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KINETIC PROPERTIES OF PHOSPHOFRUCTOKINASE OF *NEUROSPORA CRASSA*

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SUMMARY

1. The kinetic properties of phosphofructokinase (ATP: D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) extracted from *Neurospora crassa* were studied to determine their role in the regulation of glycolysis.

2. The relationship between concentrations of fructose 6-phosphate and the phosphofructokinase activity is characterized by sigmoidal kinetics at physiological levels of this substrate. This effect was observed at different levels of ATP concentration. Plots of log $(v/v_{max} - v)$ against log [fructose 6-phosphate] indicated a cooperative effect of this substrate on phosphofructokinase at lower ATP concentrations.

3. At a fixed fructose 6-phosphate concentration of 1 mM, the increase of ATP from zero to 0.8 mM was accompanied by a rapid rise in phosphofructokinase activity to a maximum. Further increase in the concentration of this substrate was paralleled by a sharp drop in the enzyme activity.

4. These results suggest that phosphofructokinase could indeed play a key role in the regulation of glycolysis in *Neurospora crassa*.

INTRODUCTION

Phosphofructokinase (ATP: D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11), has attracted much interest because of its regulatory function in cellular metabolism and its allosteric behavior in molecular configuration. The properties of this enzyme from over three dozen sources, including microorganisms, plants, and lower and higher animals[1,2] have been reported in recent years. We have observed previously that the level of phosphofructokinase activity in *Neurospora crassa* changes drastically when the carbohydrate concentration in the growth media is depleted[3]. Furthermore, there is evidence of a change in distribution of isoenzymes during the change in total activity[4]. In an effort to determine the role of phosphofructokinase in the control of glycolysis in *N. crassa*, we undertook the purification and characterization of this enzyme. The labile nature of this enzyme has thus far frustrated all our efforts, as well as the efforts of another laboratory[5], to purify this enzyme. With

phosphofructokinase extracts prepared under optimum conditions, we have been able to examine the kinetic properties of phosphofructokinase from *N. crassa* with regard to its substrates, fructose 6-phosphate and ATP, other nucleotides, and effectors such as adenosine phosphates. Results indicating feasibility of phosphofructokinase as a regulating factor in glycolysis are described in this communication.

EXPERIMENTAL PROCEDURE

Growth in organism

Cultures of *N. crassa*, wild type 10336, were grown in Difco Bacto-Neurospora minimal media and aerated by agitation with a rotary shaker. After 29 h of growth the mycelia were harvested, lyophilized, milled, and stored at —20° (ref. 3).

Extraction and purification of phosphofructokinase

A suspension of 100 g of lyophilized powder was sonicated in 1 l of 0.1 M Tris–HCl buffer (pH 8.0) containing 0.005 M $MgCl_2$ and 0.005 M EDTA. A solution of 4.0 g of protamine sulfate in 80 ml of water was added and the mixture stirred for 15 min, then centrifuged at 15 000 × *g* for 60 min. The supernatant solution was placed in an ice bath and continuously stirred while solid ammonium sulfate was slowly added. The precipitate of 70–90% ammonium sulfate saturation was collected and dissolved in 50 ml of 3 parts extraction buffer, 1 part glycerol and twice dialyzed overnight at 4° against 2 l of the same mixture.

Enzyme assay

The standard assay mixture had a final volume of 2.5 ml and the following composition: 40 mM Tris–HCl buffer (pH 7.4), 2 mM fructose 6-phosphate, 0.8 mM ATP, 0.4 mM $MgCl_2$, 2.6 mM mercaptoethanol, 0.0064 mM NADH and 0.05 ml auxiliary enzyme solution (2.4 units/ml rabbit muscle aldolase, 2.8 units/ml α-glycerophosphate dehydrogenase, and 4.6 units/ml triose phosphate isomerase in 0.01 M Tris–HCl, pH 8.0 and 0.2% bovine serum albumin). Enzyme activity was assayed using a Perkin–Elmer fluorescence spectrophotometer and Hewlett–Packard recorder. 1 unit of phosphofructokinase activity is defined as that amount catalyzing the formation of 1 μmole of fructose 1,6-diphosphate per min under conditions of the standard assay. Specific activity is expressed as units per mg of protein.

Materials

All reagents were obtained from Sigma Chemical Company, except the following: NADH from International Chemical and Nuclear Corp.; mercaptoethanol and EDTA from J. T. Baker Chemical Co.; and CTP and UTP from Calbiochem Co.

RESULTS

Purification of phosphofructokinase

In addition to ammonium sulfate precipitation, we have applied gel filtration using Sephadex G-100 and Sephadex G-150, ion-exchange chromatography with DEAE-Sephadex and NaCl gradient, preparative acrylamide gel electrophoresis, and isoelectric focusing. None of these gave adequate recovery of enzyme activity. For the

preservation of phosphofructokinase activity, only glycerol at 25% (v/v) was effective. 8-fold (range 4–18) purification of the crude extract was achieved using ammonium sulfate precipitation, yielding a specific activity of approximately 1.

Kinetics of phosphofructokinase

With increasing ATP concentrations from 0.5 to 8 mM, phosphofructokinase shows a change in the kinetics of the enzyme with respect to fructose 6-phosphate. The curve assumes a definite sigmoid shape at ATP concentrations of 1 and 4 mM. The phenomenon is shown in Fig. 1a.

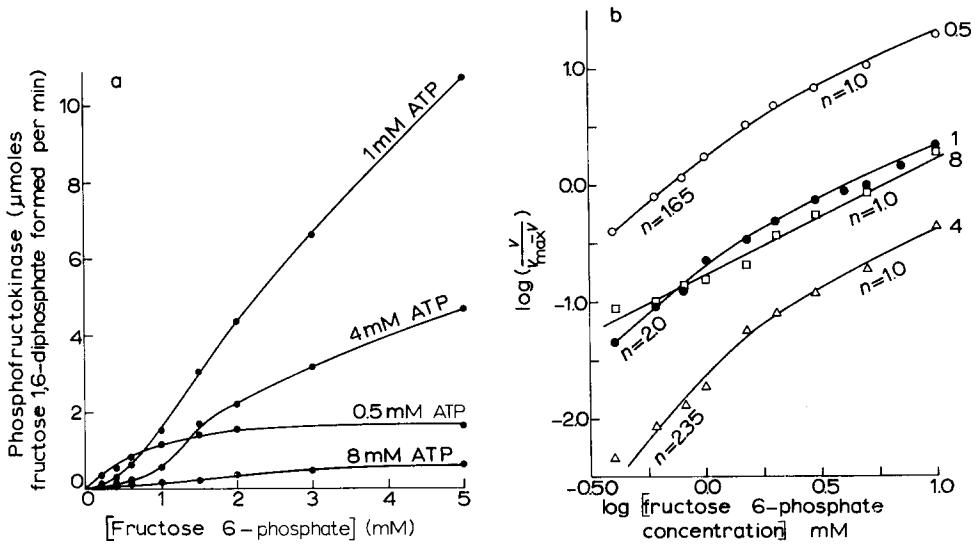

Fig. 1. (a) Effect of fructose 6-phosphate concentration on phosphofructokinase activity at various ATP levels. ATP/Mg^{2+} ratio was maintained at 2:1. (b) Plots of log $(v/v_{max}-v)$ against log [fructose 6-phosphate] showing values of Hill coefficient $n > 1$ at low substrate concentrations and $n = 1$ at higher concentrations for all ATP levels except that of 8 mM. ATP/Mg^{2+} ratio was maintained at 2:1.

In *N. crassa*, as well as in *Escherichia coli*[6], yeast[7], and rat heart[8], the fructose 6-phosphate concentration *versus* phosphofructokinase activity curve becomes more sigmoidal with increasing ATP concentrations. A plot of log $(v/v_{max}-v)$ against log [fructose 6-phosphate] (Hill plot) yields increasing slopes as the ATP concentration is increased from 0.5 mM ($n = 1.65$ at 0.5 mM ATP, 2.35 at 4 mM ATP). This is illustrated in Fig. 1b. A concomitant increase in the v_{max} of the reaction and the apparent K_m for fructose 6-phosphate is also noted. This suggests greater cooperative effects as ATP concentration is increased. At 8 mM ATP, however, the Hill coefficient is 1.0. Apparently at very low ATP concentrations, it is also 1.0 (ref. 8). It was also found that in *N. crassa* the Hill coefficient would be 1.0 at any ATP level if fructose 6-phosphate concentration was greater than 2 mM; therefore, the sigmoidal kinetics of phosphofructokinase will be apparent only at low fructose 6-phosphate concentrations and at specific ATP concentrations.

BETZ AND MOORE[7] stated that the level of fructose 6-phosphate present in yeast

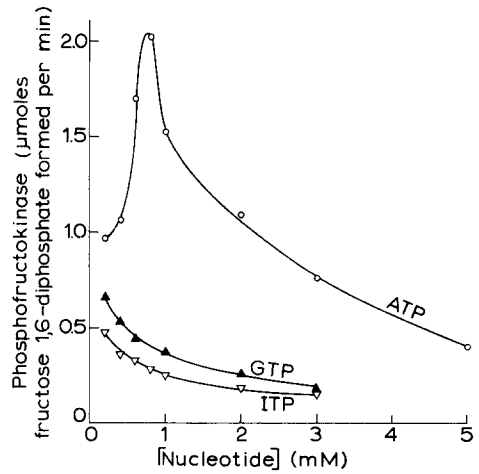

Fig. 2. Effect of ATP concentration on phosphofructokinase activity and effect of substituting GTP or ITP for ATP. Nucleotide/Mg^{2+} ratio was maintained at 2:1. Fructose 6-phosphate concentration was 1 mM.

cells was 0.2–0.4 mM. Using this as an approximation of the level of fructose 6-phosphate in *N. crassa* cells, it can be seen from Fig. 1a that any increase in ATP concentration above 0.5 mM will inhibit phosphofructokinase activity. This ATP inhibition can be overcome by increasing the fructose 6-phosphate concentration.

The concentration of ATP which gives maximum phosphofructokinase activity is dependent upon the fructose 6-phosphate concentration. Once the optimum ATP concentration is reached, a further increase will cause inhibition of phosphofructokinase activity and subsequently a sharp drop in reaction velocity. This is a characteristic common to phosphofructokinase from most sources, except slime mold[9], *Arthrobacter crystallopoietes*[10], and *Clostridium pasteurianum*[11].

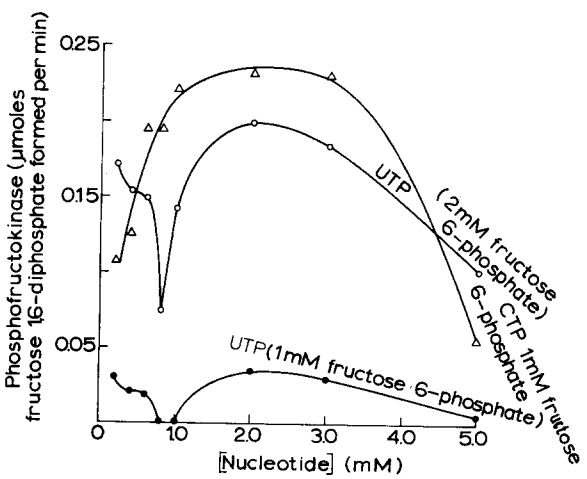

Fig. 3. Effect of substituting CTP or UTP for ATP. Nucleotide/Mg^{2+} ratio was maintained at 2:1. Fructose 6-phosphate concentration was 1 mM, except for upper UTP curve.

Other phosphate donors were tested to see if they would inhibit phosphofructokinase activity at higher concentrations in the manner of ATP. Fig. 2 demonstrates the type of kinetics seen with GTP and ITP, and Fig. 3, the type seen with CTP and UTP. GTP, ITP and CTP have been observed to be effective replacements for ATP in yeast[12,13], showing no inhibition at elevated concentrations. The response of *N. crassa* phosphofructokinase to GTP, ITP, UTP and CTP, however, was similar to that seen in brussels sprouts[14]. All were poor phosphate donors. Both UTP and CTP inhibited phosphofructokinase activity at elevated concentrations. The sharp drop of phosphofructokinase activity as the UTP concentration approaches 0.8 mM, suggests a conformational change of phosphofructokinase. At 0.2 mM level, AMP inhibits phosphofructokinase activity to the extent of 17% and ADP inhibits to a much greater degree at 85%. At 0.5 mM level, the relative inhibitions are 38% and 90%; and at 2.0 mM level, 66% and 97%, respectively. NADH has been noted to inhibit the phosphofructokinase of sheep liver[15]. However, it has been suggested that the inhibition could be due the effect of NADH on the coupling enzymes of the assay system[16]. We have also observed a moderate and linear inhibition of phosphofructokinase from *N. crassa* by NADH.

DISCUSSION

The regulation of glycolysis in fungi in general, and in Neurospora in particular, has been reviewed from the point of view of metabolic pathways[17]. We have interpreted the changes in phosphofructokinase activity in response to carbon source level in the medium as an indication of the central role of this enzyme in the control of glycolysis in *Neurospora crassa*[4]. Results of the study reported here have substantially added to the establishment of a key role for phosphofructokinase in the regulation of glycolysis in *N. crassa*. At the physiological concentrations of fructose 6-phosphate, the sigmoidal kinetics of phosphofructokinase permit this substrate to activate the enzyme; hence, phosphofructokinase could respond to an increase in fructose 6-phosphate concentration by speeding up its conversion to fructose 1,6-diphosphate. Furthermore, phosphofructokinase is sensitive to the fructose 6-phosphate levels at a wide range of ATP concentrations, and could expedite glycolysis whenever the fructose 6-phosphate concentration has been raised by the acceleration of the initial steps of glycolysis. The profound inhibitory effect of ADP on phosphofructokinase, on the other hand, leads one to believe that product inhibition of this enzyme by ADP constitutes another regulatory mechanism of glycolysis by phosphofructokinase. In other words, phosphofructokinase could slow down glycolysis when the rate of ADP formation exceeds the rate of its removal by phosphorylation resulting in an accumulation of ADP. However, one must bear in mind that ATP at concentrations higher than optimal also has an inhibitory effect on phosphofructokinase; therefore, the net increase in the inhibition of phosphofructokinase due to conversion of ATP to ADP in the reaction catalyzed by this enzyme, might not be as high as one might expect. But at ATP concentrations below optimal, the conversion of ATP to ADP could have severe retarding effect on glycolysis through inhibition of phosphofructokinase.

Some kinetic properties of *N. crassa* phosphofructokinase are common to virtually all phosphofructokinases regardless of source. These include sigmoidal kinetics (allostericity) with respect to one substrate, fructose 6-phosphate, and inhibition by

high concentrations of the other substrate, ATP. The two exceptions are slime mold[9], and *Arthrobacter crystallopoietes*[10], which exhibit Michaelis–Menten kinetics with respect to fructose 6-phosphate and no inhibition by elevated ATP concentrations.

The influence of phosphofructokinase activity by other effectors can be predicted to some extent by phylogenetic grouping. The most striking example is the activation by ADP of phosphofructokinase from animal sources[22–32] in contrast to the inhibition by ADP of phosphofructokinase from plant sources. With the phosphofructokinase from microorganisms, the effect of ADP is not predictable. The yeast enzyme is inhibited by ADP at ATP concentrations that, in themselves, are not inhibitory; at ATP concentrations that are inhibitory, ADP acts as an activator[18]. The phosphofructokinase from *N. crassa* is strongly inhibited by ADP, this property differentiates it from the enzyme from other microorganisms and animal tissues but places it in the same group as the enzyme from plants[9,14,20,33–35].

Other effectors normally associated with phosphofructokinase kinetics, AMP, cyclic AMP, phosphoenolpyruvate, fructose 1,6-diphosphate and P_i, do not share common properties in all organisms. Phosphofructokinase from plant and animal sources differs markedly in its response to AMP, being inhibited by it in the case of *N. crassa* and plants, but activated in case of animals. Two exceptions are the enzyme from Schistosoma[36] and human erythrocyte[2]. There are several activators for phosphofructokinase from animal sources: ADP, AMP, cyclic AMP, P_i and fructose 1,6-diphosphate. On the other hand, P_i seems to be the only activator of the plant enzyme such as those from pea seed[34], brussels sprouts[14], and parsley[20]. However, this is not without exceptions, as P_i inhibits the corn phosphofructokinase[35] and has no effect upon the avocado enzyme[20]. Among the phosphofructokinases of microorganisms, those from *Clostridium perfringens*[20], *Aerobacter aerogenes*[19], and *Clostridium pasteurianum*[11] are activated by AMP. The *Staphylococcus aureus* enzyme is activated, not only by AMP, but also by cyclic AMP and P_i (ref. 20).

A change in the kinetic properties of phosphofructokinase has also been noted under a variety of environmental and experimental conditions[37–40].

The kinetic properties of phosphofructokinase reported here permit consideration of this enzyme as the key regulatory enzyme for glycolysis in *N. crassa*. However, an activator for this enzyme has yet to be found, without which the response of phosphofructokinase to changes in cellular conditions would be rather limited. The profound effect of adenine nucleotides on phosphofructokinase suggests an interaction between the enzyme and the adenylate pool that contributes to the regulation of energy metabolism.

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