PURIFICATION OF ESCHERICHIA COLI PHOSPHOFRUCTOKINASE*

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Received March 20, 1967

Phosphofructokinase (PFK) catalyzes the phosphorylation of fructose-6-phosphate (F6P) to form fructose-1,6-diphosphate (FDP) in the presence of adenosine-5'-triphosphate (ATP) and magnesium ions. Recent investigations have suggested that PFK may play an important role in the regulation of glycolysis in mammalian tissues (1,2), yeast (3,4), bacteria (1,5) and other organisms (1,6). The salient features of PFK regulation are the inhibition of enzyme activity induced by high concentrations of one substrate, ATP, and the relief of this inhibition by various effector metabolites. At inhibitory ATP concentrations, a plot of initial velocity versus F6P concentration yields a sigmoidal curve indicative of a reaction order greater than one. In the presence of an effector metabolite, the plot approaches the rectangular hyperbola of simple Michaelis behavior.

PFK has been crystallized (7,8) from rabbit muscle and initial studies of its physical and biochemical properties have been reported (8,9). Also, the kinetic properties of a partially purified preparation of PFK from E. Coli B have been examined by Atkinson and Walton (5,10). In their earlier investigation, they observed that adenosine-5'-monophosphate (AMP) and adenosine-5'-diphosphate (ADP) were both effective in relieving the inhibition of PFK activity produced by high ATP concentrations. However, with an enzyme preparation free of adenylate kinase activity, Walton and Atkinson (10) found that only ADP was effective in this regard.

* Supported by N.I.H. Research Grant GM 11632-04 and Training Grant 5-F1-GM-23,575. Taken in part from a Ph.D. thesis to be submitted by C.C.G. to the Johns Hopkins University, Baltimore, Maryland. Contribution No. 495 of the McCollum-Pratt Institute.

It was felt that the response of E. Coli PFK towards various effectors differed sufficiently from that of the rabbit muscle enzyme to warrant further purification. The present communication describes the preparation of an apparently homogeneous PFK from E. Coli B and presents several kinetic properties of the purified enzyme.

EXPERIMENTAL AND DISCUSSION

The purification of phosphofructokinase from three pounds of E. Coli B (Grain Processing Corp., Muscatine, Iowa) is shown in Table I. Unless otherwise noted, all steps in the procedure were carried out at 4°C. Centrifugations were for 15 minutes at 13,200 x g.

TABLE I

Purification of phosphofructokinase from E. Coli B

Fraction	Volume	Activity	Specific Activity
	ml	units/ml	units/mg protein
Crude extract	8,150	2.80	0.215
Protamine supernate	10,700	2.03	0.325
Dialyzed ammonium sulfate fraction I	980	16.6	0.413
Protamine precipitate-dialyzed ammonium sulfate fraction II	120	79.2	8.58
Dissolved pH 4.5 precipitate	120	73.5	12.5
Calcium phosphate eluate	120	47.4	22.6
DEAE column I	36.5	71.0	123.5
DEAE column II	19.5	66.6	190.2

Phosphofructokinase activity was estimated from the rate of change of absorbance at 340 mμ in a standard assay system containing 240 μ moles Tris (pH 8.2), 3 μ moles $MgCl_2$, 0.44 μ moles reduced diphosphopyridine nucleotide (DPNH), 13.5 μ moles F6P, 3 μ moles ATP, 450 μg crystalline aldolase and 150 μg crystalline α-glycerophosphate dehydrogenase - triose-phosphate isomerase in a final volume of 2.9 ml at 23-24°C. The reaction was initiated by 0.1 ml of enzyme preparation suitably diluted in 0.09 M sodium phosphate -- 0.1 M ammonium chloride pH 7.4. One unit of enzyme is defined as that amount catalyzing the formation of one μmole of FDP per minute under the conditions of the standard assay. Protein was estimated by the method of Lowry et. al (11) employing serum albumin as a standard.

One pound of partially thawed cells were disrupted for one hour and fifteen minutes at low speed in a five-quart Waring Blendor in the presence of 2 liters of 0.1 M sodium phosphate - 10^{-3}M ethylenediaminetetraacetic acid (EDTA) pH 7.4 and 1 kg of acid-washed glass beads. The supernatant fluid was decanted and the residue was washed by blending for 5 minutes with 1 liter of buffer. The wash liquor was combined with the first supernate and centrifuged. This procedure was repeated for two additional pounds of E. Coli.

The crude extract (8.15 l) was treated with 3.49 liters of 1.0% protamine sulfate (pH 7.0) and allowed to stir for 15 minutes before centrifugation. PFK activity was precipitated from the resulting 10.7 liters of protamine supernate by the slow addition of 4.28 kg of solid ammonium sulfate at 0°C. After a 15 minute equilibration, the precipitate was collected by centrifugation, dissolved in 0.075 M Tris-phosphate pH 7.5 to a volume of 875 ml and dialyzed overnight against 15 liters of 0.075 Tris-phosphate buffer pH 7.5 containing 1.2 x 10^{-5}M FDP, 10^{-4}M ATP, 10^{-3}M EDTA and 5 x 10^{-3}M mercaptoethanol (Buffer A). The dialyzed enzyme was brought to a final volume of 980 ml with Buffer A and clarified by centrifugation (Dialyzed ammonium sulfate fraction I).

Eighty-nine ml of neutralized 1.0% protamine sulfate were added slowly to 960 ml of the dialyzed material at 0°C and stirred for 15 minutes. The precipitate was discarded and 94 ml of 1.0% protamine sulfate were added to the supernatant fluid. After a 15 minute equilibration, the PFK-containing precipitate was collected by centrifugation and extracted overnight with 500 ml of 0.1M Tris-sulfate-0.5 M ammonium sulfate pH 7.5 (Buffer B). The resulting suspension was brought to a final volume of 960 ml with Buffer B and 163 g of ammonium sulfate were added slowly at 0°C. After 15 minutes, the precipitated protein was collected by centrifugation and discarded. PFK activity was precipitated from the supernates at 0°C by slowly adding 115 g of ammonium sulfate. The resulting precipitate was dissolved in 0.1 M

Tris-sulfate pH 7.5 to a final volume of 90 ml and dialyzed overnight against 6 liters of Buffer A. The dialyzed material was brought to a final volume of 120 ml with Buffer A (Dialyzed ammonium sulfate fraction II).

One normal acetic acid was added cautiously at 0°C until the pH obtained was 5.5 After 10 minutes, the precipitated protein was collected and discarded. The supernate was then taken to pH 4.5 with acetic acid and stirred for 10 minutes. The precipitate was dissolved in 120 ml of 0.1M Tris-sulfate pH 7.5.

The enzyme solution was adjusted to pH 5.4 with acetic acid and 30 ml of 0.1 M sodium acetate pH 5.8 were added. This solution was treated at 0°C with 1.4 g (dryweight) of aged calcium phosphate gel (previously washed with 0.02 M sodium acetate pH 5.8). Adsorption was allowed to proceed for 20 minutes after which the gel was eluted twice with 60 ml portions of 0.025M phosphate pH 6.6 for 20 minutes.

The calcium phosphate eluates were combined, adjusted to pH 7.7 with dilute ammonium hydroxide and applied to a 2.5 x 19.7 cm column of DE 52 (Reeve Angel, Clifton, N.J.) equilibrated with 0.025 M phosphate - 0.125 M ammonium sulfate pH 7.6. The enzyme was eluted from the column with a linear gradient device containing 725 ml of 0.025 M phosphate - 0.125 M ammonium sulfate pH 7.6 in the mixing chamber and 725 ml of 0.025 M phosphate - 0.125 M ammonium sulfate pH 6.2 in the second resevoir. Fractions containing greater than 3 units of enzyme activity per ml were combined and concentrated by reduced pressure dialysis (collodion bag apparatus, Carl Schleicher and Shuell Co., Keene, New Hampshire) to a volume of 36.5 ml. The concentrated material (DEAE column I) was dialyzed for 3 hours against 2 liters of 0.1 M Tris-sulfate pH 7.5.

Thirty-five ml of DEAE I were applied to a 1.8 x 18.6 cm column of DE 52 equilibrated with 0.1 M Tris-sulfate pH 7.5. A linear ionic strength gradient was employed to elute PFK from the column. The mixing chamber contained 625 ml of 0.1 M Tris-sulfate pH 7.5 and the second resevoir contained 625 ml of

Buffer B. Fractions with more than 10 units per ml were combined and concentrated to 19.5 ml (DEAE column II) by reduced pressure dialysis and the concentrated, purified PFK was dialyzed overnight against 1 l of 0.1 M Tris-sulfate pH 7.5 containing 5 x 10^{-3} M mercaptoethanol and 10^{-3}M EDTA.

The purified enzyme preparation migrated as a single band during acrylamide gel electrophoresis (12). At high protein concentrations, two extremely faint satallite bands were observed. In the ultracentrifuge, PFK sediments with a symmetrical peak.

As shown in Fig. 1, the purified enzyme from E. Coli still exhibits the regulatory characteristic of substrate inhibition at high ATP concentration. Under the conditions of the experiment, maximal activity is observed near 1mM ATP and gradually declines as the ATP concentration is

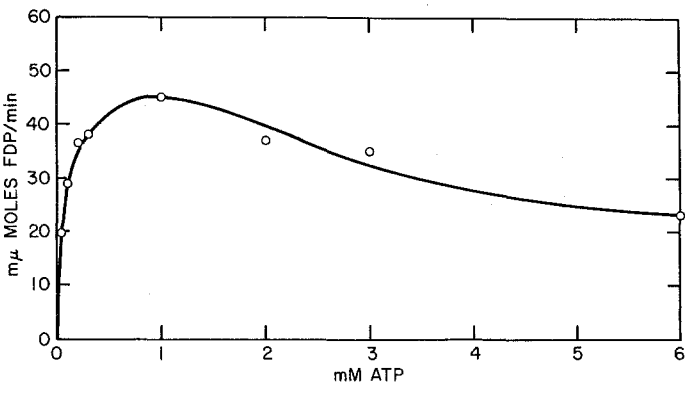

Fig. 1. Effect of ATP concentration on E. Coli PFK. F6P concentration was 0.9 mM. Other additions were as indicated in legend to Table II with no nucleoside diphosphate added.

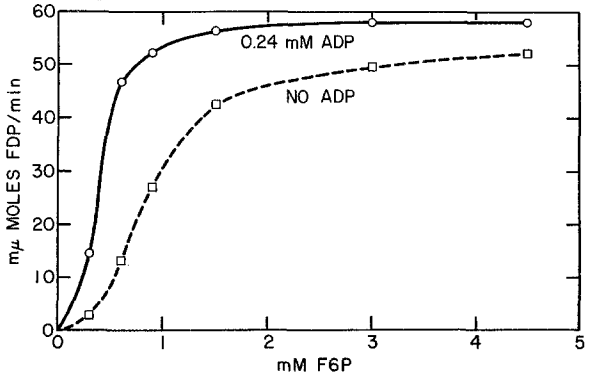

Fig. 2. Effect of ADP on the sigmoid character of initial velocity versus F6P concentration. ATP concentration was 3 mM. Other conditions were the same as for Fig. 1

raised. A ten-fold increase in Mg^{+2} ion concentration substantially prevents this inhibition over the range of concentrations examined.

At 3mM ATP, a plot of initial velocity versus F6P concentration yields a sigmoidal curve with half-maximal velocity at a concentration of 9×10^{-4} M F6P (Fig. 2). In the presence of 0.24 mM ADP, the sigmoidicity of the velocity versus substrate plot is reduced; and half-maximal velocity is attained at a F6P concentration of only 4×10^{-4}M. AMP, even at a concentration of 3 mM, was not effective in reducing ATP inhibition.

The effectiveness of other nucleoside diphosphates in relieving the ATP inhibition of PFK activity was tested in the presence of 3 mM ATP and 0.9 mM F6P. The results shown in Table II indicate that at concentrations around 2 mM, guanosine-5'-diphosphate (GDP), inosine-5'-diphosphate (IDP),

TABLE II

Effect of nucleoside diphosphates (NDP) on ATP inhibition of E. Coli PFK

NDP	Concentration	Activity
	mM	Millimicromoles FDP formed/min
None	0	23.2
ADP	0.24	53.2
GDP	1.81	64.8
	0.30	59.0
IDP	2.04	69.2
	0.34	65.3
CDP	2.02	55.7
	0.34	23.2
UDP	2.24	55.2
	0.37	42.0

Each cuvette contained in a final volume of 2.9 ml, 240 μmoles Tris pH 8.2, 9 μmoles ATP, 6 μmoles $MgCl_2$, 0.44 μmoles DPNH, 2.70 μmoles F6P, 450 μg crystalline aldolase, 150 μg crystalline α-glycerophosphate dehydrogenase-triosephosphate isomerase, and the nucleoside diphosphates in the concentration stated in the table. The addition of 0.1 ml of purified PFK containing 0.35 μg of protein in 0.09 M Tris-SO_4 buffer containing 0.045 M Na_2SO_4 and 0.1 M NH_4Cl pH 7.6 initiated the reaction.

cytidine -5'-diphosphate (CDP), and uridine-5'-diphosphate (UDP) were at least as effective in increasing enzyme activity in the sigmoid region of

the Figure 2 curve as was ADP at a concentration of 0.24 mM. With these nucleoside diphosphates at a lower concentration, however, differences in their relative effectiveness were more easily observed. IDP and GDP were significantly more effective at lower concentration than were the pyrimidine nucleotides.

The authors wish to thank Miss Gertrude Karr for the ultracentrifugation studies on the purified protein.

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