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PURIFICATION AND CHARACTERIZATION OF PHOSPHOFRUCTOKINASE FROM RHODOTORULA GLUTINIS.

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Abstract:
Phosphofructokinase has been identified and purified from extracts of Rhodotorula glutinius. Kinetic studies of the enzyme indicated high cooperativity with respect to fructose 6-phosphate. The kinetics for ATP shows no cooperativity as indicated by the hyperbolic behavior of the enzyme. The enzyme is inhibited by ADP. Citrate and phosphate have no effect on the enzyme activity. The role of ATP, fructose 6-phosphate, and ADP is discussed.

Introduction

Yeasts of the genus Rhodotorula have been reported to lack phosphofructokinase (1,2,3) and therefore unable to utilize the glycolytic pathway for the catabolism of glucose. The apparent absence of phosphofructokinase in species of Rhodotorula led to a proposal that phosphoketolase, catalyzing the reaction:

Xylulose 5-PO4 ——————→ Glyceraldhyde 3-PO4 + C_2 unit

or Fructose 6-PO4 ——————→ Erythrose 4-PO4 + C_2 unit

was present as an adjunct to the pentose cycle(4,5) such a proposal as suggested by Colin Ratiedge(6), was needed as the pentose cycle produces only one C_2 unit from each molecule of glucose. Ratiedge group has failed to find phosphofructokinase in several Rhodotorula species, and were able to detect phosphoketolase in these yeasts. The contribution which phosphoketolase makes to glucose catabolism may be only slight. Its properties as indicated by low activity, sensitivity to intracellular metabolites and its competition with transketolase for

Abbreviations: DTT: Dithioerythritol
PMSF: Phenyl Methyl Sulfonyl Fluoride

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its substrate are not the hallmarks of a major controlling enzyme. As its purpose is to divert carbon from the pentose cycle into the tricarboxlic acid cycle, it could only play a major role if Embden Meyerhof pathway was absent or was functioning at a low level. During a study of the possible pathway(s) of glucose catabolism in these yeasts, evidence was obtained which suggested that Embden-Meyerhof pathway was operative(7). Why phosphoketolase should be present at all is a puzzle?. It appears that phosphofructokinase has unusual properties, which makes it difficult to detect. In this investigation, we report our results showing the existence and some important properties of phosphofructokinase from Rhodotorula glutinis.

Materials and Methods:

ATP, ADP were obtained from P.L. Biochemicals. Fructose 6 phosphate (Grade 1), aldolase, NADH, and mixed crystals of triose phosphate isomerase and glycerophosphate dehydrogenase were products of Sigma Chemical Company. QAE-sephadex A-25 was a product of Pharmacia Fine Chemicals. Crystalline bovine serum albumin was obtained from Pentex Biochemicals. Dialysis tubing(union carbide)was prepared by the method of Paul and Lehman(8). The tubing was stored in 0.1 M Tris buffer PH 7.6 and extensively rinsed before use.

Preparation of Rhodotorula Glutinis Yeast.

Cells were grown in a medium which consised of glucose(10 grams), yeast nitrogen base without amino acids(0.7 grams) and water in one liter. The cells were harvested after 40 hours of culturing and are then suspended in cold 0.1 M Tris-HCl Buffer, PH 7.2. The cells were placed in a 50 ml Braum Homogenizer flask (Pre-chilled) containing 20 grams of glass beads(0.45-0.5 cm). The cells were ruptured in a Brqum model MSK mechanical cell.

Fifteen liters of Rhodotorula glutinis cells were prepared according to the procedure mentioned above. The cells were collected by centrifuging at 10,000 RPM for 20 minutes. The total cells weight was 187.8 grams suspended in 240 ml of 0.1 M Tris-HCl PH 7.6 buffer. The cells were broken for 4 minutes with intervals 30 secon passed after each minute. The total homogenate was centrifuged at 15,000 RPM for 15 minutes. Pellets were kept for future studies.

Enzyme Purification.

The total extract was heated to 52°C for 1.5 minute(1 minute was allowed to reach 52°C), then chilled in ice and centrifuged at 15,000 RPM for 15 minutes. The total volume after this step was 210 ml. Amonium sulfate fractionation from 0-30%, 30-40%. 40-50%, 50-60% and 60-70% saturation were carried out. The enzyme was obtained mostly from 40-50% fraction according to the procedure mentioned above. The partially purified enzyme preparation was dissolved in 60 ml 0.1 M Tris containing 10% Glycerol, 0.5 mM DTT, and 1 mM PMSF). The enzyme was dialyzed against 500 ml of 0.1 M Tris containing 0.5mM DTT, 1mM PMSF, 1mM ATP, and 10% Glycerol PH.7.5, with a change of the buffer three times. The dialyzed enzyme was then applied to QAE shadex column after it has been equilibrated for

TABLE I- Purification of Phosphofructokinase from Rhod. Glutinus

FRACTION	TOTAL VOLUME (ml.)	ACTIVITY (units/ml.)	TOTAL ACTIVITY (units)	SPECIFIC ACTIVITY (units/mg.)	% YIELD
Crude Extract	220	0.32	70.4	.048	100
Heat Step	210	0.31	65.1	.076	92.5
Ammonium Sulfate	55	0.90	49.5	.387	70.3
Q.A.E. Sephadex	150	0.29	43.9	1.45	62.0
Ultrafiltration	28	1.07	29.9	1.21	42.0

four hours with 0.1 M Tris containing 0.5 mM DTT, 1 mM PMSF, 1 mM ATP, and 10% Glycerol. A gradient of ammonium sulfate was established in 0.1 M Tris buffer containing 0.5 mM DTT, 1 mM PMSF, 1 mM ATP, 6 mM fructose 6- phosphate, and 10% Glycerol PH 7.5. Fractions were collected at a flow rate of 60 ml/hour. The purified enzyme was concentrated and stored in the cold after addition of 1 mM ATP, and 20 mM frucose 6-phosphate. The enzyme was extensively dialyzed against the same buffer mentioned above before each study.

Protein was estimated by the method of Lowry et al (9) employing bovine serum albumin as a standard.

During the purification of phosphofructokinase, enzyme activity was estimated from the rate of change in absorbance at 340 nm by following NADH disappearance in a coupled system. The reaction mixture contained 1mM ATP, 6mM fructose 6- phosphate, 450 ug of aldolase, 150 ug -glycerolphosphate dehydrogenase/triose phosphate isomerase in a final volume of 2.9 ml at 23-24^{o}C. (Aldolase and -glycerolphosphate dehydrogenase were freed of ammonium sulfate befor use by dialysis against 0.01 Tris PH 7.6). The reaction was initiated with 0.1 ml of phosphofructokinase suitably diluted in 0.1 M Tris PH. 7.6.

Results and Discussion

Table 1 outlines the results of a typical purification of phosphofructokinase from Rhodotorula glutinis. The enzyme was purified about two fold after heating the crude extract at 55^{o}C. Fivefold purification was achieved after ammonium sulfate fractionation, and a further thirty fold by chromatography through QAE-sephadex A-25 with an overall recovery of 62%.

Stability studies have indicated that the pure enzyme can maintain 95% of its activity if it is incubated with 20 mM fructose 6-phosphate and 1 mM ATP in the cold at 5°C for a period of four weeks. Freezing of the enzyme at this conditions resulted in a loss of activity. Addition of stablizing agents such as ATP, fructose 6-phosphate, 10% Glycerol, DTT, and PMSF in the buffer system was found to be essential throught the purification steps in order to keep the enzyme stable. The specific activity of the enzyme decreased about 15% upon concentrating the enzyme, and increased about twofold upon dilution to 1 mg/ml. The change in specific activity on dilution can be reasonably attributed to changes in the aggregation state of the enzyme.

Binding of fructose 6-phosphate to the enzyme is presented in Fig 1. The enzyme exhibits clearly sigmoidal dependence on fructose 6-phosphate which suggests that rodotorula PFK contains two types of subunits which may have distinct functions in the regulation of the enzyme(10). Our results suggest that fructose 6-phosphate acts as a normal substrate binds to the catalytic site, and also to the regulatory site and stablizes the active conformation of the enzyme. The idea that a specific ligand binding is directly responsible for the stabilization of particular aggregation is supported by the fact that reducing the concentration of fructose 6-phosphate below its apparent Michael's constant results in dissociation and inactivation of the enzyme. Binding of ATP to the enzyme showed a hyperbolic plot (Fig 2) which suggests that ATP acts as a substrate and binds only to the active site. Our stability studies have indicated that the enzyme is more stable if it is kept with fructose 6-phosphate(10 mM) + ATP(2mM), and actually can be left in the cold for four weeks with an increase of 20% in specific activity than either fructose 6-phosphate or ATP alone. These results with the hyperbolic behavior of the enzyme toward ATP suggests that fructose 6-phosphate induces first a conformational change to the enzyme by binding to its regulatory subunit, and this increases significantly the binding affinity of its cosubstrate ATP which will bind only to the catalytic site.

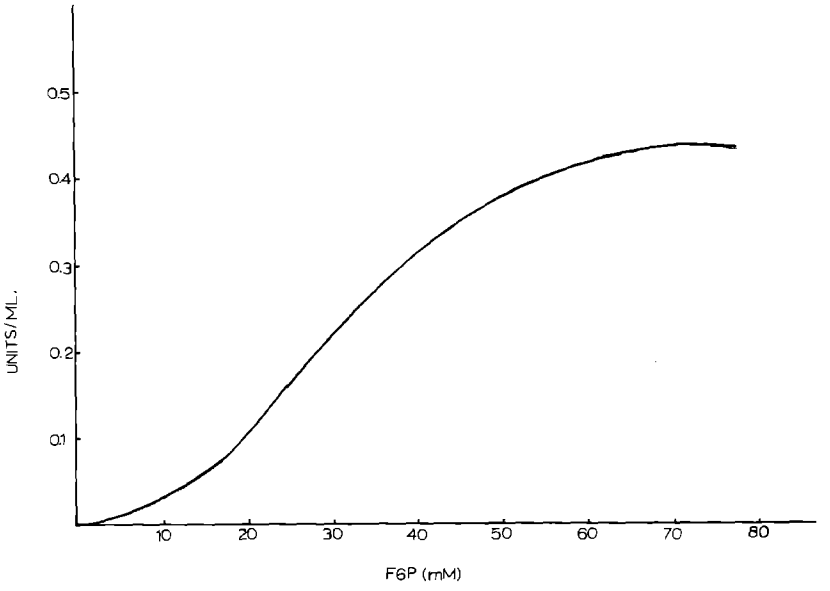

Fig 1: Initial velocity as a function of fructose 6-phosphate concentration. The enzyme was dialzed extensively before its use. Specific activity assay as described under " Materials and Methods".

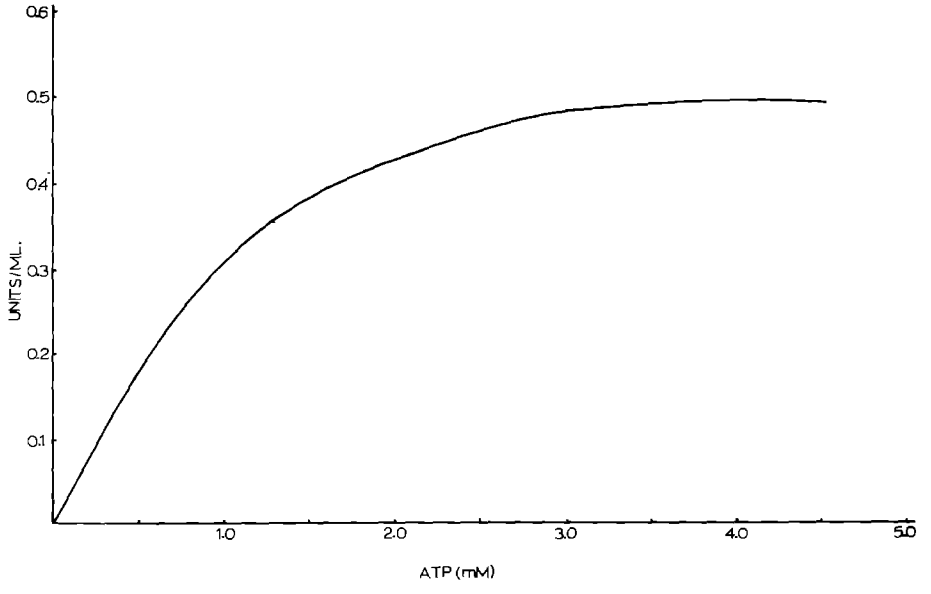

Fig 2: Initial velocity as a function of ATP concentration. Specific activity assay as described under " Materials and Methods".

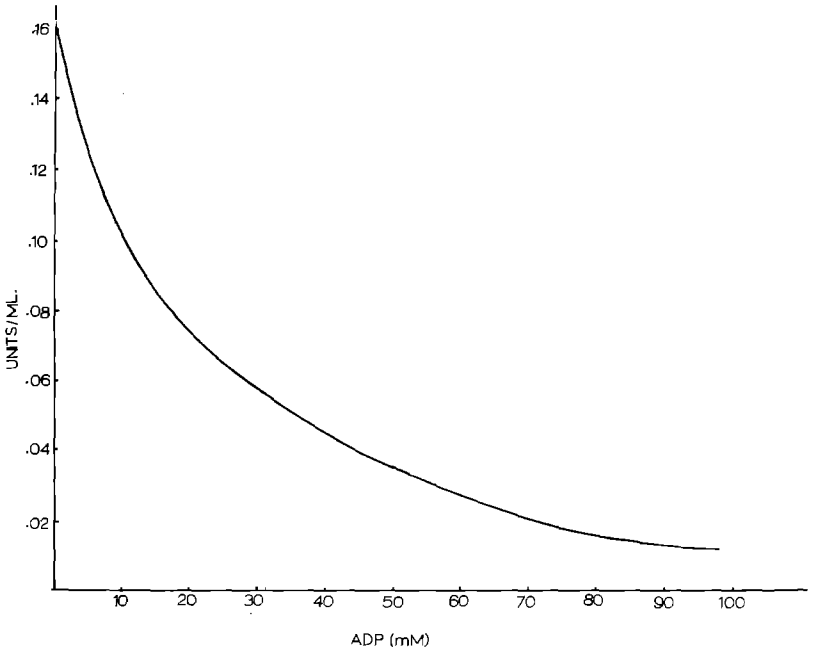

Fig 3: Initial velocity as a function of ADP concentration. Initial velocity of phosphofructokinase was measured at different ADP concentrations in regular reaction mixture as described under " Materials and Methods". The concentration of fructose 6-phosphate, and ATP were fixed at 3mM, 1 mM respectively.

A surprising result is the behaviour of ADP binding to the enzyme as it is presented in Fig 3. As we know for most of the phosphofructokinase enzymes ADP acts as a positive modifier. Our studies here show an inhibition of the enzyme by ADP. The results can suggest that ADP may suppress the interaction of the ATP, fructose 6-phosphate with the enzyme at the active site by interference with the binding of ATP. This interpretation accounts for the peculiar shape of the hyperbolic curve with respect to ATP. Thus the role of ADP can be either a competitive inhibitor or it binds to a regulatory site and acts as a negative modifier. The occurence of a specific site for ADP binding to Rhodotorula PFK should be tested with other analogs.

The effect of phosphate ion and citrate on the enzyme activity have been tested. Our results have indicated there was no effect(results not shown).

Our research now is in progress to further characterize the enzyme, and to study the role of the essential residues in catalysis.

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