Activation of yeast 6-phosphofructo-2-kinase by protein kinase and phosphate

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6-Phosphofructo-2-kinase (PFK2) is activated by a cAMP-dependent protein kinase, and inactivated by phosphatase, indicating the interconversion of PFK2. Inorganic phosphate also activates PFK2, and the optimum pH for the PFK2 activity varies with the concentration of phosphate. Phosphate also enhances the inactivation of PFK2 by citrate, suggesting that phosphate acts as a regulator of PFK2.

Yeast *6-Phosphofructo-2-kinase* *Protein kinase* *Phosphatase*

1. INTRODUCTION

F26P is synthesized by PFK2 and acts as a powerful activator for PFK1 in rat liver [1,2], yeast [3,4] and plants [5,6]. In yeast, the activity of PFK2 and the concentration of F26P are known to depend on the growth conditions. For example, F26P is not detected in yeast grown on pyruvate, but is detected in yeast grown on glucose [7]. PFK2 activity and the concentration of F26P in yeast grown anaerobically in glucose are higher than in those grown aerobically [8]. The mechanism for this regulation is unknown, although rat liver PFK2 is known to be regulated by cAMP-dependent protein kinase and by some glycolytic metabolites [9–11]. Here, the regulation of yeast PFK2 by cAMP-dependent protein kinase and glycolytic metabolites is reported.

Abbreviations: PFK1, 6-phosphofructo-1-kinase; PFK2, 6-phosphofructo-2-kinase; FBPase1, fructose-1,6-bisphosphatase; FBPase2, fructose-2,6-bisphosphatase; F6P, fructose 6-phosphate; F16P, fructose 1,6-bisphosphate; F26P, fructose 2,6-bisphosphate; PEP, phospho*enol*pyruvate; cAMP, cyclic AMP; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; 2-ME, 2-mercaptoethanol; Mes, 4-morpholineethanesulfonate

2. MATERIALS AND METHODS

F6P, F26P, catalytic subunit of cAMP-dependent protein kinase (bovine heart) and alkaline phosphatase (calf intestinal mucosa) were purchased from Sigma. Other chemicals were purchased from Boehringer Mannheim.

2.1. *Assay for F26P*

F26P was assayed by using rabbit-muscle PFK1 as in [12].

2.2. *Assay for PFK2*

The activity of PFK2 was assayed using the method in [12].

2.3. *Preparation of PFK2*

Baker's yeast was washed 3 times with water, and suspended in buffer A: 50 mM sodium phosphate (pH 6.8), 5 mM 2-ME, 1 mM EDTA, 5 mM $MgCl_2$ and 0.2 mM PMSF. Yeast cells were broken with glass beads. PFK2 was partially purified as in [12].

2.4. *Activation of PFK2 by cAMP-dependent protein kinase*

The reaction mixture (200 μl) was 40 mM Mes (pH 6.2), 1 mM EDTA, 5% glycerol, 10 mM

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$MgCl_2$, 1.2 mM ATP, 10 mM DTT, 0.2 mM PMSF and PFK2 (400 μU). The reaction was initiated by the addition of catalytic subunit of cAMP-dependent protein kinase (500 U). The reaction temperature was kept at 30°C. The aliquots of the reaction mixtures were used for the assay of PFK2 at 5-min intervals.

2.5. *Inactivation of PFK2 by alkaline phosphatase*

The reaction mixture (200 μl) was 500 mM Tris–HCl (pH 8.4), 5 mM phosphate, 0.5 mM $MgCl_2$, 0.2 mM PMSF, 0.5 mM 2-ME, 0.1 mM EDTA and PFK2 (400 μU). The reaction was initiated by the addition of alkaline phosphatase (200 U). The reaction temperature was kept at 30°C. The aliquots of the reaction mixtures were used for the assay of PFK2 at 5-min intervals.

2.6. *CM-Sephadex C-50 chromatography of PFK2*

Intact PFK2 or PFK2 inactivated partially by alkaline phosphatase was dialyzed against buffer A, and applied to a CM-Sephadex C-50 column (1 ml) equilibrated with buffer A. PFK2 was eluted with a stepwise KCl gradient. Each volume was 10 ml. After the concentration of each fraction the activity of PFK2 was determined.

2.7. *Activation of PFK2 by phosphate*

The reaction mixture (100 μl) was 400 mM Tris–HCl (pH 6.5, 7.5, 8.5), 0.5 mM 2-ME, 5 mM F6P, 5 mM ATP, 5 mM $MgCl_2$, 0.5–30 mM phosphate and 200 μU PFK2. The reaction temperature was kept at 30°C.

2.8. *Inactivation of PFK2 by citrate*

The reaction mixture (100 μl) was 400 mM Tris–HCl (pH 7.5), 5–20 mM phosphate, 5 mM ATP, 5 mM F6P, 5 mM $MgCl_2$, 0.5 mM 2-ME, 0–15 mM citrate and 200 μU PFK2. The reaction temperature was kept at 30°C.

3. RESULTS

Upon incubation of PFK2 with the catalytic subunit of cAMP-dependent protein kinase, the activity of PFK2 is enhanced 2-fold as shown in fig.1. Both the control PFK2, being in its original state, and the activated PFK2 are inactivated upon addition of phosphatase. The activity of the inactivated PFK2 was 10- and 20-times lower than the activities of the original PFK2 and the activated PFK2, respectively. These experiments clearly indicate a phosphorylation-dephosphorylation mechanism in the regulation of the activity of this enzyme. In addition, it is suggested that the original PFK activity is composed of a phosphorylated and dephosphorylated form in approximately equal distribution.

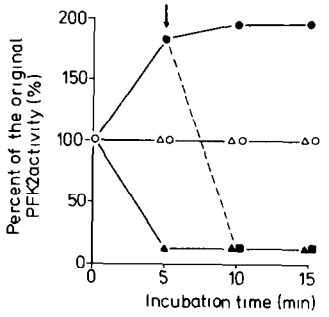

Fig.1. Activation of PFK2 by protein kinase and reinactivation by phosphatase. (○) Boiled catalytic subunit of cAMP-dependent protein kinase; (●) intact catalytic subunit of cAMP-dependent protein kinase; (△) boiled alkaline phosphatase; (▲, ■) intact alkaline phosphatase. The arrow shows the addition of phosphatase (■). Reaction conditions and enzyme activities are described in section 2.

If PFK2 after partial inactivation by phosphatase is applied to a column of CM-Sephadex C-50, and eluted with a stepwise KCl gradient, the inactivated and activated form of the enzyme can be separated as shown in fig.2. The slow elution of the inactivated PFK2 suggests that the dephosphorylation of the enzyme causes a removal of negative charges in terms of phosphate groups from PFK2 enhancing the affinity of the dephosphorylated form for the cation exchange column. Whereas PFK2 in its phosphorylated form is eluted prominently at 100/150 mM KCl, the dephosphorylated form is eluted between 200 and 250 mM KCl. In an analysis of stimulating ligands it was found that inorganic phosphate activates the PFK2 eluted at 100/150 mM KCl, namely the phosphorylated form of the enzyme. This activation is pH-dependent, and is proportional to the concentration of phosphate at pH 7.5. Phosphate has no effect on the activity at pH 8.5. The optimum pH for the activity of PFK2 in its

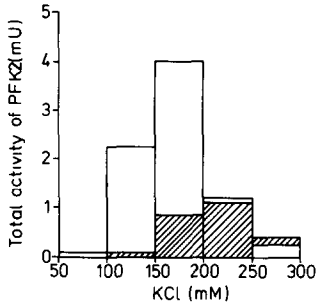

Fig.2. CM-Sephadex chromatography of PFK2 inactivated partially by alkaline phosphatase. The unshaded and shaded bars represent the original PFK2 and the partially inactivated PFK2, respectively. The original PFK2 (8 mU) or partially inactivated PFK2 (3 mU) was applied to the column of CM-Sephadex C-50. The conditions of the chromatography are described in section 2.

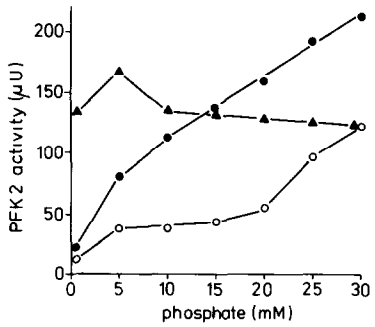

Fig.3. Activation of PFK2 by inorganic phosphate. (○) pH 6.5, (●) pH 7.5, (▲) pH 8.5. The reaction conditions are described in section 2.

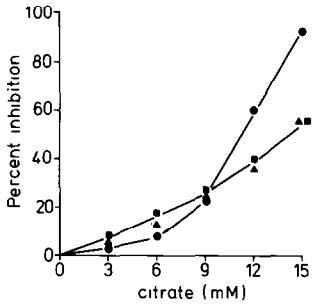

Fig.4. Inactivation of PFK2 by citrate. (▲) 5 mM phosphate, (■) 10 mM phosphate, (●) 20 mM phosphate. The reaction conditions are described in section 2.

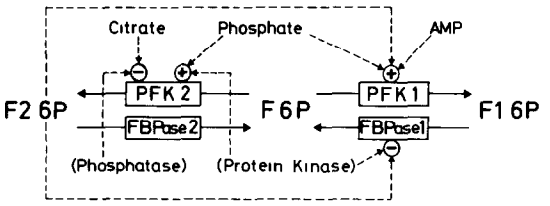

Fig.5. Regulation of PFK1, PFK2 and FBPase1.

phosphorylated form in the presence of 5 mM phosphate is pH 8.4, whereas the optimum pH at levels above 15 mM phosphate is shifted to pH 7.5 (see fig.3). These results show that protons and phosphate regulate the activity of PFK2.

The activity of this enzyme eluted in its phosphorylated form is little effected at pH 7.5 and 20 mM phosphate by the following glycolytic metabolites: 5 mM F16P; 2 mM pyruvate; 1 mM PEP; 5 mM 2-P-glycerate; 2 mM citrate; 2 mM ADP; 0.5 mM AMP; 0.5 mM cAMP. All these concentrations are above the physiological level [13–16]. If the concentration of citrate in yeast is in the range 5–15 mM (see [14]), PFK2 is inactivated by citrate as shown in fig.4. This inactivation by citrate is furthermore enhanced at levels above 10 mM citrate by 20 mM phosphate.

4. DISCUSSION

The activation of PFK2 by cAMP-dependent protein kinase and its inactivation by phosphatase as observed here using a partially purified PFK2 indicate the interconversion of PFK2 by phosphorylation and dephosphorylation. It is known that cAMP-dependent protein kinase activates yeast PFK2 and inactivates yeast FBPase1 [17,18], whereas protein kinase inactivates the rat PFK2 [9–11] and activates rat FBPase1 [19]. Our experiments support this notion by clearly showing that in contrast to the liver enzyme the cAMP-dependent protein kinase activates PFK2. Indeed, the mechanism by which glucose increases the concentration of F26P in *Saccharomyces cerevisiae* has been identified in independent experiments as a cAMP-dependent activation process (Hers et al., personal communication). Thus, cAMP-dependent protein kinase stimulates glycolysis in yeast, but gluconeogenesis in rat liver.

Our experiments show that the partially purified PFK2 consists of both the phosphorylated and dephosphorylated forms. The ratio of both forms in the partially purified PFK2 may depend on the conditions of growth as well as cell extraction procedure. Thus, interaction of the phosphorylated form of PFK2 with phosphatases causes its inactivation and an enzyme with low specific activity. Also, the association of PFK2 with a membrane fraction in yeast has been observed (unpublished). In addition it must be noted that PFK2 must be protected from the action of phosphatase as well as proteinase during the isolation of PFK2.

Although inorganic phosphate is known to activate yeast PFK1 [13], it is shown here that PFK2 is also activated by phosphate in a pH-dependent manner. The maximum activation of both PFK1 and PFK2 is observed at pH 6.4 [13] and pH 7.5, respectively. Phosphate may play an important role in the regulation of both PFK1 and PFK2 in glycolysis. On the other hand, phosphate enhances the inactivation of PFK2 by citrate.

Whereas the concentration of ATP is constant in yeast, the concentration of F6P depends on the states of glycolysis, gluconeogenesis or the Pasteur effect [13–16]. Thus, a change in the concentration of F6P may affect the activity of PFK2 [12]. Our results indicate that a cyclic AMP-dependent protein kinase as well as phosphatase cause the interconversion of PFK2 in yeast and that phosphate, citrate, F6P and protons may be regulators of the enzyme. Fig.5 shows the reciprocal control of the two pairs of enzymes coupling F6P with F16P and F26P, respectively, on the basis of data currently available [3,4,13,17,18]. A possible activation of phosphatase on FBPase2 has not yet been analyzed. Furthermore a phosphorylation mechanism for yeast PFK1 has not been identified with certainty. This figure illustrates the difference of the control of generation of F26P and F16P, respectively, in yeast being opposite to the control mechanism described for liver. Currently no explanation for the different behaviour can be given.

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