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REVERSIBLE ACTIVATION AND INACTIVATION OF PHOSPHOFRUCTOKINASE FROM *ASCARIS SUUM* BY THE ACTION OF TISSUE-HOMOLOGOUS PROTEIN PHOSPHORYLATING AND DEPHOSPHORYLATING ENZYMES

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In the presence of ATP-Mg^{2+}, purified phosphofructokinase from *Ascaris suum* muscle was effectively phosphorylated and activated *in vitro* by a protein kinase purified from the same tissue. Both effects were reversed by the action of a purified protein phosphatase from the same tissue. The findings suggest the presence of a highly potent interconversion mechanism for phosphofructokinase in the muscle of the parasitic nematode.

Phosphofructokinase has been shown to be present as a phosphoprotein in several tissues [1-3]. A role of reversible phosphorylation in the activity regulation of the enzyme has been postulated [4, 5] in spite of the fact that *in vitro* phosphorylation of the mammalian enzyme by cyclic AMP-dependent protein kinase did not result in a significant change of the catalytic or regulatory properties [6, 7].

In a previous report [3], we demonstrated that phosphofructokinase purified from *Ascaris suum* muscle in the presence or absence of F^- ions contained different amounts of phosphate and exhibited different kinetic properties. *In vitro* phosphorylation of the enzyme by the action of the purified catalytic subunit of cyclic AMP-dependent protein kinase from rabbit muscle increased the activity of the enzyme purified in the absence of F^- ions to the level of phosphofructokinase obtained in the presence of F . A similar *in vitro* effect of mammalian protein kinase was later also described for phosphofructokinase from *Fasciola hepatica* [8].

In this report, we demonstrate that a purified protein kinase from *Ascaris* muscle is considerably more effective in activating the phosphofructokinase from the nematode than the mammalian protein kinase. We also show that phosphorylation and activation are reversed by a protein phosphatase also purified from *Ascaris* muscle.

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MATERIALS AND METHODS

Materials.

Phosphofructokinase from *Ascaris suum*, catalytic subunit of cyclic AMP-dependent protein kinase and [γ-^{32}P]ATP were prepared as described previously [9, 10]. The purification and characterization of a 40 kDa protein kinase to electrophoretic homogoneity was described by Thalhofer [11, 12] and will be published in detail elsewhere. Purification of a Mn^{2+}-activated protein phosphatase was accomplished by ion exchange and affinity chromatography by a modification of the method outlined previously [13]. Protein kinase assays were performed as described in [5]. The protein phosphatase was assayed in 10 mM triethanolamine-HCl (pH 7.6) in the presence of 2 mM Mn^{2+} and 2 µg [^{32}P]phosphate labeled *Ascaris* phosphofructokinase by determining the release of radioactive phosphate in trichloroacetic acid soluble form.

Methods.

Phosphofructokinase assays were performed at pH 7.0 (30 °C) in a Gilford 250-S spectrophotometer at 340 nm. Unless otherwise indicated, 1 ml of the assay mixture contained 50 µmol imidazole - HCl buffer, 4.0 µmol $MgCl_2$, 0.15 µmol NADH, 10 µg aldolase, 1 µg triosephosphate isomerase, 5 µg glycerol 1-phosphate dehydrogenase, 1 µmol ATP, and 1 µmol fructose 6-phosphate. The reactions were initiated by the addition of 0.8 µg phosphofructokinase.

The phosphorylation of phosphofructokinase was performed in the presence of 1 mM labeled ATP (specific radioactivity 9 MBq/µmol), 5.0 mM $MgCl_2$, 2 mM dithioerythritol, and 0.1 M triethanolamine - HCl buffer (pH 7.6). Dephosphorylation was effected in the same buffer in the presence of 2.0 mM Mn^{2+} and 15 mM 2-mercaptoethanol. The phosphorylation reactions were assayed by spotting 40 µl of the reaction mixtures on Whatman 3 M filter paper followed by several washes with 5 % trichloroacetic acid as described previously [14]. The radioactivity released by the dephosphorylation was determined after precipitating the protein with trichloroacetic acid (final concentration 10 %).

Protein determinations were performed according to Bradford [15] using chemicals supplied by BioRad (Munich, Germany). The values obtained using bovine serum albumin as a standard were corrected by a factor of 0.88 to provide the concentrations of phosphofructokinase.

RESULTS

Fig. 1 shows saturation curves for fructose 6-phosphate of phosphofructokinase from *Ascaris* obtained at pH 7.0 after preincubation with or without protein kinases in the presence of ATP and Mg^{2+} ions. The control experiment (Curve A), performed in the absence of a protein kinase, resulted in a sigmoid saturation curve of phosphofructokinase confirming our previous report [16]. A 16 millimolar concentration of fructose 6-phosphate was not sufficient to saturate the enzyme. The addition of albumin did not influence the saturation kinetics. Preincubation of the phosphofructokinase in the presence of purified catalytic subunit of 3',5'-cyclic AMP-dependent protein kinase from beef heart led to a slight increase of the activity (Curve B). In spite of slightly different assay conditions used in the experiments described here, the saturation curves in the control experiment and in the experiment after treatment with the catalytic subunit of the protein kinase show a similar behaviour as that reported previously [3].

A distinctly greater influence on the saturation curve for fructose 6-phosphate was observed when the protein kinase from beef heart in the preincubation was

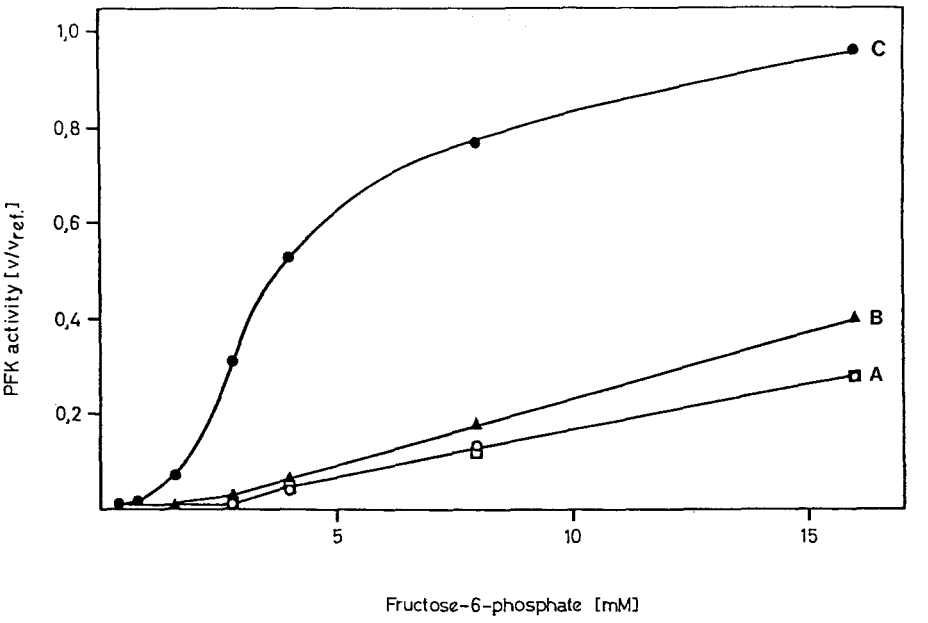

Fig. 1

Saturation curves of phosphofructokinase from *Ascaris* for fructose 6-phosphate. Phosphofructokinase (20 µg/ml) was preincubated for 60 min with 10.0 mM ATP-Mg^{2+} either in the absence (Curve A) or presence of the catalytic subunit of cAMP-dependent protein kinase from beef heart (280 pkatal/ml, Curve B) or a protein kinase purified from *Ascaris* muscle (90 pkatal/ml, Curve C). (The data for Curve A were obtained by incubation in the absence (○) and in the presence (□) of 20 mg albumin/ml).

replaced by a protein kinase purified from *Ascaris* muscle (Curve C). The saturation curve was still sigmoid. The phosphofructokinase activity, however, was increased 18-fold in the presence of 4 mM and 3.5-fold in the presence of 16 mM fructose 6-phosphate.

The time-dependence of the increase of phosphofructokinase activity and of the incorporation of phosphate into the phosphofructokinase protein in the presence of ATP and the protein kinase from *Ascaris* is shown in Fig 2 a. The phosphorlation reaction was biphasic. One phosphate per subunit was incubated within ca. 10 min followed by a much slower phase. Maximum activity of phosphofructokinase was obtained when approximately 1 phosphate per subunit was incorporated into the enzyme. The increase of the catalytic activity was slower than the increase of the phosphate content.

Fig. 2 b represents an experiment in which the phosphorylation of phosphofructokinase by the protein kinase from *Ascaris* was partly reversed by a purified protein phosphatase from *Ascaris* muscle. The decrease in phosphate content in the enzyme was accompanied by a loss of activity. In this experiment the time-dependence of phosphate release and inactivation was almost parallel.

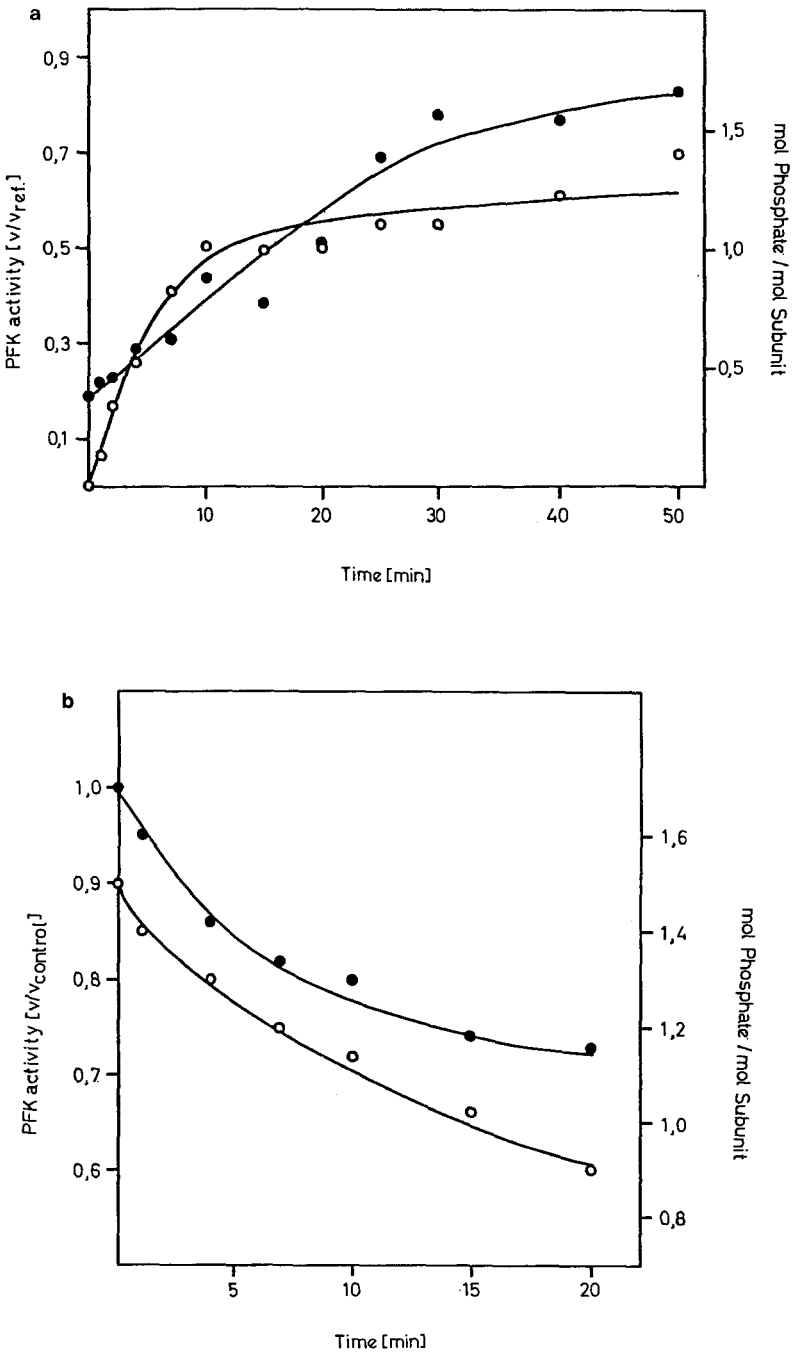

Fig. 2

Phosphofructokinase activity (●) in the presence of fixed concentrations of the substrates (1 mM ATP, 1 mM fructose 6-phosphate, pH 7.0) and phosphate content (○) after incubating 0.16 mg/ml of the enzyme in the presence of 30 pkatal/ml of the protein kinase (Fig. 2a) and 1 mM ATP-Mg^{2+} or 1.6 pkatal/ml of the protein phosphatase (Fig. 2b) from *Ascaris* muscle. In the experiment shown in Fig. 2 b, phosphofructokinase had been previously preincubated in the presence of the protein kinase from *Ascaris* and [γ-^{32}P]ATP for 4 h and then dialyzed overnight. The prolonged preincubation had resulted in the incorporation of 1.5 phosphates per subunit.

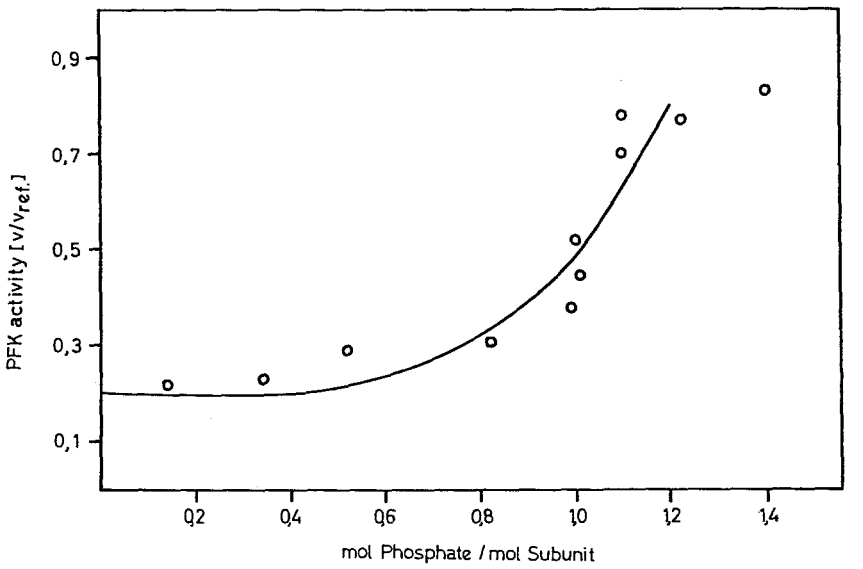

Fig. 3

Correlation between activity and phosphate content in *Ascaris* phosphofructokinase. The data plotted were taken from Fig. 2 a. The line is a fitted theoretical curve showing the moiety of phosphofructokinase tetramers containing 4 phosphates.

In Fig. 3, the phosphate contents of Fig. 2 a were plotted against the respective phosphofructokinase activities. The data points suggest that the stimulation of phosphofructokinase activity is maximal at a level of about 1.1 mol phosphate per mol subunit. The drawn curve represents the stochastic probability of the occurrence of phosphofructokinase tetramers containing 1 mol phosphate per subunit. The line has been fitted assuming the presence of phosphate-free phosphofructokinase at the beginning of the incubation and a non-specific incorporation of 0.2 mol phosphate per mol subunit.

DISCUSSION

The anaerobic degradation of glucose and glycogen provide the sole known source of energy of the parasitic nematode *Ascaris suum* [17]. Hence the regulation of glycolysis is crucial for the adaptation of energy requirements to functional demands. It has been demonstrated [16] that many properties of *Ascaris* phosphofructokinase are similar to the properties observed for the enzyme from higher animals. A significant difference, however, had been detected for the influence of phosphorylation on the regulatory properties of phosphofructokinase from *Ascaris* and mammalian sources [3]. Phosphorylation of purified phosphofructokinase from mammalian tissues by cyclic AMP-dependent protein kinase led to a slight increase of the inhibition of the enzyme by ATP [6, 7] and the significance of the phosphorylation in regulating the activity of the enzyme *in vivo* was questioned.

In contrast, phosphofructokinase from *Ascaris* was activated by treatment with the catalytic subunit of mammalian cyclic AMP-dependent protein kinase [3]. A similar effect of in vitro phosphorylation in the presence of the mammalian catalytic subunit was recently described for phosphofructokinase purified from another helminth, *Fasciola hepatica* [8].

The present study demonstrated that the extent of activation induced in *Ascaris* phosphofructokinase by the mammalian protein kinase was greatly exceeded by phosphorylation with a protein kinase purified from *Ascaris* muscle. Since the protein kinases from both sources catalyze the incorporation of 1 phosphate per subunit of phosphofructokinase [3, 11], the different extent of activation must be based on the phosphorylation of distinct serine residues. This indicates a non-identical substrate specificity of the protein kinase from *Ascaris* and of the cyclic AMP-dependent protein kinases from mammalian skeletal and heart muscle.

The phosphorylation and activation of phosphofructokinase was reversed by a protein phosphatase obtained from *Ascaris* muscle. Therefore, this is the first report describing a complete interconversion system that strongly affects the activity of phosphofructokinase. The molecular mass of the *Ascaris* protein kinase (40 kDa) and the fact that it was inhibited by the heat stable inhibitor protein from rabbit skeletal muscle [11] suggest that the enzyme is related to the catalytic subunit of cyclic AMP-dependent protein kinase from other tissues. Little is known about the regulation of the protein kinase in the parasite except that it is stimulated by increased levels of cyclic AMP induced by the administration of serotonin [18].

The differences in the dependence of the change in phosphofructokinase activity and phosphate content during the phosphorylation reaction may be explained by two assumptions. First, the activation of the enzyme may result from a conformational change induced by phosphorylation. This change may be slower than the phosphorylation reaction and thus limit the rate of activation. Second, the conformational change may be most effectively induced when all subunits of the phosphofructokinase tetramer are phosphorylated. This would introduce cooperativity into the activation of phosphofructokinase by phosphorylation as shown in Fig. 3 and render it a highly sensitive mechanism of regulation of glycolysis.

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