Pyrophosphate:
Fructose 6-Phosphate 1-Phosphotransferase

A NEW ENZYME WITH THE GLYCOLYTIC FUNCTION OF 6-PHOSPHOFRUCTOKINASE*

Richard E. Reeves, Dorothy J. South, Harold J. Blytt, and Lionel G. Warren

From the Departments of Biochemistry and Medical Parasitology and Tropical Medicine, Louisiana State University Medical Center, New Orleans, Louisiana 70112

SUMMARY

The major enzyme of Entamoeba histolytica which produces fructose 1,6-diphosphate from fructose 6-phosphate utilizes inorganic pyrophosphate as phosphate donor. In its reverse reaction the enzyme utilizes orthophosphate and fructose diphosphate to yield inorganic pyrophosphate and fructose 6-phosphate. The enzyme has been purified 100-fold from an amebal homogenate. At pH 7 with 2.5 mM fructose 6-phosphate, the enzyme has been purified 100-fold from an amebal homogenate. At pH 7 with 2.5 mM MgCl₂ the following Kₐ values were observed: pyrophosphate, 14 μM; fructose 6-phosphate, 38 μM; fructose 1,6-diphosphate, 18 μM; and orthophosphate, 800 μM. At this pH and magnesium ion concentration the maximum velocity of the reaction was approximately the same in each direction. The average intracellular concentration of pyrophosphate in the ameba is 0.18 mM, a value 13-fold greater than the pyrophosphate Kₐ for the new enzyme. This enzyme raises to the ameba is 0.18 mM, a value 13-fold greater than the pyrophosphate Kₐ for the new enzyme. This enzyme raises to the number of known enzymes in the glycolytic pathway of E. histolytica which utilize or produce pyrophosphate. The trivial name proposed for the new enzyme is 6-phosphofructokinase (PP₁).

EXPERIMENTAL PROCEDURE

Entamoeba histolytica, strain H200, was grown in flasks in Diamond's axenic medium (1). Cells in growth medium packed by centrifugation for 3 min at 800 X g weighed 1.04 g per ml of packed volume. They were twice washed by centrifugation at room temperature in a solution containing 100 mM NaCl, 20 mM K₂HPO₄, 0.5 mM MgCl₂, and 0.1 mM Ca(NO₃)₂ adjusted to pH 7 with HCl.

Assay and Linking Enzymes—Pyruvate, orthophosphate dikinase (EC 2.7.9.1) was prepared from Bacteroides symbiosus by the method of Reeves (3). Other assays and linking enzymes were Boehringer products. Their suspensions in ammonium sulfate solution were centrifuged and each pellet was dissolved in 1 ml sodium EDTA, pH 7. Alkaline phosphatase and the glycerol-3-phosphate dehydrogenase-triosephosphate isomerase mixture were further treated by dialysis overnight against 300 volumes of the EDTA solution. These two enzymes were free from inorganic pyrophosphatase activity, but the yeast glucosephosphate isomerase was contaminated with a significant amount of this enzyme. The aldolase solution contained about 0.5% of glucosephosphate isomerase activity.

Substrate and Product Assays—The concentrations of some of the substrates and products of the reactions were assayed spectrophotometrically using coupled enzymatic assays and employing a millimolar absorbance (340 nm) of 0.23 for DPNH and TPNH. Fructose 6-phosphate was assayed with glucose-6-phosphate dehydrogenase followed by glucosephosphate isomerase; fructose diphosphate, with glycerol-3-phosphate dehydrogenase and triosephosphate isomerase followed by aldolase; inorganic pyrophosphate (PP₁) by lactate dehydrogenase followed by pyruvate, orthophosphate dikinase by a procedure adapted from the method of Reeves and Malin (3). Orthophosphate was assayed by the method of Lowry and Lopez (4).

Standard Assay—The standard assay for the pyrophosphate-specific phosphofructokinase contained 50 mM imidazole-HCl, pH 7.9, 5.5 mM MgCl₂, 1 mM sodium PP₁, 1.4 mM fructose 6-phosphate, 0.2 mM DPNH, 50 μg of aldolase, 13 μg of the mixture of glycerol-3-phosphate dehydrogenase-triosephosphate isomerase, enzyme, and water to a final volume of 0.40 ml. Reaction was started by the addition of the enzyme. Samples were monitored in 1 cm quartz cuvettes at 340 nm and 30° in a model 200 or model 240 Gilford spectrophotometer equipped with a thermostatted sample compartment, sample changing device, and a recorder readout. Correction was made for any reaction noted in a control cuvette lacking PP₁. After the first column fractionation step this correction was negligible.

The one unit of activity is that amount of enzyme which produces 1 μmol per min of fructose diphosphate under the conditions of the standard assay. Specific activity is units of enzyme per mg of protein.

Other Assays—The assay for the ATP-utilizing amebal phosphofructokinase was the same as the standard assay, except that 1 mM ATP was substituted for the PP₁. Two types of assays were
used to study the reverse reaction catalysed by the pyrophosphate-utilizing enzyme. Fructose 6-phosphate formation was assayed by glucosephosphate isomerase and glucose-6-phosphate dehydrogenase in the presence of TPN. The details of this assay are given in the legend to Fig. 3. PP formation was assayed by pyruvate, orthophosphate dikinase and lactate dehydrogenase in the presence of phosphoenolpyruvate, AMP, and DPNH, as detailed in the legend to Fig. 4.

Protein was determined by the method of Lowry et al. (5) with bovine serum albumin as the standard. After purification through the DEAE-cellulose column it was found that 1 mg per ml of colorimetrically determined protein was equivalent to an absorbance of 1 unit at 280 nm. The relationship, $A_{280}^{\text{protein}} = 1$, was employed to calculate some of the protein concentrations during the last stages of the enzyme purification.

The nucleotide triphosphates were commercial products. Assays on freshly prepared solutions from two different lots of P-L Laboratories GTP showed them to contain 10% PP, on a molar basis. CTP and UTP from the same source contained about 3% PP. ATP from Sigma was essentially free from this contaminant. The assays on the first three substances indicate a greater PP contamination than was found previously (3) with other lots of these substances. Except when another buffer composition is specifically indicated in the following text, the buffer is 20 mM imidazole-HCl, pH 7.

RESULTS

Identification of Two 6-phosphofructokinases in Amoeba—The total activity of the ATP-dependent enzyme in amebal homogenates is influenced by the method of preparation of the homogenate while that of the PP-utilizing enzyme is not. To achieve the maximum yield of the ATP enzyme, 1.25 ml of fresh cells were suspended in 5 ml of 20 mM α-glycerol phosphate, pH 7, containing 2 mM dithiothreitol, and the cells were ruptured by 15 strokes with the close-fitting pestle of a Dounce homogenizer (Kontes Glass Co.). After centrifugation for 30 min at 36,000 × g the supernatant solution contained 3.1 and 28 units of the ATP and PP enzyme, respectively. The fractionation of this supernatant solution from cells ruptured in a Dounce homogenizer (Kontes Glass Co.) after centrifugation for 30 min at 36,000 × g the supernatant solution contained 3.1 and 28 units of the ATP and PP enzyme, respectively. The fractionation of this supernatant solution on a column of Bio-Gel P-300 is shown in Fig. 1. The results suggest that the two enzyme activities belong to distinct proteins.

Evidence That Two Enzyme Activities Represent Distinct Proteins—In addition to the evidence shown in Fig. 1 we have made other preliminary observations which indicate that the activities with ATP and PP are due to distinct proteins. The ATP enzyme is sedimented by 4 hours centrifugation at 100,000 × g while the PP enzyme is not. The latter activity is undiminished by lyophilization of whole cells while the former is completely destroyed by this treatment. The ATP enzyme activity is not present in cell homogenates prepared by vigorous sonication while the PP enzyme is not diminished by this treatment. When a fraction from a Bio-Gel P-300 column similar to Fraction 5 of Fig. 1 was subjected to sonication for 15 s with the microtip of a Bronson Sonifier at power setting 5, the ATP activity was reduced by 84% while the PP enzyme activity remained unchanged.

Purification of PP-utilizing Enzyme—Nine-tenths milliliter of fresh cells was suspended in 3.6 ml of α-glycerol phosphate-dithiothreitol buffer and treated, with cooling, for a total of 30 s with the microtip of a Bronson Sonifier at power setting 5. The ATP enzyme could not be detected in this sonicated homogenate. After centrifugation at 36,000 × g for 30 min the PP-utilizing enzyme in the supernatant solution was applied to the same Bio-Gel P-300 column (Fig. 1) and eluted as described above. The elution pattern of this enzyme was sharper than that obtained with the Dounce homogenate, possibly because of the more effective cell disruption provided by sonication. The enzyme-rich fractions from this column were combined and applied to a column containing hydroxylapatite (Bio-Gel HT), bed volume 35 ml. Enzyme was eluted with a linear gradient formed by placing 50 ml of 0.4 M potassium phosphate in 20 mM imidazole-HCl, pH 7, in the reservoir and 50 ml of the imidazole buffer at the same pH in the mixing chamber. Enzyme appeared in the effluent solution after 56 ml of the gradient had entered the column. The peak fractions were combined and dialyzed overnight against two 500-ml portions of the imidazole buffer. The dialyzed solution was applied to a column of DEAE-cellulose, bed volume 12 ml, which had been equilibrated with the buffer. Enzyme was eluted from this column by a linear gradient formed with 50 ml of 0.4 M NaCl in the imidazole buffer in the reservoir and 50 ml of the buffer in the mixing chamber. Enzyme activity eluted with the first protein peak. The two fractions with greatest activity were combined and concentrated to 3.5 ml by vacuum dialysis against 150 ml of the imidazole buffer. This solution was then applied to a column containing 112 ml bed volume of Sephadex G-100 and enzyme was eluted with the same buffer. The ratio of enzyme activity to $A_{280}$ was equal in the two fractions which comprised the enzyme peak. Fractions were assayed for glucose phosphate isomerase throughout the purification and selected for the subsequent step with a view to excluding this activity as far as possible. This resulted in the deliberate discarding of much of the phosphofructokinase in order to achieve low isomerase activity in the final product. Enzyme from the DEAE-cellulose column contained isomerase activity to about 1% that of the kinase and this contamination was cut in half by the subsequent fractionation on Sephadex. The purification procedure is summarized in Table 1.

Stoichiometric Experiment—The results of an experiment shown in Table 2 clearly reflect the stoichiometric conversion of fructose 6-phosphate and inorganic pyrophosphate to fructose diphosphate and orthophosphate during incubation. Glucose 6-phosphate and triose phosphate were separately assayed in this experiment and their quantities were found to have increased by only 0.1 μmol each during the 15-min incubation.

Kinetic Studies—The effects of substrate concentrations upon the initial velocity of the forward and reverse reactions were de-
Purification of pyrophosphate enzyme from 0.9 g of E. histolytica

| Treatment          | ml | Units | Recovery % | Protein mg | Specific Activity |
|--------------------|----|-------|------------|------------|------------------|
| Homogenate         | 4.5| 29    | 84         | 0.43       |                  |
| Supernatant        | 4.0| 32    | 100        | 3.2        | 1.0              |
| Bio-Gel P-300      | 32.0| 25   | 78         | 6.2        | 4.0              |
| Hydroxylapatite    | 16.7| 17   | 53         | 2.4        | 7.1              |
| DEAE-cellulose     | 3.5| 11    | 34         | 0.8        | 13.7             |
| Sephadex G-100     | 11.0| 6.4  | 20         | 0.14       | 45.7             |

Protein determined by the relationship $A_{650nm} = 1.0$.

Quantitative changes in substrates and products of enzyme-catalyzed reaction

One-half unit of enzyme from the DEAE-column, 38 μg of protein, was added to substrates of the forward reaction in 20 mM imidazole buffer, pH 7.0, containing 2.5 mM MgCl₂. The final volume was 9 ml. A sample of the solution was withdrawn and added to an equal volume of cold 6% perchloric acid at an arbitrary zero time. The remainder of the solution was then incubated at 25°C for 15 min, at which time a second sample was treated similarly. Both acidified samples were centrifuged. The supernatant solutions were then neutralized with 1 N KOH, chilled, and again centrifuged. The final supernatant solutions were assayed for substrates and products by the methods described under "Experimental Procedure.”

| Amount in total incubation mixture | Fructose-6-P | PPᵢ | Fructose diphosphate | Pᵢ |
|-----------------------------------|-------------|-----|---------------------|----|
| Initial                           | 8.7         | 12.4| 0.1                 | 2.3|
| Incubated                         | 3.1         | 6.0 | 5.7                 | 7.5|
| Change                            | -5.6        | -5.5| +5.6                | +5.2|

That hyperbolic kinetics was obeyed with each of the forward reaction substrates is indicated by the linear plots in double reciprocal coordinates shown in Fig. 2. From Fig. 2A we calculate the $K_m$ for PPᵢ to be 14 μM and we note that the true $V_{max}$ is about 0.25 μmol of fructose diphosphate formed per min per ml of the enzyme solution. From Fig. 2B we calculate the $K_m$ for fructose 6-phosphate to be 38 μM.

The same enzyme solution was used to study the kinetics of the reverse reaction. Again, hyperbolic kinetics was obeyed by each substrate. Fructose 1-phosphate was not a substrate for the enzyme. Glucose 6-phosphate reacted with crude enzyme which was heavily contaminated by isomerase, but enzyme from the DEAE- or Sephadex columns failed to react appreciably when this substance was substituted for fructose 6-phosphate in the standard assay. In conducting these experiments, a no enzyme control is essential because the assay enzyme system contains a small amount of glucosephosphate isomerase.

That added Pᵢ is essential for the reverse reaction is illustrated by the curves or Fig. 4. Curves A and B are plotted from the ordinate intercepts of B and A, respectively.
FIG. 3. Double reciprocal plots of reaction velocities in the reverse direction. The enzyme solution was the same as that used in Fig. 2. A, Pi as the varied substrate. Cuvettes contained 50 mM imidazole-HCl, pH 7, 2.5 mM MgCl₂, 0.5 mM TPN, 4 μg of glucose-6-phosphate dehydrogenase, 2 μg of glucosrophosphate isomerase, potassium phosphate, pH 7, as indicated on the abscissa, fructose 1,6-diphosphate, and 20 μl of enzyme solution in a final volume of 0.4 ml. Reaction was initiated by the addition of enzyme. The micromolar concentrations of fructose diphosphate are indicated by numbers above the respective curves. The ordinate is the reciprocal of micromoles of fructose 6-phosphate formed per min per ml of the enzyme solution. B, the same data with fructose 1,6-diphosphate (FP₂) as the varied substrate. The fixed micromolar concentrations of Pi are shown as numbers above each respective curve. Ordinate values are as in A. The broken curves in A and B are plotted from the ordinate intercepts of the solid line curves of B and A, respectively.

which the assayed product was PP₁. It is evident that this product was not formed at a significant rate until Pi was added to the reaction mixture. After the addition of Pi, all of the fructose diphosphate contained in cuvette B was recovered as PP₁. The slight activity noted before the addition of Pi is believed to be due to traces of Pi in the rather complex assay system. Curves C and D record an experiment in which fructose 6-phosphate was the assayed product. It was not formed until Pi was added to the reaction mixture. Almost all of the fructose diphosphate in cuvette D was recovered as fructose 6-phosphate within the time span of the experiment.

Metabolite Concentrations in Amoeba—Cells harvested from growth medium were taken up in medium lacking serum, incubated anaerobically 30 min at 37°, sedimented, and quickly washed twice with a balanced salt buffer which had been bubbled with nitrogen containing 5% CO₂. Supernatant fluid was removed from the final cell pellet and cold 6% perchloric acid was stirred into the pellet with a thin glass rod. The cells were disrupted by two 15-s treatments, with cooling, with the microtip of a Bronson Sonifier at power setting 5. After centrifugation for 20 min at 36,000 × g the supernatant fluid was neutralized and assayed for metabolites by methods described under "Experimental Procedure." The assayed concentrations were corrected to intracellular concentration by taking the value 0.80 to represent the ratio of intracellular fluid to packed cell volume. Average intracellular concentrations from four or five assays on each metabolite are as follows, in millimolar concentration with standard deviation: PP₁, 0.18 ± 0.02; fructose diphosphate, 0.13 ± 0.04; fructose 6-phosphate, 0.16 ± 0.06; and ATP, 0.65 ± 0.14. The average of two closely agreeing determinations on orthophosphate (in cells washed twice with phosphate-free buffer) was 2.8 mM.

DISCUSSION

The pyrophosphate-dependent enzyme was first observed acting in its reverse direction in a phosphate-containing buffer. We saw evidence of what appeared to be a specific fructose diphosphate 1-hydrolase with great affinity for its substrate. This was puzzling for two reasons: amoeba grow only when glucose is supplied and have no apparent need for a gluconeogenic pathway; secondly, this anaerobic organism could ill afford the energy dis-
ipation implicit in such great hydrolase activity. Later work proved that the supposed hydrolase activity was that of the new enzyme which, in the reverse direction, produced PP\textsubscript{i} and fructose 6-phosphate from orthophosphate and fructose 1,6-diphosphate. This enzyme is now called 6-phosphofructokinase (PP\textsubscript{i}) to distinguish it from the ATP-utilizing 6-phosphofructokinase also present in ameba.

Among the substances tested this enzyme is specific for fructose 6-phosphate and PP\textsubscript{i} in the forward reaction. Each of the four common nucleoside triphosphates failed to show activity as phosphate donor. In the reverse reaction both P\textsubscript{i} and fructose diphosphate are essential in order for the reaction to proceed.

We have not found prior reference to a phosphofructokinase which employs PP\textsubscript{i} as phosphate donor. Bar-Tana and Cleland (6) recently found that MgPP\textsubscript{i} and MgPPPP\textsubscript{i} are not substrates, but are inhibitors of rabbit muscle phosphofructokinase. They were competitive against ATP and had $K_i$ values of less than 1 mM.

Wood, Davis, and Lochmüller (7) studied the free energy of hydrolysis of the PP\textsubscript{i} bond. Their results show the apparent value, based on sums of all ionic species, to vary markedly with magnesium ion concentration. They report $\Delta F'_\text{mol}$ to be $-8.0 \pm 0.3$ Cal per mol in the absence of magnesium and $-4.5 \pm 0.3$ Cal per mol in the presence of 5 mM magnesium ion. In view of this it is probable that the reaction catalyzed by the enzyme described here will be profoundly influenced by magnesium ion concentration, particularly the ratio of rates of reaction in the forward versus reverse directions.

Unpublished observations from this laboratory indicate that the glycolytic flux of ameba may reach 3 \( \mu \)mol of glucose per min per ml of packed cell volume.\footnote{1} This value exceeds the assayed activity of the ATP-utilizing phosphofructokinase. Further, the ATP enzyme requires 0.3 mM fructose 6-phosphate for half-maximal activity.\footnote{2} These findings together with the observed intracellular metabolite levels imply that the physiological activity of the ATP enzyme would be much lower than the glycolytic flux in ameba. On the other hand, the 6-phosphofructokinase (PP\textsubscript{i}) could account for the required net synthesis of fructose diphosphate.

This enzyme raises to three the number of reversible enzymes utilizing inorganic pyrophosphate that are known to operate in the glycolytic pathway of \textit{E. histolytica} (8-10). The other two are pyruvate, orthophosphate dkinase and phosphoenolpyruvate carboxykinase (pyrophosphate) (EC 4.1.1.38). The repeated substitution of pyrophosphate for a nucleoside triphosphate suggests that amebal metabolism may have diverged very early from the evolutionary pattern of development followed by most other investigated organisms. The physiological significance of these pyrophosphate-utilizing enzymes is emphasized by our observation that pyrophosphate is present in ameba at a level comparable to that of other metabolites.

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\footnote{1} R. Serrano, unpublished observations.

\footnote{2} R. E. Reeves, unpublished evidence.
