Ca^{2+} and Mg^{2+} stimulate the enzymatic activity by inducing changes in the tertiary and quaternary structure of the phosphorylase kinase molecule [8, 9]. In the absence of Ca^{2+} and Mg^{2+} the enzyme exists as a monomer and a dimer with sedimentation coefficients $s_{20,w} = 23$ S (which corresponds to the molecular mass 1320 kD) and $s_{20,w} =$ 36.5 S, respectively [1, 10]. It should be noted that in this case the monomer of phosphorylase kinase is assumed to be a hexadecamer $(\alpha\beta\gamma\delta)_4$. Upon addition of 0.1 mM Ca^{2+} and 10 mM Mg^{2+}, higher-order oligomers are formed [10, 11].

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In skeletal muscle, about 40% of phosphorylase kinase together with other enzymes of glycogen metabolism is localized on the surface of glycogen granules [12]. It is known that glycogen increases 2-3-fold the affinity of phosphorylase kinase to its protein substrate, glycogen phosphorylase *b* [13, 14]. The α-subunit of the enzyme plays an important role in the contact of phosphorylase kinase with glycogen [15]. In addition, the regulatory α-subunit together with the catalytic γ-subunit can interact with the phosphorylase kinase substrate, glycogen phosphorylase *b* [16]. Previously we proposed a model of an ordered binding of glycogen phosphorylase *b* and phosphorylase kinase on the glycogen particle, which explains the increase in the tightness of the binding of phosphorylase kinase with glycogen in the presence of glycogen phosphorylase *b* [17].

It is known that some enzymes of the protein–glycogen complex in skeletal muscle have affinity to flavins. The flavin-binding sites in glycogen phosphorylase *a* were discovered by Sprang et al. [18]. Localization of the FMN-binding sites in the molecule of glycogen phosphorylase *a* was determined by X-ray analysis. We have studied in detail the interaction of flavins (riboflavin, FMN, and FAD) with glycogen phosphorylase *b* [19-23]. All flavins studied have inhibitory effect on the enzyme, with FMN having the strongest

influence. According to the data of Sudakov and Solovyeva [24], FMN has a strong inhibitory effect on rabbit skeletal muscle glycogen synthase. It was of interest to study the possibilities of interaction of flavins with other enzymes of the protein–glycogen complex, in particular with phosphorylase kinase, the key regulatory enzyme of glycogenolysis. We selected FAD as a flavin, because it is the prevailing form of flavins in animal tissues [25].

All biochemical processes in the cell proceed under conditions of molecular crowding (highly concentrated medium with a significant part of the volume occupied by macromolecules). Molecular crowding has a strong influence on the interaction of the molecules, on the rates and equilibrium of the biochemical processes in the cell [26-31]. It is known that under stress conditions, cells accumulate high concentrations of osmolytes, which have a stabilizing effect on proteins, and thus protects the organism against stress [32, 33]. The use of high concentrations of osmolytes *in vitro* allows one to imitate the conditions of molecular crowding [10, 34-36]. Previously we demonstrated that high concentrations of the natural osmolyte trimethylamine N-oxide (TMAO) stimulate self-association of phosphorylase kinase [10, 35].

The goal of the present work was to study the influence of FAD on the oligomeric state of phosphorylase kinase, on its interaction with glycogen, as well as to examine the effect of FAD on self-association of phosphorylase kinase under conditions of molecular crowding imitated by high concentrations of TMAO.

MATERIALS AND METHODS

HEPES, ATP, FAD, and TMAO were purchased from Sigma (USA). Glycogen from pig liver with an average molecular mass $5.5 \cdot 10^6$ daltons was from Biolar (Latvia); [γ-^{32}P]ATP was from Obninsk (Russia). DEAE-Toyopearl 650M was purchased from Tosoh (Japan).

Phosphorylase kinase was obtained from rabbit skeletal muscle according to Cohen [1] using ion-exchange chromatography on DEAE-Toyopearl 650M at the final step of purification [37]. The purity of the enzyme was checked electrophoretically according to Laemmli [38]. The preparations of phosphorylase kinase were practically homogeneous. Prior to experiments, phosphorylase kinase was dialyzed against 40 mM HEPES buffer, pH 6.8, at 4°C for 2 h.

Glycogen phosphorylase *b* was obtained from rabbit skeletal muscle according to Fisher and Krebs [39], using dithiothreitol instead of cysteine during recrystallization of the enzyme. AMP was removed from the enzyme solution by adsorption on a column of Norit A. Phosphorylase kinase and glycogen phosphorylase *b* concentrations were determined spectrophotometrically at 280 nm, using absorbance coefficients of 12.4 and 13.2 for 1% solution, respectively [1, 40].

The kinetics of phosphorylase kinase–glycogen complex formation was followed by an increase in the optical absorbance at 600 nm using a Hitachi-557 spectrophotometer (Japan) equipped with a thermostatted cell holder (1 cm optical path length). The kinetics of complex formation was studied at 20°C in 40 mM HEPES buffer, pH 6.8, containing 1 mM β-mercaptoethanol. The complex formation was initiated by addition of Ca^{2+} and Mg^{2+} to the solution containing phosphorylase kinase (50 μg/ml) and glycogen (0.25-1.0 mg/ml) in the absence and in the presence of various concentrations of FAD. The absorbance of FAD at the wavelength used (600 nm) is practically absent. The final concentrations of Ca^{2+} and Mg^{2+} were 0.1 and 10 mM, respectively.

The kinetics of phosphorylase kinase association induced by 1 M TMAO were studied in the absence and in the presence of different concentrations of FAD under the same conditions as for the formation of phosphorylase kinase–glycogen complex.

The sedimentation studies were carried out on a Spinco model E analytical ultracentrifuge (Beckman, Austria) equipped with absorbance optics, a photoelectric scanner, and on-line computer. A six-hole An-G Ti rotor and 12 mm double sector cells were used in runs. Sedimentation profiles were recorded by measuring the enzyme absorbance at the wavelength 280 nm, and FAD absorbance at 450 nm. For digital data acquisitions La-n20-12 PCI and La-1.5 PCI plates, and software specially written by A. G. Zharov (www.ADClab.ru) were used. The sedimentation coefficients were calculated from the dependence of $\ln r$ versus $\omega^2 t$, where r is the radial position of the boundary, ω is the angular velocity, and t is time of sedimentation; or the sedimentation coefficients were estimated from a differential sedimentation coefficient distribution {$c(s)$ versus s} using the SEDFIT program [41, 42].

The enzymatic activity of phosphorylase kinase was detected by the incorporation of ^{32}P into glycogen phosphorylase *b* in the absence and in the presence of various concentrations of FAD. The composition of the reaction mixture was the following: 40 mM HEPES buffer, pH 6.8, glycogen phosphorylase *b* (0.4 mg/ml), phosphorylase kinase (0.8 μg/ml), 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, [γ-^{32}P]ATP (50 μM, specific activity 100 cpm/pmol). The reaction was initiated by the addition of ATP, and the reaction mixture was incubated at 26°C. After different time intervals, aliquots of the reaction mixture were placed on Whatman 3 MM filters, which were than treated according to Reimann et al. [43]. The radioactivity was detected in liquid scintillator using the LKB 1217 Packbeta counter.

The experimental data were analyzed using the program Origin 7.0 (OriginLab Corporation, USA).

RESULTS AND DISCUSSION

Study of the interaction of phosphorylase kinase with FAD by sedimentation analysis. Figure 1 presents the sedimentation patterns of phosphorylase kinase in the presence of FAD (40 mM HEPES, pH 6.8, 0.1 mM $CaCl_2$, 10 mM $MgCl_2$). The wavelength 450 nm corresponds to the absorption maximum of FAD. The sedimentation boundary (with sedimentation coefficient 19 S) corresponds to the complex of phosphorylase kinase with FAD, because at this wavelength the enzyme absorption is negligible. To analyze the oligomeric state of phosphorylase kinase, the enzyme sedimentation was registered in the presence and in the absence of FAD also at the wavelength 280 nm (Fig. 2). Figure 2a presents the differential distribution of sedimentation coefficient $c(s)$ estimated for phosphorylase kinase in the presence of FAD. The average sedimentation coefficient was determined by integration of the $c(s)$ distribution and was equal to 17.1 S. After transformation to the standard conditions $s_{20,w} = 19.4$ S. It can be concluded from the data presented that the enzyme is in monomeric form. For comparison, the $c(s)$ distribution of phosphorylase kinase in the absence of flavin at 20°C is also shown (Fig. 2b). It is possible to see from the figure that besides the monomeric form of the enzyme (23 S) higher molecular weight forms also exist. The lower value of the sedimentation coefficient of the monomeric form of phosphorylase kinase in the presence of FAD (19.4 S), as compared to the value 23 S, points to the change in the enzyme conformation due to its binding with FAD.

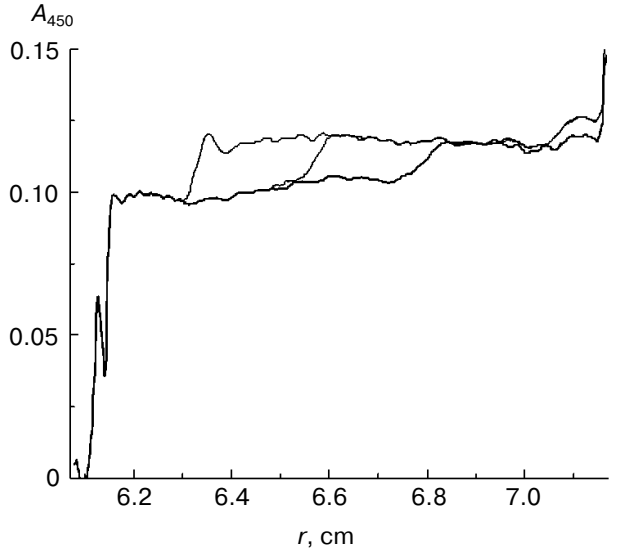

Fig. 1. Binding of FAD with phosphorylase kinase. Sedimentation patterns of the FAD–phosphorylase kinase complex. Sedimentation was recorded from left to right. Rotor speed is 48,000 rpm; sedimentation time is 12, 24, and 36 min; wavelength is 450 nm. Conditions: 40 mM HEPES, pH 6.8, 0.1 mM $CaCl_2$, 10 mM $MgCl_2$, 20°C. The enzyme concentration is 1.1 mg/ml, FAD concentration 9.7 μM.

Influence of FAD on the formation of the complex of phosphorylase kinase with glycogen. The influence of FAD on the interaction of phosphorylase kinase with glycogen has been studied under the fixed concentration of the enzyme (50 μg/ml) and various concentrations of glycogen (0.25-1.0 mg/ml). Figure 3a presents the kinetic curves of complex formation obtained by the turbidimetric method using the Hitachi-557 recording spectrophotometer. As can be seen from the data presented, FAD prevents the formation of phosphorylase kinase complex with glycogen. The initial rate of complex formation (v) can be calculated from the slope of the curve representing the dependence of absorbance at 600 nm on time. Figure 3b shows the relative initial rate of interaction of phosphorylase kinase with glycogen (v/v_0) depending on FAD concentration. As can be seen from the data presented, the change in the relative initial rate upon the increase in FAD concentration is independent of the initial concentration of glycogen (0.25, 0.7, and 1.0 mg/ml). For the quantitative description of v/v_0 dependence on FAD concentration, the Hill equation was used:

$$v/v_0 = 1/\{1 + ([FAD]/[FAD]_{0.5})^h\},$$

where h is a Hill coefficient, and $[FAD]_{0.5}$ is FAD concentration at which $v/v_0 = 0.5$. The following parameters were calculated from this equation: $h = 1.3 \pm 0.1$, and $[FAD]_{0.5} = 2.7 \pm 0.2$ μM. The fact that the Hill coefficient is higher than unity suggests positive cooperative interactions between the FAD binding sites on phosphorylase kinase.

Influence of FAD on the association of phosphorylase kinase under conditions of molecular crowding. We have previously shown that high concentrations of TMAO, imitating the crowding conditions in the cell, induce association of phosphorylase kinase [10]. It was of interest to elucidate whether FAD has any influence on TMAO-induced association of phosphorylase kinase. Figure 4 (a and b) presents the kinetic curves of phosphorylase kinase association in the presence of 1 M TMAO. The kinetic curves were registered by the turbidimetric method. The kinetics of TMAO-induced association of phosphorylase kinase were examined in the absence and in the presence of FAD (12.5-63.0 μM). As is shown in Fig. 4a, FAD prevents the association of phosphorylase kinase. The initial rate of the absorption change at 600 nm, which characterizes the association velocity of the enzyme association, was detected for each kinetic curve. Figure 4b shows that the value of the relative initial rate of phosphorylase kinase association (w/w_0) decreases with the increase in FAD concentration.

Phosphorylase kinase assay in the presence of FAD. Figure 5 presents the incorporation of ^{32}P into glycogen phosphorylase b during the kinase reaction in the absence

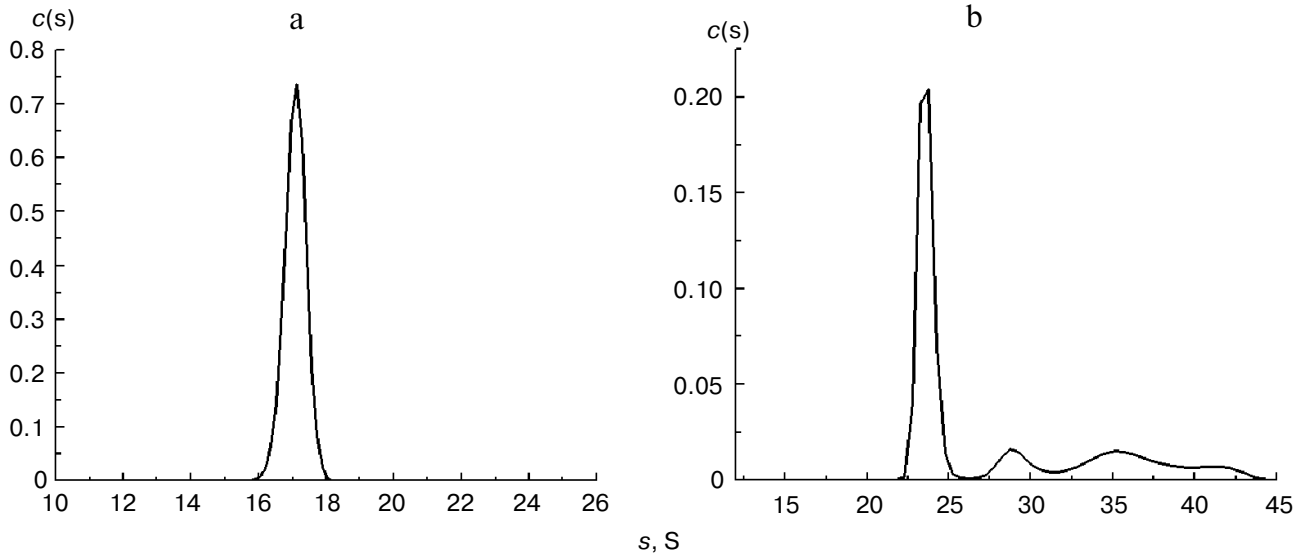

Fig. 2. Differential distribution of the sedimentation coefficient $c(s)$ of phosphorylase kinase (1 mg/ml) in the presence of 9.7 μM FAD (a) and in the absence of FAD (b). Rotor speed is 44,000 (a) and 30,000 rpm (b); wavelength is 280 nm. Conditions: 40 mM HEPES, pH 6.8, 0.1 mM $CaCl_2$, 10 mM $MgCl_2$; 17°C (a), 20°C (b).

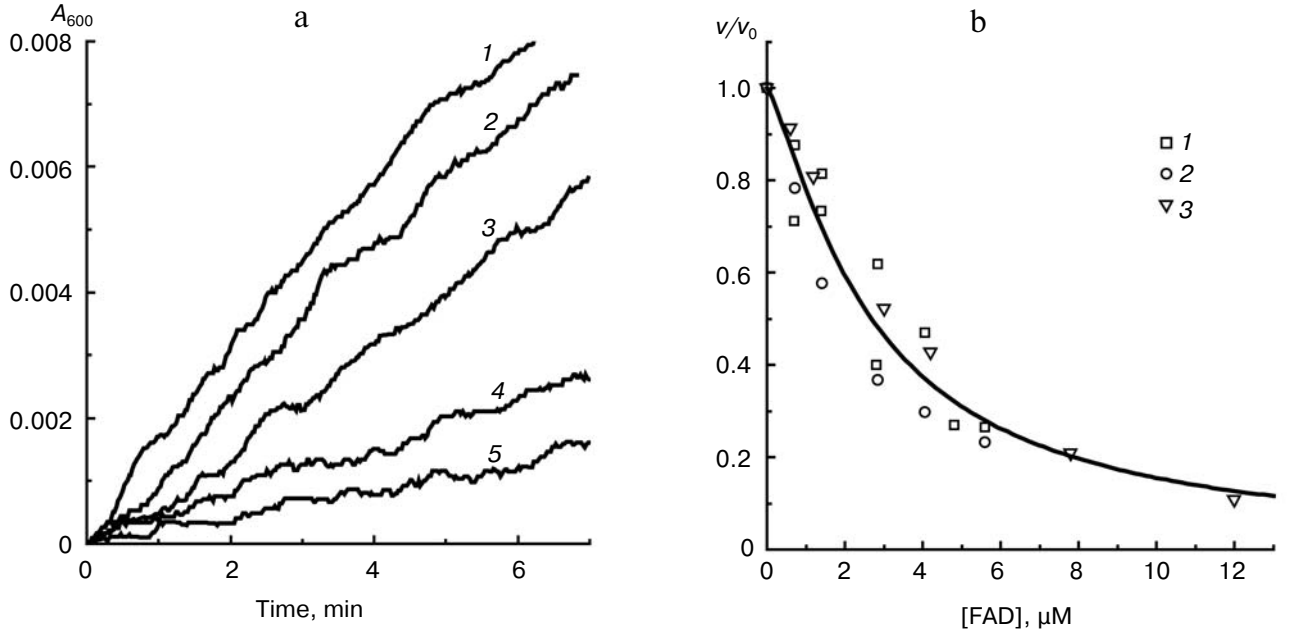

Fig. 3. Influence of FAD on the complex formation of phosphorylase kinase with glycogen in 40 mM HEPES, pH 6.8 (22°C). a) The kinetic curves of the change in the optical absorption at 600 nm (A_{600}), obtained at various concentrations of FAD (μM): 0 (*1*), 1.2 (*2*), 4.2 (*3*), 7.8 (*4*), and 12 (*5*). Complex formation was initiated by addition of Ca^{2+} and Mg^{2+} to the solution containing phosphorylase kinase (50 μg/ml) and glycogen (0.7 mg/ml). The final concentrations of Ca^{2+} and Mg^{2+} were 0.1 and 10 mM, respectively. b) The dependence of the relative initial rate of interaction of phosphorylase kinase with glycogen (v/v_0) on FAD concentration; v_0 and v are the initial rates of the complex formation in the absence and in the presence of FAD. Glycogen concentrations: 0.25 (*1*), 0.7 (*2*), and 1.0 (*3*) mg/ml.

and in the presence of various FAD concentrations (18, 92, 184, and 280 μM). As is seen from the data presented, the kinetics of phosphorylation in the presence of various concentrations of FAD do not differ substantially from those in the absence of FAD. Thus, it is possible to suggest that FAD does not have any influence on the phosphorylase kinase activity relative to its protein substrate, glycogen phosphorylase *b*.

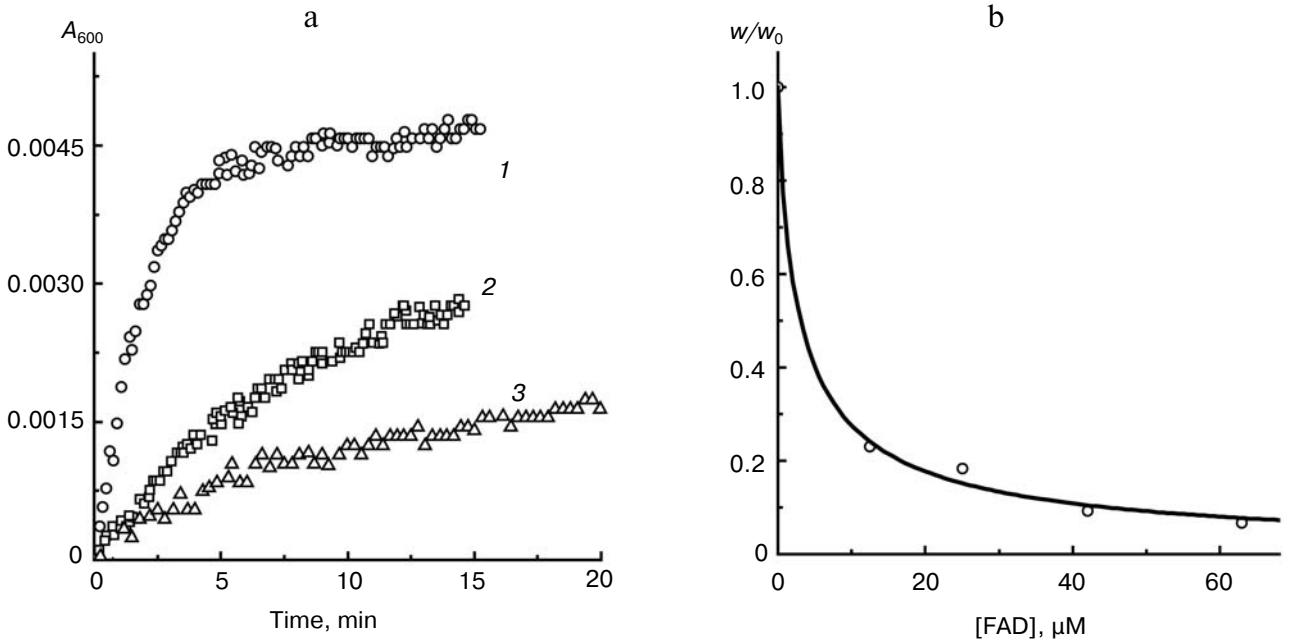

Fig. 4. Influence of FAD on the association of phosphorylase kinase induced by 1 M TMAO in 40 mM HEPES buffer, pH 6.8 (22°C). a) The kinetic curves of the change in the optical density at 600 nm (A_{600}) obtained at various FAD concentrations: 0 (*1*), 12.5 (*2*), and 63 (*3*) μM. Association was initiated by the addition of Ca^{2+} and Mg^{2+} to the solution containing phosphorylase kinase (56 μg/ml), 1 M TMAO, and various concentrations of FAD. The final concentrations of $CaCl_2$ and $MgCl_2$ were 0.1 and 10 mM, respectively. b) Dependence of the relative initial rate of phosphorylase kinase association (w/w_0) on the concentration of FAD; w_0 and w are the initial rates of phosphorylase kinase association in the absence and in the presence of FAD, respectively.

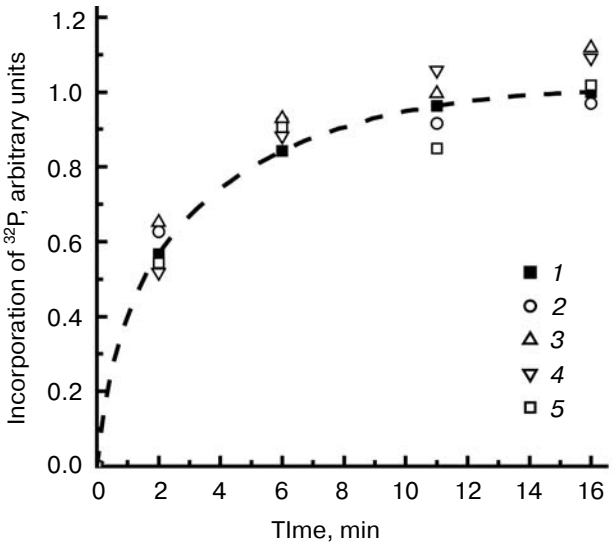

Fig. 5. Incorporation of ^{32}P into glycogen phosphorylase *b* catalyzed by phosphorylase kinase. Phosphorylation in the absence of FAD (*1*); phosphorylation in the presence of FAD (μM): 18 (*2*), 92 (*3*), 184 (*4*), and 280 (*5*). Conditions: 40 mM HEPES buffer, pH 6.8, glycogen phosphorylase *b* (0.4 mg/ml), phosphorylase kinase (0.8 μg/ml), 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, [γ-^{32}P]ATP (50 μM, specific activity 100 cpm/pmol). The reaction was initiated by the addition of ATP. The symbols denote the data obtained in three separate experiments and normalized relative to the maximal level of ^{32}P incorporation in the absence of FAD.

In conclusion, it should be noted that direct evidence of FAD binding with phosphorylase kinase has been obtained by ultracentrifugation. The data indicate that FAD influences the oligomeric state of the enzyme by shifting the equilibrium to the monomeric state, prevents phosphorylase kinase association in the crowding conditions in the presence of TMAO, and inhibits also the formation of phosphorylase kinase complex with glycogen. These data are indicative of specific binding of flavin with phosphorylase kinase. It is possible to suppose that the binding of this ligand occurs at sites spatially separated from the active site of the enzyme, because FAD has no influence on the enzymatic activity of phosphorylase kinase. According to the data on FAD influence on the interaction of phosphorylase kinase with glycogen, the binding of FAD with the enzyme is a cooperative process (Hill coefficient is equal to 1.3). This suggests the existence of positive cooperative interactions between FAD-binding sites in the molecule of phosphorylase kinase.

It is possible that FAD-binding induces a conformational state of phosphorylase kinase that is not capable of interacting with glycogen and of forming the high molecular weight associates in the presence of the osmolyte. The fact that not only glycogen phosphorylase and glycogen synthase but also phosphorylase kinase are capable of binding flavins allows us to propose that the complex of

glycogen metabolism enzymes in protein–glycogen particles may serve as a flavin "depot" in skeletal muscle.

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