Partial purification and regulatory properties of phosphofructokinase from *Aspergillus niger*

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Phosphofructokinase (EC 2.7.1.11) from a citric acid-producing strain of *Aspergillus niger* was partially purified by the application of affinity chromatography on Blue Dextran–Sephadex and the use of fructose 6-phosphate and glycerol as stabilizers in the working buffer. The resulting preparation was still impure, but free of enzyme activities interfering with kinetic investigations. Kinetic studies showed that the enzyme exhibits high co-operativity with fructose 6-phosphate, but shows Michaelis–Menten kinetics with ATP, which inhibits at concentrations higher than those for maximal activity. Citrate and phosphoenolpyruvate inhibit the enzyme; citrate increases the substrate (fructose 6-phosphate) concentration for half-maximal velocity, $[S]_{0.5}$, and the Hill coefficient, h . The inhibition by citrate is counteracted by NH_4^+ , AMP and phosphate. Among univalent cations tested only NH_4^+ activates by decreasing the $[S]_{0.5}$ for fructose 6-phosphate and h , but has no effect on V_{max} . AMP and ADP activate at low and inhibit at high concentrations of fructose 6-phosphate, thereby decreasing the $[S]_{0.5}$ for fructose 6-phosphate. Phosphate has no effect in the absence of citrate. The results indicate that phosphofructokinase from *A. niger* is a distinct species of this enzyme, with some properties similar to those of the yeast enzyme and in some other properties resembling the mammalian enzyme. The results of determinations of activity at substrate and effector concentrations resembling the conditions that occur *in vivo* support the hypothesis that the apparent insensitivity of the enzyme to citrate during the accumulation of citric acid in the fungus is due to counteraction of citrate inhibition by NH_4^+ .

The major regulatory significance of the glycolytic enzyme phosphofructokinase (EC 2.7.1.11) in carbohydrate metabolism has been established for both prokaryotic and eukaryotic cells (cf. Uyeda, 1979). A broad range of metabolic intermediates has been shown to influence the activity and allosteric behaviour of this enzyme, depending on the source of isolation. Among them, citrate has a very important role (Reinhardt & Lardy, 1980; Salas *et al.*, 1965; Dennis & Coultate, 1967).

Some groups or organisms, however, for example the filamentous fungi, have scarcely been examined with respect to phosphofructokinase and its regulation, owing to the great lability of this enzyme (Paveto & Passeron, 1977; Boonsaeng *et al.*, 1977; Tsao & Madley, 1972). It is still unclear whether citrate has the same regulatory importance in fungi as in other organisms.

The filamentous fungus *Aspergillus niger* is well known for its ability to produce and excrete large amounts of citric acid under conditions of highly

active glycolysis (Röhr & Kubicek, 1981). In a previous paper we have shown that phosphofructokinase of *A. niger* is inhibited by citrate and that this inhibition is counteracted by NH_4^+ and AMP (Habison *et al.*, 1979). The hypothesis was offered that the apparent insensitivity of the enzyme to citrate during the accumulation of citric acid is due to counteraction of citrate inhibition by NH_4^+ (Röhr & Kubicek, 1981). However, the respective results had been obtained with crude extracts, and no kinetic data had been presented. In the present paper we report a detailed examination of the kinetic and regulatory properties of a partially purified preparation of phosphofructokinase from *A. niger*.

Materials and methods

Chemicals and enzymes

Biochemicals and enzymes were obtained from C. F. Boehringer und Söhne, Mannheim, Germany.

CNBr-activated Sepharose 4B was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. All buffers and inorganic chemicals were of analytical grade from either Merck, Darmstadt, Germany, or Serva, Heidelberg, Germany.

Organism and conditions for growth

A citric acid-producing strain was used throughout these studies (*Aspergillus niger* B 60; Röhr *et al.*, 1979). The organism was kept on potato/dextrose/agar slants and subcultured each month. Mycelium for enzyme extraction was obtained by growing an inoculum of 5×10^8 conidia/l in a medium containing (g/l): glucose, 10; $(\text{NH}_4)_2\text{SO}_4$, 5; KH_2PO_4 , 5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.75; NaCl, 0.75; tap water; pH 4.5. Incubation was carried out on a rotary shaker at 250 rev./min at 30°C for 48 h, after which the organism was in the middle of the growth phase.

Preparation of cell-free extracts

Mycelia were harvested by filtration and washed thoroughly with both tap water and distilled water. Because of the high instability of phosphofructokinase, only freshly harvested mycelia were used for enzyme extractions. Cell-free extracts were then prepared as described previously (Habison *et al.*, 1979), with 50 mM-potassium phosphate buffer, pH 7.8, containing 1 mM-EDTA and 5 mM-2-mercaptoethanol. For experiments performed in the absence of phosphate, 100 mM-imidazole/HCl buffer, pH 7.8, containing 1 mM-EDTA and 5 mM-2-mercaptoethanol was used.

Dialysis of auxiliary enzymes

As $(\text{NH}_4)_2\text{SO}_4$ was found to activate phosphofructokinase from *A. niger*, it was necessary to remove NH_4^+ ions present in the auxiliary enzymes (fructose biphosphate aldolase, glycerol 3-phosphate dehydrogenase and triose phosphate isomerase), which were used for the coupled assay method given below. Dialysis against 550 vol. of buffer (100 mM-Tris/HCl buffer, pH 7.8, containing 1 mM-2-mercaptoethanol) was performed for 16 h at 4°C with one change of buffer after 8 h. The auxiliary enzymes were then stable in 50% (v/v) glycerol for many months at 4°C.

Assay of phosphofructokinase activity

The activity of phosphofructokinase was measured spectrophotometrically by an optimization of the method described by Racker (1947). Unless otherwise stated, the assay mixture contained 300 μmol of Tris/HCl buffer, pH 7.8, 15 μmol of 2-mercaptoethanol, 0.72 μmol of NADH, 4.5 units of fructose biphosphate aldolase, 2.4 units of triose phosphate isomerase, 2.4 units of glycerol 3-phosphate dehydrogenase, 0.6 μmol of MgCl_2 , 0.3 μmol

of ATP and 12 μmol of fructose 6-phosphate in a total volume of 3.0 ml. Reaction was always started by the addition of ATP, and the blank (indicating NADH oxidase and mannitol 1-phosphate dehydrogenase activities) was considered in the calculation of enzyme activity. All assays were done at 30°C. One unit of phosphofructokinase activity corresponds to the production of 1 μmol of fructose 1,6-bisphosphate/min under these conditions. The specific activity is defined as units/mg of protein.

Extraction of metabolites from mycelia

Determinations of concentrations of metabolites were performed on extracts from 50 h-old mycelia grown either in the absence or in the presence (50 $\mu\text{M-Mn}^{2+}$) of Mn^{2+} in a medium described by Kubicek & Röhr (1978). The mycelia were extracted by a method modified from that of Smith & Ng (1972) as follows. The mycelium was quickly separated from the medium by suction filtration through a precooled linen cloth, gathered into a pellet and immediately frozen by grinding the tissue in a mortar under liquid N_2 . The powdered tissue was homogenized in 10 vol. of 8% (v/v) HClO_4 /40% (v/v) ethanol in a Potter-Elvehjem homogenizer. The whole was then centrifuged (at 20000 g for 10 min), and the residue was extracted with 6% (v/v) HClO_4 . After centrifuging (at 20000 g for 10 min), the combined supernatants were adjusted to pH 6.0–7.0 (with 3 M- K_2CO_3 in 0.5 M-triethanolamine) and then centrifuged again, and the final supernatants were analysed within the next 20 h. Until then they were kept in ice.

For the extraction of intracellular citric acid, the mycelium was washed three times with 10 vol. of ice-cold tap water before being frozen in liquid N_2 .

Analysis of intracellular metabolites

Glycolytic intermediates were determined either photometrically or fluorimetrically, by using a Zeiss PMQII spectrophotometer with a xenon-lamp source, by enzymic standard methods as follows: fructose 6-phosphate and glucose 6-phosphate by the method of Lang & Michal (1974), and ATP and AMP by the method of Lundin & Thorne (1974). NH_4^+ was determined by means of an amino acid analyser as described by Kubicek *et al.* (1979). Phosphate was determined by the method of Jaenicke (1974). Citrate was determined by the method described by Dagley (1974).

Protein determination

Protein was determined by the Coomassie Blue method of Bradford (1976).

Partial purification of phosphofructokinase

Blue Dextran–Sepharose 4B was prepared by the method of Ryan & Vestling (1974). A 10 ml portion

of the cell-free extract (containing about 8 mg of protein) was applied to a cooled (4°C) column (100 mm × 16 mm) of the Blue Dextran–Sephadex, previously equilibrated with the extraction buffer containing in addition 1 mM-fructose 6-phosphate. Unbound protein was removed by washing with at least 50 ml of the buffer given above, and the column was then eluted with a linear gradient of 0–1 M-(NH₄)₂SO₄ in the same buffer. The enzyme was eluted as a sharp peak at 0.3 M-(NH₄)₂SO₄. The combined fractions containing activity were dialysed against 50 mM-imidazole/HCl buffer, pH 7.8, containing 35% (v/v) glycerol, 1 mM-EDTA and 5 mM-2-mercaptoethanol. The resultant preparation was stable in the refrigerator for many weeks, but was inactivated on freezing and thawing.

Kinetic investigations

Hill coefficients (*h*) were obtained from the least-squares linear regression of the plot of $\log[(V_{\max} - v)/v]$ versus $\log[\text{fructose 6-phosphate}]$, where *v* is the steady-state rate and *V*_{max} is the rate at saturating concentrations of fructose 6-phosphate. *V*_{max} is determined from the plot of substrate concentration versus velocity. Only those rates between 10 and 90% of *V*_{max} were used to calculate *h*. [S]_{0.5}, the substrate concentration for half-maximal velocity, is determined from the intercept of the Hill-plot straight line with the abscissa.

Results

Comments on the purification procedure

The result of a typical purification procedure was a 52-fold enrichment of activity with almost 100% recovery of the original activity, yielding a specific activity of 6.1 units/mg of protein. As shown below, glucose 6-phosphate (which is formed in the reaction mixture by glucose phosphate isomerase, which is very active in crude extracts) activates phosphofructokinase from *A. niger*. It is therefore impossible to calculate the recovery exactly. In order to remove the (NH₄)₂SO₄ and fructose 6-phosphate for certain kinetic experiments, dialysis was performed, but this resulted in a significant loss of activity. Thus these preparations had a recovery of 63% but only 27-fold enrichment. No further purification step was conducted, since preliminary experiments with (NH₄)₂SO₄ precipitation or gel filtration resulted in further significant losses of activity. The preparation obtained by the affinity-chromatography step showed one major and two minor bands on sodium dodecyl sulphate / polyacrylamide - gel electrophoresis (results not shown). The following potentially interfering enzyme activities were absent from the preparation: adenylate kinase, ATPase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, hexokinase, mannitol 1-phosphate

dehydrogenase, NADH oxidase, pyruvate kinase, NAD- and NADP-dependent isocitrate dehydrogenases, citrate synthase and aconitase; traces of fructose 1,6-bisphosphatase (less than 0.3 unit/mg of protein) were detected; fructose bisphosphate aldolase was present in larger amounts (4.8 units/mg of protein), but, since this enzyme was present in excess in the auxiliary-enzyme mixture, its presence in our preparation did not interfere with the kinetic investigations.

Effect of enzyme concentration

Partially purified phosphofructokinase activity was proportional to the enzyme concentration when the protein concentration in the assay system was kept above 0.3 µg/ml. Below this concentration partial inactivation (due to dilution) was observed. However, phosphofructokinase could effectively be stabilized by inclusion of fructose 6-phosphate (1 mM) in the buffer, though ATP (1 mM) or glycerol (50%, v/v) was without effect. In kinetic experiments the phosphofructokinase concentration in the assay system was always kept above 1 µg/ml.

Effect of pH

An optimum pH for phosphofructokinase from *A. niger* was shown to be between pH 7.6 and 8.0. All subsequent assays were done at pH 7.8.

Fig. 1. Effect of fructose 6-phosphate concentration on the activity of phosphofructokinase from *A. niger*. For full experimental details see the text. The concentration of enzyme was 4 µg of purified protein in a total volume of 3.0 ml. ●, 0.1 mM-ATP; ○, 0.5 mM-ATP.

Effect of concentration of substrates: fructose 6-phosphate and nucleoside triphosphates

Fig. 1 shows a plot of initial velocity against various concentrations of fructose 6-phosphate at two different concentrations of ATP. A pronounced sigmoidicity was observed in both cases. The corresponding Hill coefficients gave values of $h=4$ irrespective of the ATP concentration used. The $[S]_{0.5}$ values for fructose 6-phosphate increased with the ATP concentration, being 2 mM (for 0.1 mM-ATP) and 3.7 mM (for 0.5 mM-ATP). The interaction with the phosphoryl donor, as shown in Fig. 2, is quite different: nucleoside triphosphate other than ATP (as shown for GTP) displays a normal Michaelis-Menten function, whereas ATP becomes inhibitory above a critical concentration. The K_m for ATP and GTP was 0.08 mM in both cases. The Hill coefficient for ATP at 4 mM-fructose 6-phosphate, calculated by the method of Taketa & Pogell (1965), was $h = 1.3 \pm 0.3$.

Inhibition by citrate

Citrate was found to be a very potent inhibitor of phosphofructokinase from *A. niger*. The effect of citrate was to decrease the affinity of the enzyme for fructose 6-phosphate, thereby potentiating the in-

Fig. 2. Influence of the concentration of ATP and GTP on the activity of phosphofructokinase from *A. niger*. For full experimental details see the text. The concentration of enzyme was $4 \mu\text{g}/3.0 \text{ ml}$ total volume. The Mg^{2+} concentration was always kept at twice the nucleotide concentration. ■, ATP at 1 mM-fructose 6-phosphate; □, ATP at 2 mM-fructose 6-phosphate; ▲, ATP at 4 mM-fructose 6-phosphate; ○, ATP at 4 mM-fructose 6-phosphate and 20 mM- NH_4^+ ; ●, GTP at 4 mM-fructose 6-phosphate.

Fig. 3. Influence of citrate on the activity of *A. niger* phosphofructokinase at two different concentrations of ATP at various concentrations of fructose 6-phosphate. For full experimental details see the text. The concentration of enzyme was $4 \mu\text{g}/3.0 \text{ ml}$ total volume. ●, 0.5 mM-ATP; ○, 0.5 mM-ATP + 0.3 mM-citrate; □, 0.1 mM-ATP; ■, 0.1 mM-ATP + 0.3 mM-citrate.

hibitory effect of ATP (Fig. 3). Thus at 0.1 mM-ATP the $[S]_{0.5}$ for fructose 6-phosphate is increased in the presence of 0.3 mM-citrate from 2.0 to 3.6 mM, and at 0.5 mM-ATP from 3.7 to 5.0 mM. In these cases the Hill coefficient was found to increase to values beyond $h = 5$. As shown in Fig. 4, inhibition by citrate can partially be antagonized by NH_4^+ or inorganic phosphate. In a previous paper (Habison *et al.*, 1979) it was shown that AMP could also antagonize inhibition by citrate. The de-inhibition by NH_4^+ is due to an increase in V_{max} , as well as to a decrease of the $[S]_{0.5}$ value. The apparent K_i for citrate was 0.25 mM at 0.1 mM-ATP and 0.4 mM at 0.5 mM-ATP. De-inhibition by AMP is also due to a decrease in $[S]_{0.5}$ for fructose 6-phosphate. Phosphate also decreases the $[S]_{0.5}$ for fructose 6-phosphate, but the effect is not as pronounced as that with AMP.

Effect of NH_4^+ ions

When tested alone in the absence of citrate, NH_4^+ was a powerful activator of phosphofructokinase from *A. niger*. The activation of the enzyme by NH_4^+ as a function of the concentration of fructose 6-phosphate is shown in Fig. 5. NH_4^+ has no effect on V_{max} , but increases the affinity for fructose

Fig. 4. Inhibition of *A. niger* phosphofructokinase by citrate under various conditions

For full experimental details see the text. The concentration of enzyme was $4.8 \mu\text{g}/3.0 \text{ ml}$ total volume. The various conditions investigated were: O, $20 \text{ mM}-(\text{NH}_4)_2\text{SO}_4$; ●, $10 \text{ mM}-\text{KH}_2\text{PO}_4 + 4 \text{ mM}$ -fructose 6-phosphate; ■, $10 \text{ mM}-\text{KH}_2\text{PO}_4 + 1 \text{ mM}$ -fructose 6-phosphate; □, 0.5 mM -ATP + 4 mM -fructose 6-phosphate. The inset shows: Δ, 0.5 mM -ATP + 4 mM -fructose 6-phosphate; ▲, 0.1 mM -ATP + 4 mM -fructose 6-phosphate. Unless otherwise stated the concentration of ATP was 0.5 mM and that of fructose 6-phosphate was 4 mM .

Fig. 5. Influence on NH_4^+ on the activity of *A. niger* phosphofructokinase at various concentrations of fructose 6-phosphate

For full experimental details see the text. In all cases the concentration of ATP was 0.1 mM and the enzyme concentration was $3.6 \mu\text{g}/3.0 \text{ ml}$ total volume. ▲, Blank with no NH_4^+ ; ●, $10 \text{ mM}-\text{NH}_4^+$; O, $20 \text{ mM}-\text{NH}_4^+$.

Effect of inorganic phosphate

Although phosphate was a potent de-inhibitor of the effect of citrate on phosphofructokinase from *A. niger* when tested alone in the concentration range 0.1 – 100 mM at pH values between 6.5 and 7.8 it had no effect on the enzyme. Phosphate also had no influence on inhibition by ATP, and did not potentiate the activation by NH_4^+ .

Effects of AMP, ADP and cyclic AMP

The effect of AMP on the co-operativity of *A. niger* phosphofructokinase with fructose 6-phosphate is shown in Fig. 6: AMP activates at low concentrations of fructose 6-phosphate, but inhibits at high concentrations of fructose 6-phosphate. It is noteworthy that AMP decreases the $[S]_{0.5}$ for fructose 6-phosphate to values below 1 mM , which is important since the $[S]_{0.5}$ for fructose 6-phosphate observed in the absence of AMP significantly exceeds physiological values (0.05 – 0.3 mM) of cellular hexose monophosphate concentrations.

ADP influences the activity of phosphofructokinase from *A. niger* in a similar way, but the effect is not so pronounced. Cyclic AMP did not influence the activity of the enzyme.

Other metabolic effectors

Several metabolites, including phosphoenolpyruvate, glucose 6-phosphate and 2-oxoglutarate, were tested for a possible interaction with the activity of *A. niger* phosphofructokinase. The effect of fructose 1,6-bisphosphate could not be investigated because of the presence of fructose bis-

6-phosphate and concomitantly decreases the Hill coefficient. Inhibition by ATP was also counteracted by NH_4^+ . In the presence of $10 \text{ mM}-\text{NH}_4^+$, even 2 mM -ATP did not produce inhibition of the phosphofructokinase activity (cf. Fig. 2). Other univalent cations such as K^+ or Na^+ had no effect on the activity of phosphofructokinase from *A. niger*. The effect of NH_4^+ was specific irrespective of the anion used.

Fig. 6. Influence of AMP on the activity of *A. niger* phosphofructokinase at two different concentrations of ATP at various concentrations of fructose 6-phosphate. For full experimental details see the text. The concentration of enzyme was $4 \mu\text{g}/3.0 \text{ ml}$ total volume. ●, 0.5 mM-ATP; □, 0.5 mM-ATP + 1 mM-AMP; ○, 0.1 mM-ATP; ■, 0.1 mM-ATP + 1 mM-AMP.

phosphate aldolase in our preparation and also because of the necessity of using the coupled assay for activity determinations. Coupling with pyruvate kinase/lactate dehydrogenase was not possible because phosphoenolpyruvate is a strong inhibitor of phosphofructokinase from *A. niger*, with an apparent K_i of 0.15 mM.

Glucose 6-phosphate activated *A. niger* phosphofructokinase, the effect depending on the concentration of fructose 6-phosphate. Thus at 2 mM-fructose 6-phosphate 4.8 mM-glucose 6-phosphate increased V_{max} 1.5-fold, whereas at 4 mM-fructose 6-phosphate V_{max} was increased 2-fold. However, the concentrations of glucose 6-phosphate required are too high to be considered as being physiological.

2-Oxoglutarate had no effect on the activity of phosphofructokinase from *A. niger*.

Activity of phosphofructokinase *in vitro* at concentrations of intermediates that occur in *A. niger* *in vivo*

In order to scrutinize whether the concentrations of NH_4^+ observed *in vivo* (Kubicek *et al.*, 1979) would in fact antagonize inhibition by concentrations of citrate that occur *in vivo* in *A. niger*,

Table 1. Concentrations *in vivo* of metabolites relevant to phosphofructokinase activity in *A. niger* under conditions of high and low citric acid production

Conditions for cultivation and methods for preparing cell-free extracts are described in the Materials and methods section. A cell-water/fungal-dry-weight ratio of 2.54 ml/g was used for calculating intracellular concentrations (Slayman & Tatum, 1964), assuming an even distribution within the cell. Means $\pm$ s.e.m. are given, for the numbers of experiments given in parentheses. (A) indicates the citric acid-producing mycelium (Mn^{2+} absent from the culture medium), and (B) the non-citric acid-producing mycelium ($50 \mu\text{M-Mn}^{2+}$); data for (A) have in part been presented by Habison *et al.* (1979).

	Concn. of metabolite in mycelium (mM)	
	(A)	(B)
Fructose 6-phosphate	0.15 ± 0.05 (6)	0.10 ± 0.05 (5)
Glucose 6-phosphate	0.35 ± 0.07 (5)	0.25 ± 0.07 (7)
ATP	1.5 ± 0.22 (4)	1.5 ± 0.24 (5)
AMP	0.10 ± 0.4 (5)	0.10 ± 0.05 (3)
NH_4^+	15.0 ± 2.1 (5)	3.0 ± 0.85 (7)
Phosphate	10.0 ± 1.6 (4)	10.0 ± 1.5 (6)
Citrate	4.0 ± 0.7 (4)	1.0 ± 0.3 (5)

the activity of the purified phosphofructokinase was assayed in the presence of effectors (NH_4^+ , citrate, phosphate, AMP, glucose 6-phosphate) at concentrations of substrates resembling those that occur *in vivo* (Table 1) under both citrate-accumulating and non-citrate-accumulating cultivation conditions. The only differences observed in these two cultivation conditions occurred in the concentrations of hexose monophosphates, citrate and NH_4^+ . The phosphofructokinase activity, assayed as described in the Materials and methods section (with $2 \mu\text{g}$ of the partially purified preparation in the test system), but in the presence of glucose 6-phosphate, fructose 6-phosphate, inorganic phosphate, NH_4^+ , citrate, AMP and ATP at final concentrations resembling those given in Table 1, was 0.024 ± 0.005 unit/ml and 0.21 ± 0.004 unit/ml for 'citrate-producing' and 'non-citrate-producing' conditions respectively, showing that there are no significant differences in activity. Since hexose monophosphates at these concentrations are not capable of producing this effect, the de-inhibition against the higher concentration of citrate under 'citrate-producing' conditions must be attributed to NH_4^+ .

Discussion

The present study is the first detailed investigation of phosphofructokinase from a filamentous fungus. Previous studies of phosphofructokinase from *Penicillium chrysogenum* (Boonsaeng *et al.*, 1977), *Mucor rouxii* (Paveto & Passeron, 1977) and

Neurospora crassa (Tsao & Madley, 1972) were made with either crude extracts or $(\text{NH}_4)_2\text{SO}_4$ -precipitated material because of the 'frustrating lability' of the enzyme (Tsao & Madley, 1972). Moreover, only few aspects of the regulatory properties were examined, and their significance is uncertain, since these preparations must have contained a number of interfering enzymes (glucose phosphate isomerase, ATPase, mannitol 1-phosphate dehydrogenase). In the present study, the inclusion of fructose 6-phosphate and glycerol in the working buffer provided sufficient stability for a short, but efficient, affinity chromatography, by which most of the presumably interfering proteins could be removed.

The kinetic and regulatory properties of the partially purified preparation of phosphofructokinase are well in accordance with a regulatory function of this enzyme even in this citric acid-producing strain of *A. niger*. From a phylogenetic point of view, a relationship with the well-studied enzyme from yeast was to be expected. This was the case in many respects (kinetics with substrates, influence of AMP and citrate). However, certain exceptions are noteworthy. (a) The enzyme of *A. niger* is insensitive towards inorganic phosphate, although phosphate can counteract inhibition of the enzyme by citrate; yeast phosphofructokinase has been shown to be activated by phosphate at pH 6.5 in a manner synergistic with AMP (Banuelos *et al.*, 1977). (b) The enzyme is specifically activated by NH_4^+ , whereas other univalent cations have no effect; this differs from the observed activation by K^+ of the enzyme in yeast (Mavis & Stellwagen, 1970). Furthermore the enzyme is insensitive towards cyclic AMP, which is an activator of *Mucor rouxii* phosphofructokinase (Paveto & Passeron, 1977). In the case of (a) a possible explanation is that addition of citrate produces a change in the conformation of the phosphofructokinase in a way such that the enzyme is accessible to inorganic phosphate. A similar case has been reported for pyruvate carboxylase from *Aspergillus nidulans* in relation to acetyl-CoA-sensitivity (Osmani *et al.*, 1981). On the other hand, phosphofructokinase from *A. niger* is inactivated on dilution, which is a characteristic of the enzyme from higher eukaryotes (Reinhardt & Lardy, 1980), whereas the enzyme from yeast is not inactivated by this treatment (Reuter *et al.*, 1979). The enzyme from *A. niger* may thus be considered as a novel species of phosphofructokinase, phylogenetically located between the yeast and the mammalian enzymes.

With regard to the mechanism of citric acid accumulation by *A. niger*, at present phosphofructokinase is the only enzyme for which a regulatory function in citrate biosynthesis could be demonstrated. Other possible candidates so far

have proved less likely to play a regulatory role, e.g. pyruvate kinase (B. Monori, unpublished work), pyruvate carboxylase (Wongchai & Jefferson, 1974) and citrate synthase (Kubicek & Röhr, 1980). Thus, in order to guarantee optimal production of citric acid, it is necessary to provide a mechanism by which feedback inhibition of citrate biosynthesis by citrate at the phosphofructokinase step could be overcome. From measurements of metabolite concentrations we have previously assumed that the observed increase in the intracellular NH_4^+ pool could be the factor responsible for the antagonization of the inhibition of phosphofructokinase activity by citrate (Habison *et al.*, 1979). The results from the kinetic experiment made under conditions resembling those occurring *in vivo* with respect to metabolite concentrations show that phosphofructokinase under citrate-producing conditions is as active as under non-citrate-producing conditions. Thus, although only the total cellular concentration of citrate was used during this experiment, this gives further support for a regulatory role of NH_4^+ in citric acid accumulation.

The mechanism for elevation of the cellular NH_4^+ concentration requires further investigation. The metabolic situation responsible specifically occurs under conditions of manganese deficiency (Kubicek *et al.*, 1979) and is reflected in an increased degradation of nitrogen macromolecules during cellular turn-over (H. Ma, C. P. Kubicek & M. Röhr, unpublished work).

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