Isolation and Characterization of a Pyrophosphate-dependent Phosphofructokinase from Propionibacterium shermanii*

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A pyrophosphate dependent phosphofructokinase (pyrophosphate; D-fructose 6-phosphate 1-phosphotransferase) has been purified and characterized from extracts of Propionibacterium shermanii. The enzyme catalyzes the transfer of phosphate from pyrophosphate to fructose 6-phosphate to yield fructose-1,6-P_2 and phosphate. This unique enzymatic activity was observed initially in Entamoeba histolytica (Reeves, R. E., South, D. J., Blytt, H. G., and Warren, L. G. (1974) J. Biol. Chem. 249, 7734-7741). This is the third pyrophosphate-utilizing enzyme that these two diverse organisms have in common. The others are phosphoenolpyruvate carboxytransphosphorylase and pyruvate phosphate dikinase. The PP_i-phosphofructokinase from P. shermanii is specific for fructose-6-P and fructose-1,6-P_2, no other phosphorylated sugars were utilized. Phosphate could be replaced by arsenate. The K_m values are: phosphate, 6.0 × 10^{-5} M; fructose-1,6-P_2, 5.1 × 10^{-5} M; pyrophosphate, 6.9 × 10^{-5} M; and fructose-6-P, 1.0 × 10^{-4} M. The s_{20,w} is 5.1 S. The molecular weight of the native enzyme is 95,000.

Sodium dodecyl sulfate electrophoresis of the enzyme showed a single band migrating with an R_f corresponding to a molecular weight of 48,000. Extracts of P. shermanii have PP_i-phosphofructokinase activity approximately 6 times greater than ATP-phosphofructokinase and 15 to 20 times greater than fructose diphosphatase activities. It is proposed that (a) PP_i may replace ATP in the formation of fructose-1,6-P_2 when the organism is grown on glucose and (b) when the organism is grown on lactate or glycerol the conversion of fructose-1,6-P_2 to fructose-6-P during gluconeogenesis may occur by phosphorylation rather than hydrolysis.

The discovery of a pyrophosphate-dependent phosphofructokinase (pyrophosphate; fructose-6-phosphate 1-phosphotransferase) in Entamoeba histolytica by Reeves et al. (1) prompted a search for a similar enzyme activity in Propionibacterium shermanii. Such an investigation was not undertaken without precedent. These two widely divergent organisms, one eukaryotic, one prokaryotic, also have in common two other unique pyrophosphate-utilizing enzymes: pyruvate phosphate dikinase, which is found not only in these two organisms (2-5) but also in some tropical grasses (6, 7) and phosphoenolpyruvate carboxytransphosphorylase, which has been demonstrated only in these two organisms (8-14).

PP_i-phosphofructokinase catalyzes the reaction shown below.

\[
\text{Fructose-6-P} + \text{PP}_i \xrightleftharpoons{\text{Me}^{2+}} \text{fructose-1,6-P}_2 + \text{P}_i
\]

This communication describes the purification and physical and chemical properties of the enzyme from P. shermanii. The possible role of pyrophosphate in the metabolism of P. shermanii is discussed.

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EXPERIMENTAL PROCEDURE

Cell Material

Propionibacterium shermanii was cultured as described by Wood et al. (15).

Enzymes

Phosphoglucoisomerase, glucose-6-phosphate dehydrogenase, aldolase, triosephosphate isomerase, glycerol phosphate dehydrogenase, and malate dehydrogenase were purchased from Boehringer. Inorganic pyrophosphatase was obtained from Worthington.

Chemicals

NADP, NADH, fructose 6-phosphate, fructose 1,6-diphosphate, fructose 1-phosphate, glucose 1,6-diphosphate, sedoheptulose 1,7-diphosphate, ribulose 1,5-diphosphate, ATP, and AMP were purchased from Sigma. All other chemicals were reagent grade.

Enzyme Assays

A. Fructose 6-Phosphate Assay—The assay mixture contained in micromoles, imidazole-HCl, pH 6.8, 20; fructose-1,6-P_2, 6.5; MgCl_2, 1.25; potassium phosphate, pH 7.0, 2.00; NADP, 0.10; and in units, glucose-6-phosphate dehydrogenase, 0.15; phosphoglucoisomerase, 0.1; in a total volume of 0.35 ml.

B. Fructose-1,6-P_2 Assay—The assay mixture contained in micromoles, imidazole-HCl, pH 7.4, 20; pyrophosphate, 0.35; MgCl_2, 1.25; fructose-6-P, 0.5; NADH, 0.06; and in units, aldolase, 0.1; triosephosphate isomerase, 1.9; glycerol phosphate dehydrogenase.
An extinction coefficient of 6.22/μmol at 340 nm was utilized for the determination of micromoles of product formed.

**C. "PP_i Assay—**This assay was adapted from that reported by Carter and Thuiller (16). The mixture contains in micromoles, fructose-1,6-P_2, 0.5; MgCl_2, 1.25; imidazole-HCl, pH 7.0, 6.8; 20 μl potassium [PP_i] phosphate 2.00 (specific activity approximately 10^6 dpm/μmol, determined daily) in a final volume of 0.5 ml. The reaction is terminated by addition of 0.2 ml of 40 mM ammonium molybdate in 2.5 N H_2SO_4 followed immediately by the addition of 0.1 ml of 20 mM sodium pyrophosphate and then mixed. Then 0.2 ml of 100 mM triethylamine-HCl, pH 9.0, is added and the reaction mixture is mixed again. The precipitate that results is removed by centrifugation and an aliquot of the supernatant solution is removed for determination of the radioactivity. The results obtained from Assays A and C were identical. All enzyme assays were conducted at 25°C.

Fructose diphosphatase assays contained in micromoles: Tris-Cl, pH 8.8, 30; MgCl_2, 1.5; EDTA, 0.5; fructose-1,6-P_2, 0.2; NADP, 0.15; and in units: glucose-6-P dehydrogenase, 0.15; and phosphoglucoisomerase, 0.1; in a final volume of 0.35 ml. ATP-phosphofructokinase assays contained in micromoles: glycylglycine, pH 8.0, 15; MgCl_2, 1.5; cysteine-HCl, 1.5; fructose-6-P, 2.0; ATP, 0.15; NADH, 0.06; and in units: glycophosphate dehydrogenase, 0.32; aldolase, 0.1; and triosephosphate isomerase, 1.9; in a final volume of 0.36 ml.

**Analytical Procedures**

Disc gel electrophoresis was performed as described by Brewer and Ashworth (17), the sodium dodecyl sulfate gel electrophoresis as described by Weber and Osborn (18), and the sucrose density centrifugation as described by Martin and Ames (19). Sedimentation behavior of the protein upon electrophoresis at pH 8.9 is depicted in Fig. 1A. The enzyme from Step 5 of Table I sediments to essentially homogeneous.

**Table I**

| Step | Total units | Total protein | Specific activity | Recovery | Purification |
|------|-------------|---------------|------------------|----------|--------------|
| i.u. | mg          | i.u./mg       |                  | %        |              |
| 1. Crude extract | 18,000 | 46,000 | 0.39 | 100 | 1 |
| 2. 35 to 55% saturated (NH_4)_2SO_4 | 17,400 | 23,200 | 0.74 | 96 | 1.9 |
| 3. First cellulose-PO_4 column | 13,200 | 13,500 | 0.98 | 74 | 2.5 |
| 4. Second cellulose-PO_4 column | 4,810 | 24 | 192 | 43 | 493 |
| 5. (NH_4)_2SO_4 back-extraction | 3,150 | 24 | 132 | 33 | 338 |

*a The protein measurements were determined by the method of Warburg and Christian (21). Protein determinations made with the biuret reaction (20) gave values which were larger by a factor of 1.4.

*b All activity measurements were obtained by the fructose-6-P assay as described under "Experimental Procedure."

**RESULTS**

**Criteria of Purity—**The enzyme from Step 5 of Table I routinely gave rise to two bands on the standard polyacrylamide gel system of pH 8.9 as shown in Fig. 1A. The behavior of the protein upon electrophoresis at pH 8.9 is perhaps due to an association-dissociation induced by the high pH of the electrophoretic system. On electrophoresis in the presence of sodium dodecyl sulfate, only one band was observed; Fig. 1B. The enzyme from Step 5 of Table I sediments as a single symmetrical peak in the analytical ultracentrifuge, Fig. 1C. Based on these criteria we believe the enzyme to be essentially homogeneous.

**Substrate Specificity and Stoichiometry of Reaction—**Table II shows the definite specificity of the enzyme for fructose-6-P and PP_i in one direction and fructose-1,6-P_2 in the reverse direction. Nucleotide triphosphates nor ADP would replace PP_i in the phosphorylating reaction. Fructose-1,6-P_2 was not phosphorylated nor dephosphorylated by the enzyme suggesting that the phosphorous atom on carbon 6 is required for binding of the substrate. Neither glucose-1,6-P_2 nor ribulose-1,5-P_2 were utilized. Although not shown in Table II, sedoheptulose-1,7-P_2 gave some activity with Assay C, but this compound had a large amount of fructose-1,6-P_2 contaminant and at the present time we are unable to draw a conclusion regarding the utilization of this compound by the enzyme. As is observed with some other enzymes which utilize inorganic
phosphate as a substrate, arsenate will replace phosphate. Although the $K_m$ for arsenate appears to be much higher, the $V_{max}$ approaches the same value as obtained with phosphate. Proof that the reaction proceeds as presented in Reaction 1 is given in Table III. There is a one to one ratio for fructose-6-P or PP$\_1$ utilization and fructose-1,6-P$_2$ formation.

**Reaction Parameters for PP$_1$-phosphofructokinase**—Table IV gives the Michaelis constants for all substrates as determined from double reciprocal plots. All kinetic patterns for the substrates were intersecting, thereby ruling out the possible involvement of an intermediate phosphorylated enzyme. Slope and intercept lines were linear. The apparent $K_m$ values for Mg$^{2+}$ and Mn$^{2+}$ (not given in Table IV) were determined to be $8.3 \times 10^{-5}$ and $6.4 \times 10^{-6}$ M, respectively, in the fructose-6-P forming reaction. The data are for total metal concentration and no attempt was made to calculate free and bound metal. The fructose-1,6-P$_2$ forming reaction has a slightly higher $V_{max}$ under the conditions of the assay.

Both the forward and reverse reactions have pH optima at 7.4 in imidazole-HCl buffer.

The enzyme activity is not affected by ATP, AMP, phosphoenolpyruvate, malate, pyruvate, or citrate at 1 mM.

**Determination of Molecular Weight and Sedimentation Coefficient for Enzyme**—The molecular weight of the enzyme was determined by high speed meniscus depletion in the model E ultracentrifuge. The log $C$ versus $r^2$ plot was linear attributing to the purity of the preparation. The molecular weight calculated from the data was 95,000. The subunit molecular weight was determined by electrophoresis in sodium dodecyl sulfate (18). These results are presented in Fig. 2. PP$_1$-phosphofructokinase migrated with an $R_f$ of 0.30 corresponding to a minimum molecular weight of 48,000 for the polypeptide chain. From these observations we conclude that the enzyme is a dimer composed of two subunits of identical molecular weight.

The effect of protein concentration on the sedimentation coefficient was investigated from 100 to 900 pg/ml. There is no effect of protein on the $s_{20,w}$ at the concentrations tested. A value of 5.1 was determined for the $s_{20,w}$.

**Substrates for Growth and Effects on Enzymes Utilizing Fructose-1,6-P$_2$**—The activity of enzymes which utilize fructose-1,6-P$_2$ was measured in cells grown on various substrates to determine whether the specific activities of the enzymes varied under different conditions of growth. The results are shown in Table V. In the case of the PP$_1$-phosphofructokinase, the highest activity was observed in glycerol.

**Table II**

| Product assayed | Possible substrates | Substrate concentration | Activity as % of normal substrate |
|-----------------|---------------------|-------------------------|----------------------------------|
| Fructose-1,6-P$_2$ by Assay A | PP$_1$ | 0.87 | 100 |
| Fructose-6-P by Assay B | ATP or ITP or GTP | 0.87 | 0 |
| Fructose-1,6-P$_2$ by Assay C | Glucose-6-P | 0.87 | 100 |

* Both UTP and CTP gave some activity. However, this activity was due to PP$_1$ contamination as demonstrated by complete abolition of activity by treatment of the UTP and CTP solutions with yeast pyrophosphatase.

* In this case glucose-6-P formation was assayed by merely omitting the phosphoglucoisomerase from the normal Assay A.
Protein was determined by the biuret reaction.

PI Fructose-1,6-P, pp, Fructose-6-P

The organism apparently lacks pyruvate kinase (30) but has sufficient pyruvate, phosphate dikinase activity, Reaction ii, to account for its glycolytic rate (2). Recently, Reeves et al. (1) have reported the presence of a PPi-phosphofructokinase in this organism. These authors propose that the enzyme functions in a glycolytic capacity forming fructose-1,6-P,. This hypothesis is based on their observations that the organism does not contain sufficient ATP-phosphofructokinase activity to account for its glycolytic flux. Also, the intracellular level of PPi in the amoeba is 0.18 mM which is 13 times greater than the Km of the PPi-phosphofructokinase for this metabolite.

**DISCUSSION**

The discovery of PPi-phosphofructokinase in *P. shermanii* brings to four the number of enzymes isolated from this organism that are capable of utilizing PPi as a phosphate donor in reactions in which nucleotide triphosphates normally participate. The other three enzymes are carboxytransphosphorylase; pyruvate, phosphate dikinase; and pyrophosphate, L-serine phosphotransferase (22). The reactions are given below.

\[
\text{Fructose-6-P + PPi} \rightarrow \text{fructose-1,6-P, + Pi} \quad \text{(iv)}
\]

**TABLE IV**

| Substrate | Kinetic pattern | \( K_m \) (M) | \( V_{max} \) (\( \mu \text{mol/min/mg} \)) |
|-----------|----------------|---------------|----------------------------------|
| Fructose-6-P | Intersecting | \( 1.0 \times 10^{-4} \) | 118 |
| PPi | | \( 6.9 \times 10^{-5} \) | 258 |
| Fructose-1,6-P, | Intersecting | \( 5.1 \times 10^{-3} \) | 232 |
| Pi | | \( 6.0 \times 10^{-4} \) | |

**TABLE V**

| Enzyme | Substrates for growth |
|--------|-----------------------|
| PPi-phosphofructokinase | Glycerol | Glucose | Lactate |
| ATP-phosphofructokinase | 0.29 | 0.19 | 0.27 |
| Fructose diphosphatase | 0.017 | 0.010 | 0.010 |

**FIG. 2.** Determination of molecular weight by sodium dodecyl sulfate electrophoresis. The standard gel system described in Ref. 18 was employed. The standard proteins applied to the gels were (1) bovine serum albumin, 68,000; (2) aldolase, 40,000; and (3) chymotrypsinogen, 25,700. Approximately 20 \( \mu \)g of each protein were applied. The designation PT refers to the position of the PPi-phosphofructokinase. and lactate-grown cells. The activity is somewhat lower in glucose-grown cells. The activity of the ATP-phosphofructokinase and fructose diphosphatase was also investigated. There was very little fructose diphosphatase activity regardless of growth substrates. The activity of the PPi-phosphofructokinase under all conditions was 5- to 6-fold higher than the ATP-phosphofructokinase and 10- to 20-fold higher than the fructose diphosphatase. It is difficult to assess the true activity of these enzymes in crude extracts because of our lack of knowledge concerning the optimum assay conditions for the ATP-dependent phosphofructokinase and fructose diphosphatase. The highest activity of these enzymes was observed at pH 8.0 and 8.5, respectively. The addition of AMP had no effect on the activity of the ATP-phosphofructokinase. There was no change in the activity of these three enzymes upon sedimentation for 1 hour at 124,000 \( \times g \). The ATP-phosphofructokinase in *Entamoeba histolytica* was found to be membrane-bound by Reeves et al. (1).
Therefore, they suggested that high energy phosphate must be produced in the reactions. The numbers in parentheses indicate the moles of pyrophosphate (PPi) formed in the reaction. Very little PPi would be formed by the reaction. The finding of a third enzyme in the metabolic pathway which utilizes PPi re-emphasizes this possibility. It is notable that the PPi-phosphofructokinase reaction provides a means of salvaging the bond energy of the PPi which arises during the synthesis of fats, carbohydrates, proteins, and nucleic acids. This may, in part, account for the highly efficient growth of the propionic acid bacteria as compared to some other microorganisms.

If the propionic acid fermentation occurred by the following equation,

\[ 3 \text{ Glucose} \rightarrow 4 \text{ propionate} + 2 \text{ acetate} + 2 \text{ CO}_2 \]

per 3 mol of fermented glucose, 6 mol of ATP would be utilized at A and B (if we assume ATP is used in B), 12 would be formed at C and D, and 2 at E, or a net of 2.66 ATP per mol of glucose, without a contribution by G. If there also is formation of ATP during succinate formation (G), one ATP would be formed for each propionate or a net total of four ATP per mol of glucose.

We have considered previously (33, 34) that the high growth efficiency of the propionic acid bacteria might result from their utilization of the energy from PPi. The finding of a third enzyme in the metabolic pathway which utilizes PPi re-emphasizes this possibility. It is noteworthy that the amount of ATP-phosphofructokinase found in crude extracts of *P. shermanii* which was used in G of Fig. 3. Such electron transport-linked ATP formation has been shown in the obligately anaerobic sulfur bacteria (32) but this has not been shown as yet in propionibacteria.

FIG. 3. Mechanism of the propionic acid fermentation and role of PPi in the reactions. The numbers in parentheses indicate the moles of substrate or product.

high growth efficiency compared to the other bacteria studied by them and they estimated that a net of at least 6 mol of ATP were formed in the fermentation per mol of fermented glucose. Therefore, they suggested that high energy phosphate must be expected if its function is primarily anaerobic as indicated in Fig. 3. The enzyme is particularly important for cells growing on lactate or pyruvate, since the P-enolpyruvate required for anaerobic purposes must be synthesized from pyruvate in this case. It is to be noted that extracts of *P. shermanii* contain pyruvate kinase in sufficient quantity to account for the formation of pyruvate from P-enolpyruvate at the required glycolytic rate. The carboxytransphosphorylase reaction (Box F) likewise serves an anaplerotic function, since the oxaloacetate of the main metabolic pathway is supplied by the transcarboxylase reaction and the carboxytransphosphorylase is only required to replenish C4-dicarboxylic acids which are withdrawn from the cycle when succinate is a fermentation product and for synthesis of aspartate and other compounds.

The crude extract contains 0.1 unit of carboxytransphosphorylase/mg of protein (36). Thus, the scheme shown in Fig. 3 does not provide a major source of PPi, unless there is formation of PPi during the reduction of fumarate to succinate (Box G of Fig. 3). It is possible that PPi may be generated in this step by an electron transport-coupled phosphorylation linked to the reduction of a flavoprotein by NADH and the reduced flavoprotein may then be reoxidized in the reduction of the fumarate to succinate. A coupled synthesis of PPi during light-induced electron transport of photosynthetic bacteria (23) provides some precedence for such consideration.

It is to be noted that the PPi-phosphofructokinase reaction provides a means of salvaging the bond energy of the PPi which arises during the synthesis of fats, carbohydrates, proteins, and nucleic acids. This may, in part, account for the highly efficient growth of the propionic acid bacteria as compared to some other microorganisms (31).

A second problem relative to the proposal that PPi-phosphofructokinase may act in both the synthesis and breakdown of fructose 1,6-diphosphate is the mechanism of control. In mammalian cells, the ATP-phosphofructokinase and fructose diphosphatase reactions provide different routes for glycolysis and gluconeogenesis which are controlled reciprocally by allosteric effectors (37). This control prevents futile cycles which would result in an ATPase-like activity by the two enzymes. Thus far, no allosteric inhibitors of PPi-phosphofructokinase have been found. In fact, if the PPi-phosphofructokinase serves in both directions, allosteric inhibition would not be expected. It is conceivable that the concentration of metabolites and the thermodynamic properties of the reaction are such as to permit the necessary control and to prevent futile cycling. The equilibrium of the PPi-phosphofructokinase reaction favors the formation of fructose-1,6-P2 and is strongly dependent on divalent metals. There probably are controls of the propionic acid fermentation at other points (9). If there were no specific controls of the PPi-phosphofructokinase the flux through fructose-1,6-P2 would depend upon the kinetic properties of the enzyme and the intracellular concentrations of the substrates and products of the reaction.

The PPi-phosphofructokinase from *P. shermanii* does not appear to have any structural similarities either with the mammalian or bacterial fructose diphosphatases (38) which are generally larger and composed of four identical subunits nor with the ATP-phosphofructokinases (39). It will be inter-

* Unpublished observations.
esting to compare the mechanism of action of the PP₁-phosphofructokinase with these other enzymes. The PP₁-phosphofructokinase from propionibacteria may be very similar not only chemically but also structurally with the corresponding enzyme isolated from *E. histolytica*. Such comparisons must await further investigations of these enzymes.

It is tempting to speculate about the evolutionary significance of finding such unique enzyme activities as pyruvate, phosphate dikinase, carboxytransphosphorylase, and now PP₁-phosphofructokinase in such diverse organisms as an amoeba and a bacteria. It is possible that these two organisms evolved from a common ancestor, one that utilized PP₁ instead of nucleotide triphosphates as its high energy phosphate donor. Alternatively, these organisms might have evolved independently but in similar environment in which PP₁ was abundant and hence the enzyme systems were developed for its utilization. Although the possibility of comparing the amino acid sequences of these enzymes from the two organisms is not presently feasible, a comparison of their immunological properties might provide evidence concerning the evolutionary hypotheses.

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