Stimulation of Phosphofructokinase from *Phycomyces blakesleeanus* and Some Other Fungi by Micromolar Concentrations of Fructose 2,6-bisphosphate

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The phosphofructokinase in crude extracts of *Phycomyces blakesleeanus* required the presence of ammonium salts, AMP or fructose 2,6-bisphosphate for its activity. The enzyme had slightly sigmoidal kinetics with respect to fructose 6-phosphate as substrate. It was slightly inhibited by high ATP concentrations and by citrate. Fructose 2,6-bisphosphate stimulated the *Phycomyces blakesleeanus* phosphofructokinase; the K_m for fructose 6-phosphate decreased, the inhibition by ATP was completely relieved and the affinity for the activator ammonia was increased. AMP also stimulated the catalytic activity but there was poor co-operation with fructose 2,6-bisphosphate. Preliminary experiments showed that fructose 2,6-bisphosphate also stimulated the phosphofructokinase from *Mucor rouxii*, *Neurospora tetrasperma* and *Agaricus bisporus*.

INTRODUCTION

Dormant sporangiospores of *Phycomyces blakesleeanus* can be induced to germinate by several treatments including heating at 50 °C for a few minutes (Robbins *et al.*, 1942) or by treatment with acetate (Borchert, 1962). The most prominent biochemical feature of germinating spores is a large increase in glycolytic activity. This is manifested by an increased oxygen uptake (Van Laere *et al.*, 1980) and the production of large amounts of glycerol (Furch *et al.*, 1976), pyruvate (Rudolph *et al.*, 1966; Van Laere *et al.*, 1982) and lactate (Furch, 1972) during early germination. An increased glycolytic activity was also found after activation of *Neurospora* ascospores (Eilers *et al.*, 1970).

PFK is believed to regulate the glycolytic flow in a variety of organisms and special attention has recently been focused on the effects of Fru-2,6-P₂ on this enzyme (Van Schaftingen *et al.*, 1980). Fru-2,6-P₂, in less than micromolar concentrations, activates PFK from several mammalian sources (Hers & Van Schaftingen, 1982), *Saccharomyces cerevisiae* and two *Rhodotorula* species (Bartrons *et al.*, 1982) as well as the PFK from *Ricinus communis* plastids (Miernyk & Dennis, 1982). Fru-2,6-P₂ has no effect on bacterial PFKs (H. G. Hers, personal communication). In plants it stimulates inorganic pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase (Sabularse & Anderson, 1981); this enzyme may contribute significantly to glycolytic flow in green tissues of several plants (Carnal & Black, 1983).

This paper describes an investigation of the effects of Fru-2,6-P₂ on the PFK of *Phycomyces blakesleeanus* as part of an attempt to gain a better understanding of the regulation of glycolysis in *Phycomyces* spores.

METHODS

Organisms. Experiments were done with sporangiospores of *Phycomyces blakesleeanus* Burgeff (Strain K) and *Mucor rouxii* (CBS 416.77), with ascospores of *Neurospora tetrasperma* (CBS 377.74) or commercially available *Agaricus bisporus* caps.

Abbreviations: Fru-2,6-P₂, fructose 2,6-bisphosphate; PFK, ATP:D-fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.11).

Preparation of extracts. The spores (50 mg dry wt) or caps were homogenized (Van Laere *et al.*, 1976) in 1.5 ml 50 mM-HEPES/KOH, pH 7.5, containing 5 mM-mercaptoethanol. After homogenization, the extract was diluted to 2.5 ml and centrifuged for 15 min at 58000 *g* (3 °C). The clear supernatant was used immediately to assay enzyme activity.

Assay of PFK activity. Enzyme activity was monitored spectrophotometrically at 25 °C by measuring the disappearance of NADH. The reaction mixture contained, except when stated otherwise, 25 mM-HEPES, 12.5 mM-KOH, 2.5 mM-mercaptoethanol, 12.5 mM-(NH₄)₂SO₄, 5 mM-MgSO₄, 1 mM-ATP, 2 mM-fructose 6-phosphate, 0.2 mM-NADH, 0.18 units aldolase, 0.6 units glycerolphosphate dehydrogenase and 2 units triosephosphate isomerase (all from Boehringer). The final pH was 7.5. Since the extract contained large amounts of phosphoglucose-isomerase activity, the fructose 6-phosphate was added as an equilibrium mixture with glucose 6-phosphate (ratio 1:3). Control samples were measured without fructose 6-phosphate; these yielded low values which were subtracted from the reaction velocities. Tests in the absence of ammonium salts were done with coupling enzymes which had been desalted by passing through Sephadex G-10 equilibrated with extraction buffer. It was ascertained that under all conditions assayed the reaction velocity was not limited by the activity of the coupling enzymes. Although a crude extract was used, the 2500-fold dilution used during the assay should have diluted the concentration of endogenous components that might affect the assay to negligible levels. Some variation in specific activity occurred among different extracts and so the results presented in each figure were obtained with the same extract. All experiments were repeated and gave consistent results.

Fru-2,6-P₂ was a gift from Professor H. G. Hers.

RESULTS AND DISCUSSION

Ammonium ions stimulated PFK activity considerably (e.g. Fig. 3), so most experiments were done in the presence or absence of 12.5 mM-(NH₄)₂SO₄. *Phycomyces blakesleeanus* PFK has a pH optimum between pH 7.5 and 8.5 in HEPES buffer. Activity dropped sharply on the acid side and was only some 10% of its maximum at pH 6. This pH optimum differs markedly from that reported for PFK from yeast cells of the related fungus *Mucor rouxii* (6.4 in phosphate buffer; Paveto & Passeron, 1977). Although the use of phosphate buffer could affect the pH optimum observed (Banuelos *et al.*, 1977), the pH optimum of PFK from *P. blakesleeanus* spores was not markedly shifted by using phosphate instead of HEPES buffer. The pH activity curve was not shifted in the presence of ammonium sulphate. Since near optimal activity was obtained at pH 7.5 and since this pH is likely to reflect the physiological pH, all experiments were done at pH 7.5.

In the absence of ammonium ions and Fru-2,6-P₂ hardly any PFK activity could be detected in the spore extracts. Under these conditions 5 mM-AMP greatly stimulated PFK activity, mainly by a fivefold increase in $V_{\max}$. The dependence on fructose 6-phosphate concentration was slightly sigmoidal (Hill coefficient, 1.7). Fru-2,6-P₂ was found to be a much more potent stimulator than AMP. Its presence increased $V_{\max}$ 23-fold and lowered the K_m for fructose 6-phosphate (from 0.25 mM with AMP to 0.1 mM with Fru-2,6-P₂), resulting in less hyperbolic kinetics (Fig. 1*a*). Addition of AMP had no further stimulatory effect in the presence of 1 μ M-Fru-2,6-P₂.

In the presence of 12.5 mM-(NH₄)₂SO₄ considerable PFK activity was found (more than tenfold stimulation) without addition of any activator (Fig. 1*b*). The dependence on fructose 6-phosphate concentration remained clearly sigmoidal (Hill coefficient, 1.7). Addition of AMP (5 mM) or Fru-2,6-P₂ (1 μ M) increased $V_{\max}$ (about twofold) and decreased the K_m (from 0.5 mM to 0.27 mM with AMP and to 0.15 mM with Fru-2,6-P₂).

The effect of ATP concentrations on PFK activity is shown in Fig. 1. In the absence of ammonium ions (but in the presence of AMP to obtain significant activity) an ATP concentration of 0.2 mM was optimal, although higher concentrations were only slightly inhibitory (Fig. 1*c*). In the presence of NH₄⁺ ions (Fig. 1*d*) a broader optimum was obtained: about 0.5 mM-ATP in the absence and about 2 mM in the presence of AMP. The presence of 1 μ M-Fru-2,6-P₂, however, removed all inhibitory effects of ATP and yielded a nearly hyperbolic concentration dependence.

AMP stimulated the enzyme not only by a decrease in the K_m for fructose 6-phosphate but also by an increase in the $V_{\max}$. Depending on the fructose 6-phosphate concentration, optimum

Fig. 1. Effect of fructose 6-phosphate concentration on PFK activity in the absence (a) or presence (b) of 12.5 mM- $(\text{NH}_4)_2\text{SO}_4$ (ATP, 1 mM; Mg^{2+} , 5 mM) and the effect of ATP concentration on PFK activity in the absence (c) or presence (d) of 12.5 mM- $(\text{NH}_4)_2\text{SO}_4$ (fructose 6-phosphate 2 mM; Mg^{2+} , 5 mM). ●, Control; ■, + 5 mM-AMP; ▲, + 1 μM -Fru-2,6- P_2 ; ▼, + 5 mM-AMP + 1 μM -Fru-2,6- P_2 .

stimulation by AMP was obtained at 2 to 5 mM. However, AMP had no effect in the simultaneous presence of 1 μM -Fru-2,6- P_2 .

Citrate, an inhibitor of several PFKs (Bloxham & Lardy, 1973), was only slightly inhibitory (30% inhibition at 10 mM). Fru-2,6- P_2 relieved this inhibition.

The most clearcut stimulation of PFK by Fru-2,6- P_2 was obtained in the absence of ammonium salts (Fig. 2). In this case activity was nearly dependent on the presence of Fru-2,6- P_2 (or AMP). In the presence of AMP the stimulation by Fru-2,6- P_2 was less important. AMP slightly increased the affinity of the enzyme for Fru-2,6- P_2 . Similar though less pronounced stimulations were obtained in the presence of ammonium ions (results not shown). As in liver (Van Schaftingen *et al.*, 1981) and yeast (Bartrons *et al.*, 1982) Fru-2,6- P_2 decreased the K_m for fructose 6-phosphate. As in yeast, but unlike liver, $V_{\max}$ was also affected. Poor synergism of the Fru-2,6- P_2 response with AMP was found compared with the yeast and liver enzymes; the inhibition by high ATP concentration was completely removed by Fru-2,6- P_2 with the *Phycomyces* enzyme, resulting in hyperbolic kinetics towards ATP concentration.

Enzyme activity was also dependent on the presence of Mg^{2+} salts. Optimal activity with 1 mM-ATP was obtained at 1 mM- Mg^{2+} . Higher concentrations inhibited to a variable degree depending on the conditions of the assay.

The activity of *Phycomyces* PFK was stimulated by the presence of ammonium salts (Fig. 3). Na_2SO_4 up to 20 mM had little effect on PFK activity. In the absence of other effectors, saturation was only obtained at ammonium concentrations above 40 mM. In the presence of Fru-2,6- P_2 the dependence on ammonium ions was not so absolute but a substantial stimulation was still obtained. Moreover, AMP, and especially Fru-2,6- P_2 , increased the sensitivity of ammonium salts, since near maximal stimulation was obtained at 10 to 20 mM (with 1 mM-AMP) or 2 to 4 mM (with 1 μM -Fru-2,6- P_2). The PFK from *Dictyostelium discoideum* was also dependent on the presence of ammonium ions (Baumann & Wright, 1968). Several other PFKs, especially the yeast enzyme, are activated by ammonium (Mavis & Stellwagen, 1970). In yeast, however, the effect of ammonium can be mimicked by K^+ , but this is not true for the *Phycomyces* enzyme and only partly for the *Dictyostelium* enzyme (Baumann & Wright, 1968). All our

Fig. 2

Fig. 2. Effect of Fru-2,6-P₂ on PFK (ATP, 1 mM; Mg²⁺, 5 mM; fructose 6-phosphate, 1 mM). ●, Control; ▲, + 0.2 mM-AMP; ▼, + 1 mM-AMP.

Fig. 3

Fig. 3. Effect of (NH₄)₂SO₄ concentration on PFK activity (ATP, 1 mM; fructose 6-phosphate, 2 mM; Mg²⁺, 5 mM). ●, Control; ■, + 1 mM-AMP; ▲, + 1 μM-Fru-2,6-P₂; ▼, + 1 mM-AMP + 1 μM-Fru-2,6-P₂.

experiments were done in 25 mM-K⁺/HEPES with a K⁺ concentration above 10 mM. Moreover, addition of supplementary K⁺ did not stimulate enzyme activity.

Preliminary experiments indicated that the PFKs from *Mucor rouxii* (Zygomycetes), *Neurospora tetrasperma* (Ascomycetes) and *Agaricus bisporus* (Basidiomycetes) were activated by Fru-2,6-P₂ in micromolar concentrations. The PFK from *Mucor* was most sensitive, whereas stimulation in *Agaricus* was less pronounced and occurred at slightly higher concentrations.

Conclusion

The *in vivo* activity of *Phycomyces blakesleeanus* PFK is probably regulated by a combination of Fru-2,6-P₂, ammonium ions and the concentration of fructose 6-phosphate. Citrate and ATP are poor effectors, certainly in the presence of Fru-2,6-P₂. Regulation by AMP is probably not involved *in vivo* since high concentrations are needed and the AMP effect is overshadowed by the effector Fru-2,6-P₂. The increased glycolytic activity of *Phycomyces* spores during early germination could be due to an increased availability of substrate, to increases in the concentration of Fru-2,6-P₂ or ammonia. Although preliminary experiments confirm the presence of Fru-2,6-P₂ in *Phycomyces* spores (E. Van Schaftingen & A. Van Laere, unpublished), its role in the germination of *Phycomyces* spores remains to be investigated. The effect of Fru-2,6-P₂ in extremely low concentrations on the PFK of both yeast (Bartrons *et al.*, 1982) and mycelial metabolism.

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