Regulation of Phosphofructokinase from *Aspergillus niger*: Effect of Fructose 2,6-Bisphosphate on the Action of Citrate, Ammonium Ions and AMP

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(Received 2 September 1986; revised 28 November 1986)

The role of fructose 2,6-bisphosphate in the regulation of glycolysis in the citric acid accumulating fungus *Aspergillus niger* was investigated. Fructose 2,6-bisphosphate stimulated the activity of partially purified phosphofructokinase by increasing the affinity of the enzyme for fructose 6-phosphate and relieving inhibition by ATP. Fructose 2,6-bisphosphate acted synergistically with AMP, but not with NH_4^+ ions, which otherwise also activate phosphofructokinase. Fructose 2,6-bisphosphate also partially antagonized citrate inhibition of phosphofructokinase; complete deinhibition against high (5 mM) concentrations of citrate (as occur during citric acid accumulation), however, required the simultaneous presence of fructose 2,6-bisphosphate (0.1 μM), AMP (0.1 mM) and NH_4^+ ions (20 mM).

INTRODUCTION

The discovery that Fru 2,6- P_2 (fructose 2,6-bisphosphate) is an activator of PFK in liver cells opened new insights into the mechanism of glycolytic regulation (Van Schaftingen *et al.*, 1980*a, b*). The formation of Fru 2,6- P_2 is triggered in response to glucose supply; it is active at nanomolar concentrations and it stimulates PFK [ATP:D-fructose-6-phosphate 1-phosphotransferase ('phosphofructokinase'; EC 2.7.1.11) by shifting its $[S]_{0.5}$ value for Fru 6-P (fructose 6-phosphate) to lower, physiological concentrations and abolishing inhibition by ATP (Van Schaftingen *et al.*, 1981). Since most other modifiers of PFK (citrate, AMP) act by affecting ATP binding (Ogawa & Atkinson, 1985), Fru 2,6- P_2 occupies a key position in the regulation of this enzyme.

We are investigating the regulation of glycolysis in the fungus *Aspergillus niger* in relation to its ability to accumulate high concentrations of citric acid, and have shown that PFK is a major control point (Habison *et al.*, 1979; 1983; Meixner-Monori *et al.*, 1984; Schrefel *et al.*, 1986). Several lines of evidence have led to the assumption that citrate accumulates in this fungus because the feedback control of PFK by citrate is relieved by high intracellular concentrations of NH_4^+ ions (Röhr & Kubicek, 1981; Kubicek & Röhr, 1986). The influence of Fru 2,6- P_2 , however, has so far not been investigated.

Effects of Fru 2,6- P_2 that are similar, but not identical, to those that occur in higher eukaryotic systems have been demonstrated with purified PFK from *Saccharomyces cerevisiae* (Bartrons *et al.*, 1982) and with crude extracts from *Phycomyces blakesleeana* (Van Laere, 1983). In the present paper we report studies on the effect of Fru 2,6- P_2 on partially purified PFK from *A. niger*.

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Abbreviations: Fru 6-P, fructose 6-phosphate; Fru 2,6- P_2 , fructose 2,6-bisphosphate; PFK, ATP:D-fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.11).

METHODS

Organism and conditions for growth. *Aspergillus niger* B60, a citric acid producing strain, was used throughout these studies. Its maintenance, as well as conditions for growth under citric acid producing and non-producing conditions, were described by Kubicek & Röhr (1978) and Habison *et al.* (1983).

Preparation of cell-free extracts and partial purification of PFK. This was done by following exactly procedures described by Habison *et al.* (1983). The final preparation had a specific activity of $6 (\pm 0.5)$ U (mg protein)⁻¹ and was essentially free of several enzymes capable of reacting with the substrates or potential effectors of PFK. The preparation was used for kinetic studies within 72 h, during which time it did not lose more than 25 (± 7)% of its activity (4 °C). It was shown in separate experiments that the partial loss in activity did not lead to a change in the regulatory properties.

Assay of PFK activity and kinetic investigations. PFK activity was measured spectrophotometrically by coupling with aldolase, triose phosphate isomerase and glycerol-3-phosphate dehydrogenase, and measuring the decrease in NADH at 340 nm. [One unit (U) is defined as the decrease of 2 μ mol NADH per min under the conditions of the assay.] Unless stated otherwise, the conditions reported by Habison *et al.* (1983) were used. Mg²⁺ ions were always maintained at twice the ATP concentration. Kinetic constants were determined as reported by Habison *et al.* (1983).

Chemicals. Fru 2,6-P₂ was obtained from Calbiochem; Dextran-Blue Sepharose 4B was from Pharmacia. Enzymes and substrates were from Boehringer. All other reagents were of analytical grade.

RESULTS

The effect of Fru 2,6-P₂ on the saturation curve of *A. niger* PFK for its two substrates, Fru 6-P and ATP, is shown in Fig. 1(a, b). It appears that Fru 2,6-P₂ at less than 1.0 μ M increased the affinity of PFK for Fru 6-P and decreased inhibition by ATP. In contrast to reports on *S. cerevisiae* and *P. blakesleeana* PFK, but in agreement with reports on the enzyme from several mammalian tissues (Bartrons *et al.*, 1982; Van Laere, 1983; Van Schaftingen *et al.*, 1981; Heylen *et al.*, 1982), the presence of Fru 2,6-P₂ did not increase $V_{\max}$. With Fru 6-P concentrations between 0.47 and 1 mM half maximum stimulation was obtained at 0.1–0.2 μ M-Fru 2,6-P₂ (Fig. 1c). This value is similar to that reported for the enzyme from most other sources, but significantly lower than that for *S. cerevisiae* PFK.

We reported recently that the affinity of *A. niger* PFK for its substrate Fru 6-P can be modulated by AMP, NH₄⁺ ions and citrate (Habison *et al.*, 1983), as is the case for PFK from several other eukaryotic tissues and cells (Uyeda, 1979). We have now investigated the effect of Fru 2,6-P₂ on the action of these effectors of *A. niger* PFK (Figs 2 and 3). Again, in contrast to the reports on *S. cerevisiae* and *P. blakesleeana* PFK but in agreement with reports on the mammalian enzyme (Bartrons *et al.*, 1982; Van Laere, 1983; Heylen *et al.*, 1982; Hers & Van Schaftingen, 1982), clear synergism was seen with AMP and Fru 2,6-P₂ (Fig. 2c). Most importantly, this synergism was seen at AMP concentrations of about 0.1 mM, which actually occur *in vivo* in *A. niger* (Habison *et al.*, 1983). Synergistic modulation of PFK affinity for Fru 6-P by AMP and Fru 2,6-P₂ is thus probably of physiological importance. In contrast NH₄⁺ ions, which also increase PFK affinity toward Fru 6-P, exhibited no synergism with Fru 2,6-P₂ (Fig. 2c).

Citrate is a strong inhibitor of *A. niger* PFK, even at concentrations as low as 0.1 mM (Habison *et al.*, 1983); it acts by decreasing the affinity of PFK for Fru 6-P. At 5 mM-citrate, [S]_{0.5} for Fru 6-P was 12 mM, indicating complete inhibition at physiological Fru 6-P concentrations (0.1–0.2 mM; Habison *et al.*, 1983). Fru 2,6-P₂ and AMP were both able to considerably relieve PFK from this inhibition (Fig. 3a); however, even when AMP and Fru 2,6-P₂ were present simultaneously, [S]_{0.5} for Fru 6-P was 0.5 mM, indicating considerable inhibition under physiological conditions.

Citrate inhibition of *A. niger* PFK is counteracted by high concentrations of NH₄⁺ ions (Habison *et al.*, 1983); such concentrations are found in mycelia of *A. niger* under conditions of citric acid fermentation (cf. Röhr & Kubicek, 1981). We have now shown that the simultaneous presence of high concentrations of NH₄⁺ ions, Fru 2,6-P₂ and AMP completely abolishes the effect of citrate (Fig. 3b) when compared to the control in the absence of citrate (cf. Fig. 2b). Under these conditions, the [S]_{0.5} value for Fru 6-P was 0.1 mM, which closely resembles the Fru 6-P concentration in *A. niger* mycelia *in vivo* (Habison *et al.*, 1983).

Fig. 1. (a) Effect of Fru 6-P concentration on PFK activity in the absence (■) or presence of 0.2 μM (▲) or 0.4 μM (△) Fru 2,6-P₂; (b) effect of ATP concentration on PFK activity in the absence (■) or presence (□) of 0.2 μM-Fru 2,6-P₂; (c) effect of Fru 2,6-P₂ concentration on PFK activity in the presence of 0.47 mM (△) or 1 mM (▲) Fru 6-P. All results shown were obtained from a single experiment but repetition yielded consistent results. The concentrations of ATP and Fru 6-P were 0.1 mM and 5 mM, respectively, unless otherwise indicated.

Fig. 2

Fig. 3

Fig. 2. Effect of Fru 6-P concentration on PFK activity as influenced by various effectors in the absence (a) or presence (b) of 20 mM-NH₄⁺: ○, control; ●, control plus 0.1 mM-AMP; △, control plus 0.2 μM Fru 2,6-P₂; ▲, control plus 0.1 mM-AMP and 0.2 μM-Fru 2,6-P₂. ATP concentration in all experiments was 0.1 mM. All results shown were taken from a single experiment, but repetition yielded consistent results.

Fig. 3. Effect of Fru 6-P concentration on citrate-inhibited PFK activity as influenced by various effectors in the absence (a) or presence (b) of 20 mM-NH₄⁺. Symbols as in Fig. 2. All conditions were as described in Fig. 2, except that all assays were done in the presence of 5 mM-citrate.

DISCUSSION

The effects reported here of Fru 2,6-P₂ on *A. niger* PFK are very similar to the effects observed with PFK from human erythrocytes, liver or muscle (Van Schaftingen *et al.*, 1981; Uyeda *et al.*, 1981; Heylen *et al.*, 1982), but are at some variance with reports on the PFKs from *S. cerevisiae* (Bartrons *et al.*, 1982) and *P. blakesleeanus* (Van Laere, 1983). This agrees with our previous assumption that PFK from *A. niger* is 'phylogenetically located between the yeast and the mammalian enzymes' (Habison *et al.*, 1983). Differences between our results and those for *P. blakesleeanus* (Van Laere, 1983), the only other filamentous fungus so far investigated, could indicate further differences between lower and higher fungi; it must, however, be emphasized that Van Laere (1983) reported results obtained exclusively with crude cell-free extracts.

Our interest in the regulation of PFK from *A. niger* is related to the ability of this fungus to accumulate and secrete high concentrations of citrate (Röhr & Kubicek, 1981). We have previously postulated that the ability of the fungus to maintain a high glycolytic hexose metabolism while accumulating intracellular concentrations of citrate of 4 mM depends on the intracellular accumulation of NH₄⁺ ions, which are derived from increased proteolysis, and can antagonize citrate inhibition of PFK (Röhr & Kubicek, 1981). In the light of our present results we are now able to support and detail this model: by looking at the effect of metabolites on the [S]_{0.5} value of PFK for Fru 6-P, we can clearly state that the simultaneous presence of Fru 2,6-P₂, AMP and NH₄⁺ is required to completely abolish the effect of citrate at concentrations resembling those found in *A. niger* mycelia during citric acid accumulation. This is not necessarily a contradiction of our previous postulation on the role on NH₄⁺ ions in relation to the requirement for manganese-deficient cultivation conditions to enable *A. niger* to accumulate citrate, since little difference has so far been detected in the mycelial contents of Fru 2,6-P₂ and AMP under manganese-deficient and -sufficient conditions (unpublished results). The requirement for Fru 2,6-P₂ and AMP to allow PFK to be active in the presence of citrate may, however, limit glycolytic flow to citric acid under other physiological conditions. As an example, it is well documented (Kubicek & Röhr, 1986) that citric acid accumulation in excessive amounts only occurs upon cultivation on media containing glucose or another readily metabolizable carbon source. Little acidogenesis occurs on galactose or lactose. This coincides well with Fru 2,6-P₂ only being synthesized in the presence of extracellular glucose (Hers & Van Schaftingen, 1982). It might thus be speculated that the effect of carbon source on citric acid accumulation is mediated by Fru 2,6-P₂.

We believe that closer investigations of the metabolism of Fru 2,6-P₂ and AMP in *A. niger* should contribute considerably to the elucidation of the fine regulation of carbon flow and acidogenesis in this fungus.

This work was supported by Schwerpunktsprogramm Biotechnologie of the Österreichisches Bundesministerium für Wissenschaft und Forschung, project P 9261. E. A. gratefully acknowledges a grant from the Austrian-Netherlands exchange programme.

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