CONTROL OF MUSCLE PHOSPHORYLASE PHOSPHATASE BY PHOSPHORYLASE KINASE

G. BOT, Magdolna VARSÁNYI and P. GERGELY
Institute of Medical Chemistry, University of Medicine, Debrecen, Bem J. Place 18/B, Hungary

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1. Introduction

Meyer et al. observed that the properties of the enzymes in the glycogen particles isolated from rabbit skeletal muscle are different from those of the purified enzymes [1]. In glycogen particles the activation and inactivation of phosphorylase (alpha-1,4-glucan: orthophosphate glycosyltransferase EC 2.4.1.1.) occur with a similar rate as in vivo [2,3]. Moreover the dephosphorylation of phosphorylase *a* by phosphorylase phosphatase is inhibited during the activation process and this inhibition appears to be due to an affinity decrease of phosphorylase phosphatase for its substrate, phosphorylase *a*.

Haschke et al. attributed the inhibition to a protein–protein interaction caused by an unknown protein component of the particle [4,5]. In the present paper we provide data which show the inhibition of phosphorylase phosphatase by non-activated phosphorylase kinase.

2. Materials and methods

^{32}P-labeled phosphorylase *a* was prepared from three times crystallized phosphorylase *b* obtained from rabbit skeletal muscle [6] by incubation with phosphorylase kinase, Mg^{2+} and gamma-^{32}P-ATP (obtained from Radiochemical Centre, Amersham) according to the method of Krebs et al. [7]. The labeled phosphorylase *a* was recrystallized and then dissolved in 0.04 M Tris, 0.02 M mercaptoethanol, 0.002 M EDTA buffer, pH 6.8. AMP was removed from phosphorylase *a* by Norit-cellulose treatment reaching a ratio of $A_{260} : A_{280} = 0.54$ [8].

Phosphorylase activity was assayed by the method of Illingworth and Cori [9]. The specific activity of phosphorylase *a* was 59.5 units per mg in the presence of 16 mM glucose-1-phosphate and in the absence of AMP. The specific radioactivity was 1.89×10^5 cpm per mg protein. Protein concentration was determined spectrophotometrically using an absorbance index of $A^{1\%}_{280} = 12.5$ [10].

Non-activated phosphorylase kinase was prepared from rabbit skeletal muscle according to the method of DeLange et al. [11]. The 105 000 *g* precipitate was dissolved in 0.04 M Tris, 0.004 M EDTA, pH 6.8 (20 ml/kg of original muscle), and chromatographed on Sepharose 4B column [12]. The column was equilibrated and the enzyme was eluted with the above buffer. The fractions having the highest kinase activity were collected and concentrated through a membrane filter. The activity of phosphorylase kinase was assayed according to the method of Cohen [12]. The specific activity of kinase after Sepharose 4B chromatography was 6.90 units per mg protein at pH 8.2. The ratio of activity at pH 6.8 to 8.2 was 0.01, therefore phosphorylase kinase could be considered as a non-activated, dephosphorylated one. Moreover the kinase preparation was free of phosphorylase phosphatase.

Phosphorylase phosphatase was prepared from rabbit skeletal muscle. The initial steps were identical to those developed for the purification of phosphorylase kinase [11]. The acid precipitate obtained at pH 6.1 was suspended in 0.04 M Tris, 0.004 M EDTA, pH 8.4 (25 ml/kg of original muscle) and centrifuged at 0°C for 30 min at 10 000 *g*. The supernatant was applied to a Sephadex G-200 column, equilibrated and eluted with the above buffer. First

the fractions containing kinase were eluted and separated from the peak of phosphatase. Fractions having the highest phosphorylase phosphatase activity were pooled and served as purified phosphatase in the kinetic measurements. The specific activity of phosphatase was 7×10^{-5} units per mg, measured according to Hurd et al. [13] and it was free of phosphorylase kinase activity.

3. Results

The assumption, that phosphorylase kinase could be considered as an inhibitor of phosphorylase phosphatase, is supported by the fact that the inhibitory effect increases parallel with the specific activity of kinase (table 1).

The effect of non-activated phosphorylase kinase on the substrate saturation of phosphorylase phosphatase is shown in fig.1.

It is seen that non-activated phosphorylase kinase inhibits the phosphatase reaction and by increasing the kinase concentration the inhibition of phosphatase reaction is enhanced. Further it is concluded that phosphorylase kinase does not influence the V_{max} of phosphatase reaction, the inhibition is competitive in nature.

Table 1
Effect of phosphorylase kinase purification on the inhibition of phosphatase reaction

Purification steps	Specific activity of kinase at pH 8.2 units/mg protein	Kinase needed for 50% inhibition of phosphatase reaction mg protein/ml
pH 6.1 precipitate	0.51	2.01
78 000 *g* supernatant	1.16	1.07
105 000 *g* precipitate	1.73	0.57
Sepharose 4B eluate	6.90	0.13

Purification steps of non-activated phosphorylase kinase are described in Materials and methods. The rate of phosphatase reaction was measured as given in the legend of fig.1.

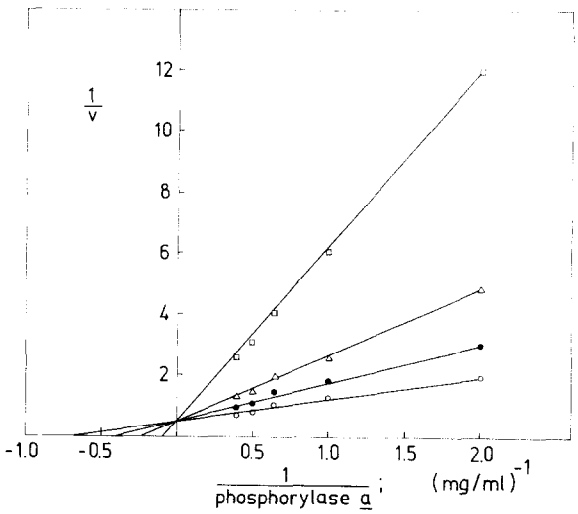

Fig.1. Effect of non-activated phosphorylase kinase on the phosphorylase phosphatase reaction. Lineweaver-Burk plot with respect to [^{32}P]phosphorylase *a* in the absence (○–○–○) and in the presence of 0.09 (●–●–●), 0.135 (△–△–△) and 0.27 (□–□–□) mg per ml kinase. ^{32}P-labeled phosphorylase *a* was incubated with phosphorylase-phosphatase in a buffer containing 0.04 M Tris– 0.02 M mercaptoethanol– 0.002 M EDTA at 30°C, pH 6.8. The concentration of phosphorylase-phosphatase was chosen so that it should not induce more than 50% dephosphorylation of phosphorylase *a* in 10 min. Aliquots (0.1 ml) were removed after 10 min from the incubation mixtures and precipitated with trichloroacetic acid. The precipitate was dissolved in 0.1 N NaOH, reprecipitated and washed once more with trichloroacetic acid, and finally redissolved in 0.25 N NaOH. The radioactivity was measured by counting an aliquot using the Cerenkov-effect of ^{32}P with a Packard Tri-Carb scintillation spectrometer [14,15]. The rate of phosphatase reaction was measured by the release of ^{32}P from labeled phosphorylase *a* (v = cpm·10^{-4} per 10 min/ml).

Fig.2 demonstrates the change in the affinity of phosphorylase phosphatase for its substrate in the presence of kinase.

The K_m of phosphorylase phosphatase for phosphorylase *a* increases to about 9-fold at the highest phosphorylase kinase concentration applied. These results are in good agreement with the observation of Haschke et al., who reported a similar change in the K_m of phosphorylase phosphatase in the suspension of glycogen particles [4]. To determine the inhibitory constant of non-activated phosphorylase kinase on phosphatase reaction the data were plotted according to Dixon.

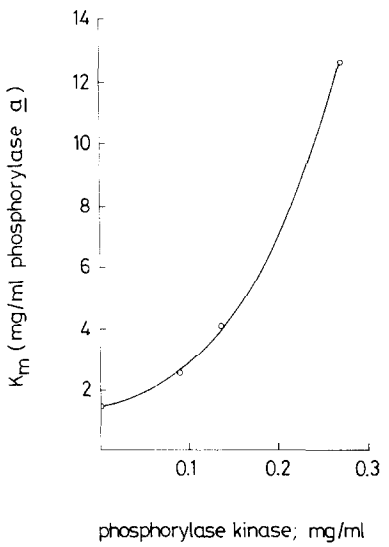

Fig.2. Influence of non-activated phosphorylase kinase on the K_m of phosphorylase phosphatase for phosphorylase *a*. Data were taken from fig.1.

When the reciprocal values of the reaction rate were taken against the phosphorylase kinase concentration the plots showed an upward curvature. When they were plotted against the square of kinase concentration (fig.3), straight lines were obtained and the inhibitory constant could be determined. This value corresponds

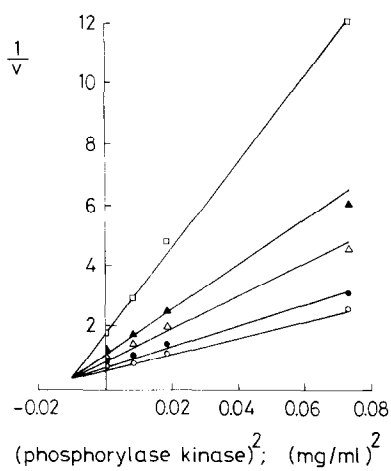

Fig.3. Dixon plot with respect to non-activated phosphorylase kinase of data from fig.1 at 2.5 (○–○–○), 2.0 (●–●–●), 1.5 (△–△–△), 1.0 (▲–▲–▲) and 0.5 (□–□–□) mg per ml phosphorylase *a*.

to 0.11 mg per ml of non-activated phosphorylase kinase (the specific activity of kinase at pH 8.2 was 6.9 units per mg).

4. Discussion

Recently Zieve and Glinsman have demonstrated that activated (phosphorylated) phosphorylase kinase inhibits the dephosphorylation of glycogen synthetase by synthetase-D-phosphatase [16]. The inhibition is competitive and can be explained by the fact that both glycogen synthetase and activated phosphorylase kinase are substrates of synthetase-D-phosphatase [16].

The inhibition of phosphorylase phosphatase by non-activated phosphorylase kinase can not be explained in the same way, because non-activated (non-phosphorylated) phosphorylase kinase is not a substrate of phosphatase. Therefore this inhibition could be considered as a protein–protein interaction. The interaction may exist between phosphorylase kinase and phosphorylase phosphatase. An interaction between kinase and phosphatase was detected by frontal gel filtration in our laboratory [17]. Another interaction might exist between kinase and phosphorylase *a*. In this case the modification of substrate phosphorylase *a* by kinase may cause the inhibition of phosphatase reaction. It would be premature to hypothesize which interaction may play a role in the inhibition of phosphatase.

According to our assumption, phosphorylase kinase has two roles in the glycogen metabolism. On the one hand, phosphorylase kinase converts phosphorylase *b* into *a* starting the mobilization of glycogen. On the other hand, non-activated phosphorylase kinase inhibits phosphorylase phosphatase and in this way it prolongs the glycogen mobilizing effect of phosphorylase *a*.

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