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# Interaction of flavin mononucleotide with dimeric and tetrameric forms of muscle phosphorylase β

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**Summary** — Interaction of flavin mononucleotide (FMN) with dimeric and tetrameric forms of rabbit muscle glycogen phosphorylase β has been studied under the conditions when allosteric activator binding sites are saturated by AMP (1 mM AMP; pH 6.8; 17°C). Simultaneous use of schlieren optical system and photoelectric scanning absorption optical system of analytical ultracentrifuge Spinco, model E, makes it possible to register the oligomeric state of the enzyme and calculate the degree of saturation of individual oligomeric enzyme forms by FMN. The apparent association constant for the equilibrium dimer ⇄ tetramer decreased with increasing FMN concentration. The microscopic dissociation constants for the complexes of dimeric and tetrameric forms of glycogen phosphorylase β with FMN have been found to be equal to 10 and 79 μM, respectively.

**glycogen phosphorylase β / dimer ⇄ tetramer equilibrium / binding of flavin mononucleotide**

## Introduction

Under certain conditions allosteric oligomeric enzymes reversibly dissociate to subunits or form supramolecular structures. Allosteric effectors influence the dissociation or association degree of the enzyme oligomer that can trigger a dissociation mechanism of the enzyme activity regulation based on the effector-induced shift of the equilibrium between oligomeric enzyme forms with different catalytic and regulatory properties [1–5]. In general, the effect of allosteric ligand on the catalytic activity of the associating enzyme system is a sum of effects stipulated by the ligand influence on the catalytic activity of individual oligomeric forms and on the enzyme association degree. In order to identify these effects and consequently, to demonstrate the existence of the dissociation mechanism for regulation of the enzyme activity, one needs the experimental methods which control the dynamic equilibrium shift between the oligomeric forms of the enzyme under the ligand action.

The paper deals with an approach that allows detection of an equilibrium position shift between oligomeric forms and calculation of a degree of saturation of individual oligomeric forms with a ligand. The method is based on the parallel application of the schlieren optical system and photoelectric scanning absorption optical system of analytical ultracentrifuge Spinco, model E. The schlieren system registers the enzyme oligomeric state, whereas the absorption system makes it possible to determine the amount of the ligand bound to each oligomeric form.

This approach was used to study the binding of flavin mononucleotide (FMN) of muscle glycogen phosphorylase β (EC 2.4.1.1) presented by interconvertable dimeric and tetrameric forms. Muscle glycogen phosphorylase β fits well our aim to reveal how the association degree of the enzyme can be controlled by an allosteric ligand. According to the X-ray analysis [6, 7] the dimeric molecule of glycogen phosphorylase β (molecular mass 194 800 Da) contains double sets of active sites, activator allosteric sites, and inhibitory allosteric sites. The AMP binding to the activator allosteric site, located near the subunits contact area in the dimer at a distance 3.2 nm from the active site, results in the enzyme conformation capable of association yielding the tetramer [8, 9]. Heterocyclic compounds bound to the inhibitory allosteric site form intercalative complexes with amino acid residues Phe 285 and Tyr 613, which belong to different domains of the enzyme subunit [10]. Among the studied heterocyclic compounds flavins show the highest affinity for the inhibitory allosteric site [11–16]. Flavin binding to the inhibitory allosteric site decreases strength of the AMP binding in the activator allosteric site [11]. Our preliminary results indicate that FMN prevents association by

means of allosteric activator AMP [11]. This study provides detailed information concerning the FMN influence on a position of equilibrium between dimeric and tetrameric forms of muscle glycogen phosphorylase β and characterizes the FMN affinity to each of oligomeric forms upon saturation of the activator allosteric site with AMP.

## Materials and methods

Glycogen phosphorylase β from rabbit skeletal muscles was obtained by the Fischer and Krebs method modified by Lissovskaya [17]. Upon crystallization cysteine was replaced by 0.05 M mercaptoethanol. The 4-fold recrystallized enzyme sample was used within 2 weeks after isolation. AMP was separated from the enzyme solution on a column filled with the activated coal Norit-cellulose powder mixture (ratio 1:1). The absence of AMP in the enzyme preparation was evaluated by the optical absorption ratio at two wavelengths ($A_{260}/A_{280}$ not exceeding 0.53). The enzyme concentration was determined spectrophotometrically at λ = 280 nm, extinction coefficient 1.32 $mg/ml^{-1}$ $cm^{-1}$.

Use was made of AMP purchased from Reanal (Hungary) and FMN (Science-Technical Association Vitamins, Moscow). The FMN concentration was detected spectrophotometrically by using molar extinction coefficient $\varepsilon_{445} = 1.25 \times 10^{-4}$ $M^{-1}$ $cm^{-1}$ [11].

Sedimentation experiments were carried out in analytical ultracentrifuge Spinco, model E, equipped with an absorption optical system, a photoelectric scanner, a monochromator, a multiplexer and an additional X-Y plotter (EMG, Hungary). We used four-channel rotor An-F, Ti and double-sector cells with 12-mm coal centerpiece No 306493 and a counterbalance for the absorption optical system. The rotor speed was 60 000 rpm. Three cells were used in each experiment. The first contained enzyme solution with AMP, the second – enzyme solution with AMP and FMN, the third (control) – FMN solution (concentration in the second and third cells was the same).

The experimental conditions were chosen in such a way (17°C, 1 mM AMP) that both enzyme forms, dimeric and tetrameric, were present on sedimentation patterns. The schlieren optics made it possible to record the enzyme oligomeric forms as two partially overlapping peaks. The schlieren pattern of FMN solution showed a line coinciding with the basal one. An empirical equation of the sum of two Gaussian functions [18] was used to treat the sedimentation curves:

$$y = F_1 \left( \frac{1}{2\pi\sigma_1} e^{(x-\alpha_1)^2/2\sigma_1^2)} \right) + F_2 \left( \frac{1}{2\pi\sigma_2} e^{(x-\alpha_2)^2/2\sigma_2^2)} \right) \quad (1)$$

To obtain the values of parameters $F_1$, $F_2$, $\alpha_1$, $\alpha_2$ ($\alpha_2 > \alpha_1$), $\alpha_1$, $\alpha_2$, the non-linear least square technique based on the Nelder-Mead algorithm was employed [19]. A square correlation coefficient for such an approximation was about 0.99. Fitting was made by an IBM PC program elaborated by us and available on request. The enzyme dimeric form was evaluated by formula: $\gamma = F_1/(F_1 + F_2)$.

At rotor speed 60 000 rpm the pressure achieved approximately 400 atm in a centrifuge cell that could affect the aggregation state of biomacromolecules [20]. To find out how the pressure influenced the equilibrium dimer ⇄ tetramer of phosphorylase β, a series of experiments with mineral oil covering was performed at 17°C (enzyme concentration 6–8 mg/ml). The relationship between peak squares corresponding to the dimeric and tetrameric enzyme forms ($F_1$ and $F_2$) on sedimentation patterns remained unchanged within the limits of experimental error at the rotor speed 30 000 to 60 000 rpm. The results suggested that the pressure did not influence self-association of phosphorylase β.

The absorption optical system and the photoelectric scanner were used to detect sedimentation of FMN-dimer and FMN-tetramer complexes by FMN absorption (λ = 471 nm). It allowed us to calculate concentrations of each ligand-oligomer complex.

## Results and discussion

The schlieren optical system is very helpful in finding the portion of dimeric and tetrameric forms of glycogen phosphorylase β. On a sedimentation pattern they are presented as partially overlapping peaks (17°C). The rapidly moving peak corresponds to the enzyme tetramer (T) and the slowly moving one – to the dimer (D). At enzyme concentration 7.6 mg/ml and AMP concentration 1 mM, glycogen phosphorylase β consists of equal T and D portions (fig 1A). If the dimer portion ($\gamma$) is known, one can easily find the association constant ($K_{ass}$) for equilibrium 2D ⇄ T:

$$K_{ass} = \frac{1-\gamma}{2\gamma^2[E]_0} \quad (2)$$

where $[E]_0$ is the molar concentration of the enzyme per dimer. Under the studied conditions the association constant is $(2.4 \pm 0.4) \times 10^4$ $M^{-1}$ (according to the results of 15 sedimentation experiments at the enzyme concentrations 6.0–11.0 mg/ml).

Addition of allosteric inhibitor FMN leads to the increase of the relative dimer portion. For instance, at FMN concentration 72 μM the dimer portion increases to 0.78 (fig 1B).

To determine the amount of FMN bound to dimeric and tetrameric forms of glycogen phosphorylase β and the concentration of free FMN, we used the absorption optical system and photoelectric scanner (fig 2). The chosen wavelength (471 nm) corresponds to the isobestic point for absorption spectra of free and enzyme-bound FMN [21]. The rapidly moving boundary corresponds to sedimentation of the FMN-tetramer complex, the slowly moving boundary – to sedimentation of the FMN–dimer complex. The plateau height ($\Delta A_1$) formed near the meniscus after precipitation of the enzyme-ligand complex is in line with the optical density of free FMN and the concentration of free ligand [L] can be calculated as follows: $[L] = [L]_0 \Delta A_1/A_0$, where $[L]_0$ is the total concentration of FMN, $A_0$ is the optical absorption of the original FMN solution which is in the third (control) cell. In a similar way the concentration of the ligand bound to the

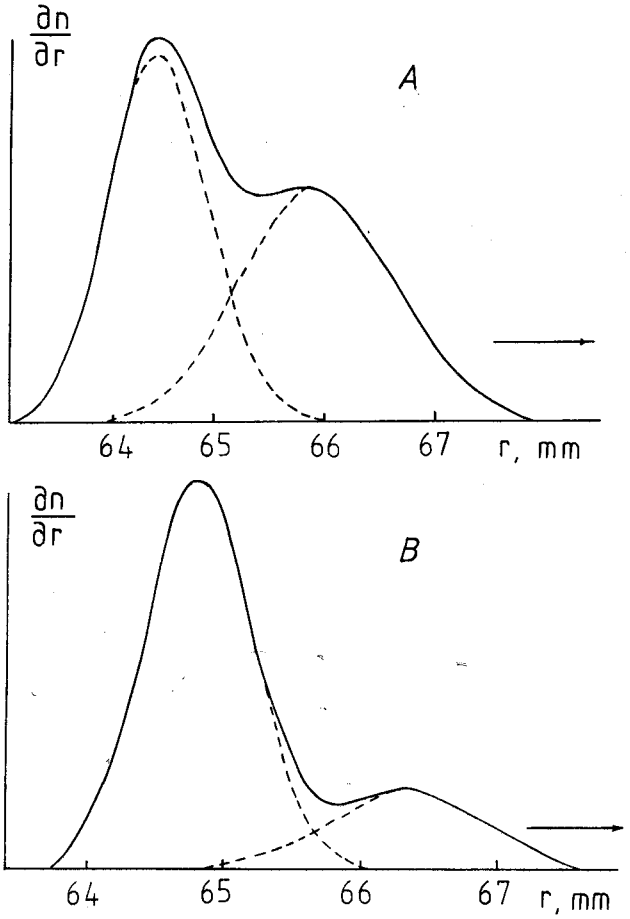


**Fig 1.** Schlieren patterns of glycogen phosphorylase β (7.6 mg/ml) in the absence (**A**) and in the presence of FMN (72 μM, **B**). The rotor speed 60 000 rpm, sedimentation time 30 min. Sedimentation is from left to right. $r$ – radial distance, $\delta n/\delta r$ – the refractive index gradient. 0.05 M glycyl-glycine buffer, pH 6.8, 1 mM AMP, 17°C.

dimeric form of the enzyme can be calculated from the height of the slowly moving boundary ($\Delta A_2$):

$$[L]^{D}_{bound} = [L]_0 \, \Delta A_2/A_0$$

Concentration of FMN bound to the tetramer was calculated as follows:

$$[L]^{T}_{bound} = [L]_0 - [L]^{D}_{bound} - [L],$$

since the extinction coefficient of FMN bound to the tetramer is unknown.

The relative portions of dimeric and tetrameric enzyme forms, concentrations of free FMN and FMN bound to certain oligomeric forms were determined in the experiments where the enzyme concentration varied from 71.3 to 115.8 μM per monomer, and the total concentration of FMN – 17.4 to 82.6 μM. The equation (2) was used to plot the dependence of the apparent constant of the dimer-tetramer association *vs* concentration of free FMN (fig 3). A decrease in the apparent association constant with an increase of FMN concentration testifies that FMN shifts the equilibrium between dimeric and tetrameric enzyme forms to the dimeric form. At concentration of free FMN equal to 29 μM $K_{ass}$ is 0.3 x $10^4$ $M^{-1}$.

The degree of saturation of the dimeric form with FMN was determined as the ratio of concentration of dimer-bound FMN (the value was estimated by means of the absorption optical system) to molar concentration of dimer per monomer (the value was estimated by means of schlieren optical system). Similarly we calculated the degree of saturation of the tetramer with FMN. Figure 4 shows the dependence of the saturation degree ($Y$) of dimer (1) and tetramer (2) with FMN depending on free FMN concentration. The saturation functions were quantitatively treated by taking into account the proposal that ligand-binding sites of each oligomeric form are equivalent and noninteracting. In our papers [11, 14] we showed that this assumption is true for FMN binding to the enzyme dimeric form. In this case the dependence of

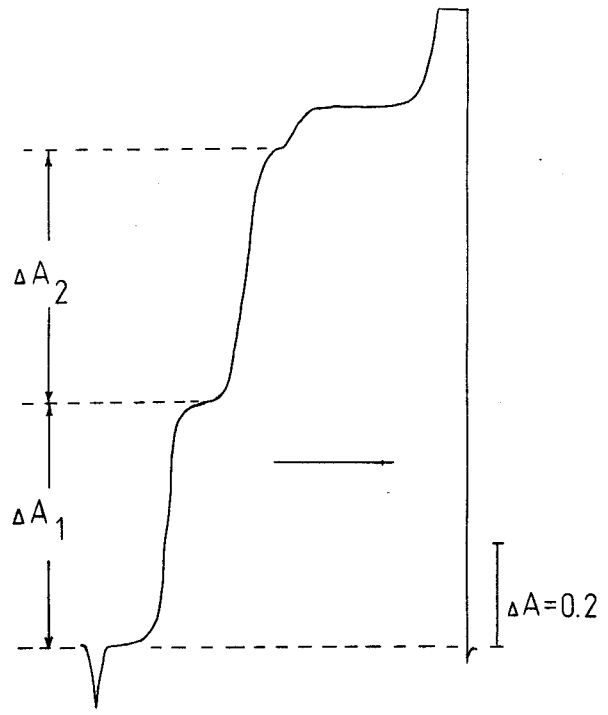


**Fig 2.** Sedimentation of FMN-dimer and FMN-tetramer complexes. Registration with absorption photoelectric scanning system, 471 nm. Conditions as in figure 1.

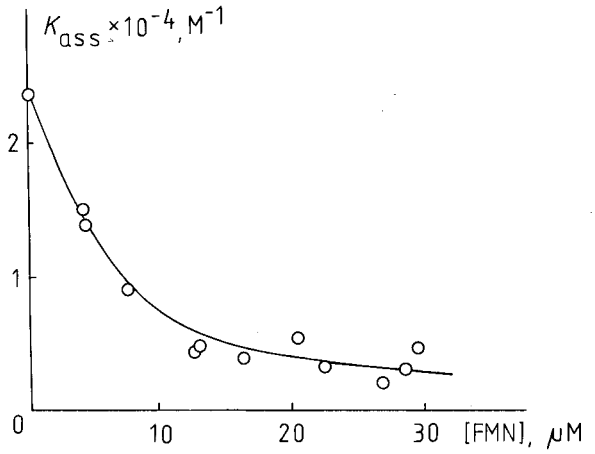


**Fig 3.** Dependence of apparent association constant $K_{ass}$ for the dimer-tetramer equilibrium on concentration of free FMN (in the presence of 1 mM AMP).

saturation for the dimer ($Y_D$) and the tetramer ($Y_T$) with ligand (L) on free ligand concentration has a hyperbolic shape:

$$Y_D = \frac{[L]/K_{DL}}{1 + [L]/K_{DL}} \quad (3)$$

and

$$Y_T = \frac{[L]/K_{TL}}{1 + [L]/K_{TL}} \quad (4)$$

where $K_{DL}$ and $K_{TL}$ are the microscopic dissociation constants for the complexes of dimeric and tetrameric forms of glycogen phosphorylase β and FMN, respectively. The values of $K_{DL}$ and $K_{TL}$ were found by plotting the linear anamorphoses $[L] = K_{DL}Y_D/(1 - Y_D)$ and $[L] = K_{TL}Y_T/(1 - Y_T)$: $K_{DL} = 10.0 \pm 0.5$ μM and $K_{TL} = 79 \pm 8$ μM.

It is noteworthy that the obtained values of microscopic dissociation constants characterize the affinity of the studied allosteric inhibitor to oligomeric forms of the enzyme, the activator allosteric site of which is saturated with allosteric activator AMP. Earlier [11] we have arrived at the conclusion that the formation of the ternary complex of glycogen phosphorylase β with AMP and FMN, where the activator and inhibitory sites are occupied by proper ligands, is rather possible. The conclusion was based on the measurements of the rates of the enzymic reaction catalysed by glycogen phosphorylase β in the presence of both ligands. The microscopic constant of dissociation of the enzyme-FMN complex, where the activator allosteric site is occupied by AMP (75 μM; pH 6.8; 30°C), was found from the dependence of the limiting rate of the enzymic reaction achieved at saturating AMP concentrations (and calculated for ascendant branch of the reaction rate *vs* AMP concentration curve) on the FMN concentration. It should be noted that under conditions of the enzymic activity assay glycogen phosphorylase β is in the dimeric form (because of the enzyme low concentration and the presence of glycogen that prevents the tetramer formation [22]). The microscopic constant of dissociation of the enzyme-FMN complex ($K_{DL}$ = 75 μM) is lower than the corresponding value obtained here by the sedimentation method at slightly lower temperature ($K_{DL}$ = 10 μM at 17°C). This depends not only on the temperature difference in the kinetic and sedimentation experiments but also on the presence of substrates (glycogen and glucose-1-phosphate) in the kinetic experiments (the mentioned substrates decrease the strength of FMN binding to glycogen phosphorylase β [14]).

Nowadays the difference between the active R-state of glycogen phosphorylase β stabilized by an allosteric activator and the inactive T-state induced by allosteric inhibitors is characterized in detail by the X-ray method [23, 24]. Upon the T⇄R transition, *ie*, allosteric transition, each subunit rotates by 5° around an axis perpendicular to the two-fold symmetry axis of the dimeric enzyme molecule. The quaternary structure changes are accompanied with alterations of the tertiary structure of the subunits [23]. A least-square superimposition [25] of the R-state subunit onto the T-state subunit reveals the extent of the tertiary conformational differences between the two states. Those regions that deviate significantly between the two

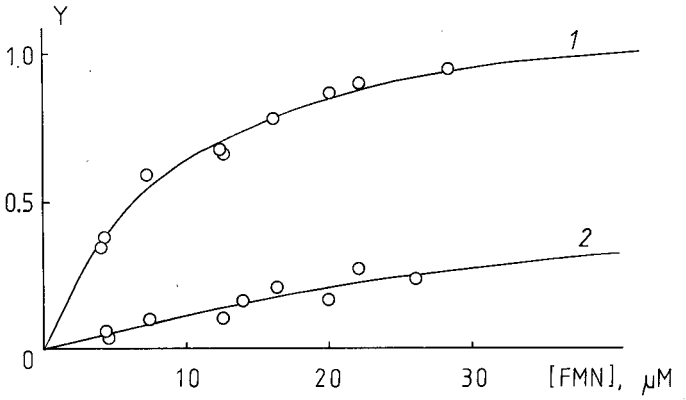


**Fig 4.** Function of saturation of dimeric (1) and tetrameric (2) forms of glycogen phosphorylase β with FMN. Dots – experimental results. Solid lines are calculated from equations (3) and (4).

structures are either important for the allosteric response, the tower helix (residues 262-276), or form a part of the new tetramer interface (helices α12, α13 and α23-α27). At the T⇄R transition the antiparallel association of the two tower helices is disturbed. The tower helices in the R-state protrude from the main body of the enzyme. Movement of the tower helix indirectly leads to a replacement of an aspartate by an arginine residue at the catalytic site, and the opening of tunnel to allow access for substrate [23]. Conformational changes induced by AMP binding in the activatory site favour self-association of phosphorylase β. On the contrary, allosteric inhibitors (flavins, NADH, caffein, ADP, ATP) inducing the T-state show the dissociating effect [14–16, 26, 27, 28]. The less tetramer affinity to FMN is explained by the stabilizing action of intersubunit interaction in the tetramer on the conformation state having the high affinity for the allosteric activator and the low affinity for the allosteric inhibitor. Thus the tendency of phosphorylase β to association may be considered as a test of the enzyme conformational state.

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