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-P₂ 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 PP₂-phosphofructokinase from P. shermanii is specific for fructose-6-P and fructose-1,6-P₂, no other phosphorylated sugars were utilized. Phosphate could be replaced by arsenate. The Kₐ values are: phosphate, $6.0 \times 10^{-4}$ M; fructose-1,6-P₂, $5.1 \times 10^{-4}$ M; pyrophosphate, $6.9 \times 10^{-5}$ M; and fructose-6-P, $1.0 \times 10^{-4}$ M. The $\varepsilon_{280}$ 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 PP₂-phosphofructokinase activity approximately 6 times greater than ATP-phosphofructokinase and 15 to 20 times greater than fructose diphosphatase activities. It is proposed that (a) PP₂ may replace ATP in the formation of fructose-1,6-P₂ when the organism is grown on glucose and (b) when the organism is grown on lactate or glycerol the conversion of fructose-1,6-P₂ to fructose-6-P during gluconeogenesis may occur by phosphorolysis rather than hydrolysis.

The discovery of a pyrophosphate-dependent phosphofructokinase (pyrophosphate; D-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).

PP₂-phosphofructokinase catalyzes the reaction shown below.

\[
\text{Fructose-6-P + PP}_2 \xrightarrow{\text{Me}^{17}} \text{fructose-1,6-P}_2 + P_1
\]

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, glyceraldehyde 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-P₂, 0.5; MgCl₂, 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-P₂ Assay—The assay mixture contained in micromoles, imidazole-HCl, pH 7.4, 20; pyrophosphate, 0.35; MgCl₂, 1.25; fructose-6-P, 0.5; NADH, 0.06; and in units, aldolase, 0.1; triosephosphate isomerase, 1.5; glyceraldehyde phosphate dehydrogenase.
velocity centrifugation was done in a Beckman model E analytical ultracentrifuge, Fig. 1C. Based on these criteria we believe that the protein preparations from the previous step are extracted with solutions of ammonium sulfate (prepared in 0.1 M potassium phosphate, pH 7.0) by guest on October 15, 2017 http://www.jbc.org/ Downloaded from

TABLE I

Purification of pyrophosphate-phosphofructokinase

<table>
<thead>
<tr>
<th>Step</th>
<th>Total units</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Recovery</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.u.</td>
<td>mg</td>
<td>mg</td>
<td></td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>1. Crude extract</td>
<td>18,000</td>
<td>46,000</td>
<td>0.39</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>2. 35 to 55% saturated (NH₄)₂SO₄</td>
<td>17,400</td>
<td>23,700</td>
<td>0.74</td>
<td>96</td>
<td>1.9</td>
</tr>
<tr>
<td>3. First cellulose-PO₄ column</td>
<td>13,200</td>
<td>13,500</td>
<td>0.98</td>
<td>74</td>
<td>2.5</td>
</tr>
<tr>
<td>4. Second cellulose-PO₄ column</td>
<td>4,700</td>
<td>19,245</td>
<td>26</td>
<td>628</td>
<td></td>
</tr>
</tbody>
</table>

*The protein measurements were determined by the method of Warburg and Christian (21). Activity measurements were obtained by the fructose-6-P assay as described under “Experimental Procedure.”

**All activity measurements were obtained by the fructose-6-P assay as described under “Experimental Procedure.”**
phosphate as a substrate, arsenate will replace phosphate. Although the $K_m$ for arsenate appears to be much higher, the $V_{	ext{max}}$ 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$_i$ utilization and fructose-1,6-P$_2$ formation.

**Reaction Parameters for PP$_i$-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 $8.3 \times 10^{-4}$ and $6.4 \times 10^{-4}$ 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 $V_{	ext{max}}$ 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 96,000. The subunit molecular weight was determined by electrophoresis in sodium dodecyl sulfate (18). These results are presented in Fig. 2. PP$_i$-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$_i$-phosphofructokinase, the highest activity was observed in glycerol.

### Table II

<table>
<thead>
<tr>
<th>Product assayed</th>
<th>Possible substrates</th>
<th>Substrate concentration</th>
<th>Activity as per cent normal substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose-1,6-P$_2$ by PP$_i$</td>
<td>Assay B</td>
<td>ATP or ITP or GTP</td>
<td>0.57</td>
</tr>
<tr>
<td>Fructose-6-P</td>
<td>1.4</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Fructose-1,6-P$_2$ by PP$_i$</td>
<td>Assay A</td>
<td>PO$_4^{3-}$</td>
<td>3.6</td>
</tr>
<tr>
<td>Fructose-6-P</td>
<td>SO$_4^{2-}$</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>Fructose-6-P</td>
<td>MoO$_4^{2-}$</td>
<td>17.9</td>
<td></td>
</tr>
<tr>
<td>Fructose-6-P</td>
<td>SeO$_4^{2-}$</td>
<td>17.9</td>
<td></td>
</tr>
<tr>
<td>Fructose-6-P</td>
<td>AsO$_4^{2-}$</td>
<td>28.6</td>
<td></td>
</tr>
<tr>
<td>Fructose-1,6-P$_2$ by PP$_i$</td>
<td>Assay C</td>
<td>Glucose-1,6-P$_2$</td>
<td>1.4</td>
</tr>
<tr>
<td>Fructose-1,6-P$_2$</td>
<td>1.0</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Ribulose-1,5-P$_2$</td>
<td>1.0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Fructose-1-P</td>
<td>1.0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

* Both UTP and CTP gave some activity. However, this activity was due to PP$_i$ 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.

### Table III

<table>
<thead>
<tr>
<th>Compound</th>
<th>Initial Concentration</th>
<th>Final Concentration</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose-6-P</td>
<td>1.42</td>
<td>0.24</td>
<td>-1.18</td>
</tr>
<tr>
<td>PP$_i$</td>
<td>1.97</td>
<td>0.74</td>
<td>-1.23</td>
</tr>
<tr>
<td>Fructose-1,6-P$_2$</td>
<td>0</td>
<td>1.21</td>
<td>+1.21</td>
</tr>
</tbody>
</table>

The experiment was conducted at 20° and contained the following in micromoles: MgCl$_2$, 4.5; imidazole, 50; fructose-6-P and PP$_i$, as indicated in a final volume of 1.0 ml. Enzyme (1.8 units) was added and products determined after 30 min and 1 hour. There was no difference in these two determinations. The enzyme was still active after the 1-hour incubation period.
Protein was determined by the biuret reaction.

Fructose-1,6-P, from 0.0051 to 0.061 mM; fructose-6-P from 0.014 to 1.14 mM.

The enzyme 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 μg 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 × g. The ATP-phosphofructokinase in Entamoeba histolytica was found to be membrane-bound by Reeves et al. (1).

**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.

**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 μg of each protein were applied. The designation PT refers to the position of the PPi-phosphofructokinase.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Kinetic pattern</th>
<th>( K_m ) ( \text{μmol/min/mg} )</th>
<th>( V_{max} )</th>
<th>( \text{μmol/min/mg} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose-6-P</td>
<td>Intersecting</td>
<td>( 1.0 \times 10^{-4} )</td>
<td>( 6.9 \times 10^{-5} )</td>
<td></td>
</tr>
<tr>
<td>PPi</td>
<td>Intersecting</td>
<td>( 5.1 \times 10^{-5} )</td>
<td>( 6.0 \times 10^{-5} )</td>
<td></td>
</tr>
<tr>
<td>Fructose-1,6-P</td>
<td>Intersecting</td>
<td>( 6.9 \times 10^{-5} )</td>
<td>( 258 )</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE V**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrates for growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glycerol</td>
</tr>
<tr>
<td>PPi-phosphofructokinase</td>
<td>0.29</td>
</tr>
<tr>
<td>ATP-phosphofructokinase</td>
<td>0.052</td>
</tr>
<tr>
<td>Fructose diphosphatase</td>
<td>0.017</td>
</tr>
</tbody>
</table>

**Pyrophosphate** is produced in many biosynthetic reactions and it is generally accepted that the PPi thus formed is hydrolyzed rapidly in order to provide the driving force for these endothermic reactions. In most instances this is undoubtedly the case, however, there is mounting evidence for the concept that PPi may serve as a high energy phosphate donor in some organisms. There is increasing evidence concerning the role of PPi in energy conservation in the photosynthetic bacteria, Rhodospirillum rubrum and Rhodopseudomonas viridis. It has been demonstrated that these organisms can couple the synthesis of PPi to light-induced electron transport (23). They can also utilize PPi as an energy source for energy-linked transhydrogenation (24, 25), cytochrome reduction (26, 27) and succinate-linked NAD+ reduction (28). Extracts of *R. rubrum* also catalyze a PPi ↔ P1 exchange reaction (29).

Perhaps the strongest case for PPi serving as a phosphate donor for substrate level phosphorylation is provided in *E. histolytica*, an anaerobic amoeba, which only grows on glucose. The organism apparently lacks pyruvate kinase (30) but has sufficient pyruvate, phosphate dikinase activity, Reaction ii, 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 \( K_m \) of the PPi-phosphofructokinase for this metabolite.

The role of the PPi-utilizing enzymes in the metabolism of *P. shermanii* is not as obvious as in *E. histolytica*. In the fermentation of glucose by *P. shermanii* there are six reactions in which phosphorylations are involved. These are indicated by the boxed areas A, B, C, D, E, and F of Fig. 3. Bauchop and Elsden (31) found that the propionic acid bacteria have a very
Therefore, they suggested that high energy phosphate must
substrate or product. PP, in the reactions. The numbers in parentheses indicate the moles of
protein function in the direct metabolic pathway. Such enzymes are
compared to that of other enzymes of P. shermanii which
shermanii (0.05 unit/mg of protein, Table V) is very low
ATP-phosphofructokinase found in crude extracts of P.

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
arise not only in the usual steps of glycolysis but also during
the formation of propionate. A high energy phosphate might arise
during the reduction of tumarate to succinate as indicated in
Box 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.

If the propionic acid fermentation occurred by the following
equation,

3 Glucose → 4 propionate + 2 acetate + 2 CO2

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

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-empha-
sizes this possibility. It is noteworthy that the amount of
ATP-phosphofructokinase found in crude extracts of P. shermanii (0.06 unit/mg of protein, Table V) is very low
compared to that of other enzymes of P. shermanii which
function in the direct metabolic pathway. Such enzymes are
usually present in crude extracts at more than 0.5 unit/mg of
protein (15, 33). In view of the small amount of the ATP-phos-
phofructokinase, it appears that the PPi-phosphofructokinase
may account for a significant proportion of the glycolytic flux
with PPi, replacing ATP as the phosphorylating agent.

There are several problems which need to be considered in
relation to this suggestion. One relates to the source of the PPi
for the reaction. Very little PPi would be formed by the

pyruvate, phosphate kinase reaction (Box D) which has an
activity of about 0.04 unit/mg of protein in crude extracts (35).
This low activity is to be expected if its function is primarily
anaplerotic as indicated in Fig. 3. The enzyme is particularly
important for cells growing on lactate or pyruvate, since the
P-enolpyruvate required for anabolic purposes must be synthe-
sized 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 oxalacetate of the main metabolic pathway is supplied by
the transcarboxylase reaction and the carboxytransphosphoryl-
ase 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 carboxytransphosphoryl-
ase/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 flavo-
protein 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-phos-
phofructokinase 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-phos-
phofructokinase 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.
testing 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.

REFERENCES

Isolation and characterization of a pyrophosphate-dependent phosphofructokinase from Propionibacterium shermanii.
W E O'Brien, S Bowien and H G Wood


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