PPᵢ-Dependent Phosphofructotransferase (Phosphofructokinase) Activity in the Mollicutes (Mycoplasma) Acholeplasma laidlawii

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A PPᵢ-dependent phosphofructotransferase (PPᵢ-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.90) which catalyzes the conversion of fructose 6-phosphate (F-6-P) to fructose 1,6-bisphosphate (F-1,6-P₂) was isolated from a cytoplasmic fraction of Acholeplasma laidlawii B-PG9 and partially purified (430-fold). PPᵢ was required as the phosphate donor. ATP, dATP, CTP, dCTP, GTP, dGTP, UTP, dUTP, ITP, TTP, ADP, or Pᵢ could not substitute for PPᵢ. The PPᵢ-dependent reaction (2.0 mM PPᵢ) was not altered in the presence of any of these nucleotides (2.0 mM) or in the presence of smaller (≤300 μM) amounts of fructose 2,6-bisphosphate, (NH₄)₂SO₄, AMP, citrate, GDP, or phosphoenolpyruvate. Mg²⁺ and a pH of 7.4 were required for maximum activity. The partially purified enzyme in sucrose density gradient experiments had an approximate molecular weight of 74,000 and a sedimentation coefficient of 6.7. A second form of the enzyme (molecular weight, 37,000) was detected, although in relatively smaller amounts, by using Blue Sepharose matrix when performing electrophoresis experiments. The back reaction, F-1,6-P₂ to F-6-P, required Pᵢ arsenate could substitute for Pᵢ, but not PPᵢ or any other nucleotide tested. The computer-derived kinetic constants (± standard deviation) for the reaction in the PPᵢ-driven direction of F-1,6-P₂ were as follows: v, 38.9 ± 0.48 mM min⁻¹; Kᵢₗₑ₅₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀₋₆₀-
DEAE-Sephacel were purchased from Pharmacia Fine Chemicals, Piscataway, N.J. Coomassie blue protein dye reagent was purchased from Bio-Rad Laboratories, Richmond, Calif. Polyethyleneimine thin-layer chromatography plates containing 0.55 meq of polyethyleneimine g of cellulose \(^{-1}\) were obtained from Analtech, Inc., Newark, Del. All enzymes and chemicals were obtained from Sigma Chemical Co., St. Louis, Mo. (or other commercial sources where noted) and were of the highest purity available.

**Preparation of cell extract.** *A. laidlawii* B-PG9 and *A. florum* L1\(^2\) were grown at 37°C in a modified Edward medium, sometimes with 1% (vol/vol) heat-reactivated horse serum (3). Membrane and cytoplasmic fractions from hypotonically shocked washed cells were prepared as described previously (20). The hypotonic lysate was centrifuged at 200,000 \(\times g\) for 1 h at 4°C. The supernatant was dialyzed four times, each time in approximately 100 volumes of TBM buffer (10 mM Tris acetate [pH 7.4], 2 mM 2-mercaptoethanol, 1 mM MgCl\(_2\), 0.2 mM phenylmethylsulfonyl fluoride) at 10°C. In some cases, after dialysis in TBM buffer, the supernatant was dialyzed against 100 volumes of IBM buffer (20 mM imidazole acetate [pH 7.5], 2 mM 2-mercaptoethanol, 2 mM MgCl\(_2\)) at 10°C. The retentate was called crude lysate and was the starting material for all assays and purification studies.

**Assay for PP\(_2\)-PPF activity.** The synthesis of F-1,6-P\(_2\) from F-6-P was measured by the disappearance of NAD\(_{-}\)H at 340 nm in a series of coupled reactions with aldolase, triose phosphate isomerase, and \(\alpha\)-glycerol phosphate dehydrogenase (18). To remove (NH\(_4\))\(_2\)SO\(_4\), these enzymes were briefly dialyzed against 120 mM Tris acetate (pH 7.4) or against 50 mM imidazole acetate (pH 7.5) at 5°C. The PP\(_2\)-PPF reaction mixture for crude fractions contained (in 800 \(\mu\)l [final volume]) 50 mM imidazole acetate (pH 7.5), 1.6 U of aldolase, 2.4 U of triose phosphate isomerase, 0.2 U of \(\alpha\)-glycerol phosphate dehydrogenase, 0.2 mM NAD\(_{-}\)H, 0.75 mM MgCl\(_2\), 2 mM 2-mercaptoethanol, and enzyme (5 to 250 \(\mu\)g of protein). Each enzyme sample was tested for the presence of contaminating (and interfering) membranes and NADH oxidase activity (20). To test for NAD\(_{-}\)H oxidase activity, all reaction components except enzyme and F-6-P were combined in a cuvette. The enzyme was added next, and the reaction was monitored for about 1 min. The PP\(_2\)-PPF reaction was begun by the addition of F-6-P. NAD\(_{-}\)H oxidase activity was only detected at very low levels in crude hypotonic lysates and in fractions in the early stages of enzyme purification. The PP\(_2\)-PPF reaction rate was calculated from the change in \(A_{340}\) before and after the addition of F-6-P. Each active enzyme sample was tested at two to five concentrations to determine the maximum specific activity. All assays were conducted on a Gilford 260 spectrophotometer and recorder (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) with cells of 1-cm path length. The temperature was between 21 and 22°C. In some experiments with PP\(_{1}\), we also added either fructose 2,6-bisphosphate (F-2,6-P\(_{2}\)) (200 \(\mu\)M), (NH\(_4\))\(_2\)SO\(_4\) (250 \(\mu\)M), or both, or AMP (300 \(\mu\)M), citrate (200 \(\mu\)M), GDP (200 \(\mu\)M), or phosphoenolpyruvate (150 \(\mu\)M) to the reaction mixture.

**Purification of A. laidlawii B-PG9 PP\(_2\)-PPF.** Washed membrane fractions of *A. laidlawii* B-PG9 and *A. florum* L1\(^2\) had no detectable PP\(_2\)-PPF activity, and only the crude lysate of *A. laidlawii* B-PG9 was fractionated for PP\(_2\)-PPF activity. All purification procedures were conducted at 4°C. The crude lysate (dialyzed cytoplasmic fraction) was chromatographed on a DEAE-Sephacel (1) column (2.5 \(\times\) 30 cm) equilibrated in TBM buffer. The PP\(_2\)-PPF activity was eluted in a stepwise fashion by using increasing concentrations of KCl (0.2, 0.6, and 1 M) in TBM buffer. The enzyme eluted at a KCl concentration of 0.6 M. Fractions containing PP\(_2\)-PPF activity were pooled and dialyzed overnight against TBM buffer.

The content of the dialysis bag was rechromatographed on a DEAE-Sephacel (II) column (1.5 \(\times\) 28 cm) equilibrated in TBM buffer containing 0.2 M KCl. The PP\(_2\)-PPF activity was eluted with a linear gradient of increasing concentration of KCl by using the equilibration buffer and TBM containing 0.6 M KCl. The PP\(_2\)-PPF activity eluted at a KCl concentration of 0.28 \(\pm\) 0.03 M (n = 3). Solid (NH\(_4\))\(_2\)SO\(_4\) was added to the pooled active fraction until a final concentration of 1 M was obtained. The solution was centrifuged at 30,000 \(\times g\) for 30 min to separate any precipitate, and the supernatant was applied to a Phenyl Sepharose column (1.5 \(\times\) 28 cm) equilibrated in TMN buffer (10 mM Tris hydrochloride [pH 6.0], 1 mM MgCl\(_2\), 2 mM 2-mercaptoethanol, 1 M (NH\(_4\))\(_2\)SO\(_4\), 0.2 mM phenylmethylsulfonyl fluoride). The PP\(_2\)-PPF activity was eluted with a linear gradient of both decreasing (NH\(_4\))\(_2\)SO\(_4\) concentration and increasing concentration of ethylene glycol monomethyl ether by using TMN buffer and TBM buffer (10 mM Tris hydrochloride [pH 6.0], 1 mM MgCl\(_2\), 2 mM 2-mercaptoethanol, 50% [vol/vol] ethylene glycol monomethyl ether, 0.2 mM phenylmethylsulfonyl fluoride). The PP\(_2\)-PPF activity eluted as a single peak at a (NH\(_4\))\(_2\)SO\(_4\) concentration of 0.44 \(\pm\) 0.05 M (n = 3). The fractions containing PP\(_2\)-PPF activity were pooled and dialyzed against TBM buffer.

The content of the dialysis bag was chromatographed on a Blue Sepharose column (0.9 \(\times\) 10 cm) equilibrated in TBM buffer. The PP\(_2\)-PPF activity was eluted in a stepwise fashion, by using 5 mM ATP, then 10 mM tetradsodium PP\(_{1}\), and finally 2 mM KCl in TBM buffer. PP\(_2\)-PPF activity eluted with 2 mM KCl was used for all characterization studies after dialysis against IBM buffer or TBM buffer. One unit of PP\(_2\)-PPF was defined as the amount of enzyme which converted 1 \(\mu\)mol of F-6-P to F-1,6-P\(_2\) min\(^{-1}\) at 21 to 22°C.

Identification of protein. \(^{32}\)P-Labeled F-1,6-P\(_2\) with confidence that F-1,6-P\(_2\) was the product of the PP\(_2\)-PPF reaction, we reacted the produce with aldolase. We combined 50 mM imidazole acetate (pH 7.5) approximately 6 \(\times\) 10\(^5\) dpm of tetradsodium \(^{32}\)P-PP\(_{1}\) (>1 nmol of Na\(_2\)PP\(_{1}\)), 1 mM F-6-P, 2 mM MgCl\(_2\), and 2 mM 2-mercaptoethanol (final volume, 1.0 ml). In some reaction mixtures we added nonradioactive tetradsodium PP\(_{1}\). To each of these reaction mixtures we added 10 \(\mu\)g of our 430-fold-purified PP\(_2\)-PPF. After incubation for 30 min at 37°C, the reactions were stopped by heating at 90°C for 2 min. After cooling, samples of each reaction mixture were incubated at 37°C for 30 min with 0.3 mM NADH and the following (per milliliter of reaction mixture): 2 U of aldolase, 3 U of triose phosphate isomerase, and 0.3 U of \(\alpha\)-glycerol phosphate dehydrogenase. The reaction was stopped by heating at 90°C for 2 min. After cooling, 25 \(\mu\)l of enzyme-treated or untreated samples were chromatographed with nonradioactive samples of F-1,6-P\(_2\) and PP\(_{1}\), on polyethyleneimine plates using a solvent which contained 0.25 M LiCl\(_2\) and 1 N formic acid. In this system, F-1,6-P\(_2\) migrates ahead and clear of tetradsodium PP\(_{1}\). Resolved components were visualized by FeCl\(_3\), sulfosalicylic acid spray (29) and by radioautography. Areas identified as F-1,6-P\(_2\) were scraped off the plates and counted for radioactivity (30).

**Other methods.** (1) Method 1. Protein was assayed by the
Coomassie blue method according to the instructions of the manufacturer, by using bovine serum albumin as the standard.

(ii) Method 2. To estimate enzyme purity, samples of our purified PPi-PFP activity were concentrated by dialysis against kappa buffer (20) containing 35% (wt/vol) sucrose at 4°C. A quantity of 200 μl of the concentrate containing 7.2 μg of protein was applied to slab gels and electrophoresed. Slab gels (0.75 mm by 24 cm by 24 cm) were made by the method of Davis (8). Electrophoresis was performed with a Protean II slab gel electrophoresis system (Bio-Rad Laboratories, Richmond, Calif.) equipped with a ECPS 3000/150 power supply and a Voltzou Integrator VH-1 (Pharmacia, Inc., Piscataway, N.J.). Samples were electrophoresed initially for 30 min at 150 V gel⁻¹, then by 100 V gel⁻¹, until 900 V·h of electrophoresis was obtained (approximately 5 h). Gels were stained by using the KODAVUE Electrophoresis Visualization Kit (Eastman Kodak Co., Rochester, N.Y.) according to the instructions of the manufacturer. By using the high-molecular-weight protein standards kit (Pharmacia), we determined that in our system, the lower limit of protein detection was 10 ng per band.

(iii) Method 3. To determine the metal requirement and concentration yielding the maximum reaction velocity, chlorides of Mg²⁺, Co²⁺, Mn²⁺, Ca²⁺, and Zn²⁺ were added individually to the PPi-PFP reaction mixture at final concentrations ranging from 0.01 to 97.0 mM. Also, combinations of Mg²⁺, Co²⁺, and Mn²⁺ at 6.19, 0.20, and 0.10 mM, respectively, were tested in the reaction mixture.

(iv) Method 4. To determine the optimal reaction pH over a range from 3.49 to 10.4, we substituted different buffers in the PPi-PFP assay (50 mM final concentration): dimethylglyutaric acid-NaOH, pH 3.49 to 7.55; imidazoleacetic acid, pH 7.04 to 7.88; Tris-acetic acid, pH 7.50 to 8.94; and glycine-NaOH, pH 8.65 to 10.4.

(v) Method 5. Molecular weight determination by sedimentation analysis of the purified PPi-PFP was carried out in a linear (5 to 20% wt/vol) sucrose gradient (50 mM Tris hydrochloride [pH 7.5], 2 mM 2-mercaptoethanol, 100 mM NaCl, 2 mM MgCl₂) at 41,000 rpm for 25.0 h in an SW41 Ti rotor (Beckman Instruments, Inc., Fullerton, Calif.) at 4°C. Approximately 100 μg of protein was layered on each gradient. After centrifugation, each gradient was removed from the top of the tube with an Auto Densi-Floc IIC (Buchler Instruments Div., Nuclear-Chicago Corp., Fort Lee, N.J.). Fractions (0.4 ml each) were collected and were assayed for protein or PPi-PFP activity as described above. Standards (Sigma Chemical Co., St. Louis, Mo.) were β-amylose (sweet potato, Mr = 200,000), alcohol dehydrogenase (bakers’ yeast, Mr = 141,000), creatine phosphokinase (rabbit muscle, Mr = 81,000), bovine serum albumin (Mr = 67,000), and ovalbumin (chicken, Mr = 45,000). An estimate of the sedimentation coefficient was calculated by the method of Martin and Ames (15). We also estimated the molecular weight by polyacrylamide gel electrophoresis by using the conditions and standards described for estimation of enzyme purity.

(vi) Method 6. To calculate the Kₘ and Vₘₐₓ of the reaction, we determined the reaction velocity and moles of F-1,6-P₂ synthesized minute⁻¹ milligram⁻¹ at various concentrations of PPi and F-6-P. In one set of experiments, the PPi concentration was held at 0.078, 0.39, or 1.97 mM, and F-6-P concentration was varied at each PPi level over the range of 0.036 to 0.74 mM. In the converse set of experiments, the F-6-P concentration was held at either 0.098, 0.49, or 2.46 mM, and the PPi concentration was varied at each F-6-P level over the range of 0.005 to 0.30 mM. All reaction conditions were otherwise identical, and each assay contained 0.25 μg of the same batch of partially purified (430-fold) PPi-PFP. The Mg²⁺ concentration was held constant at 6.09 mM, and no allowance was made for bound or free Mg²⁺ (5). In initial calculations, reciprocal substrate concentrations were plotted versus the reciprocal values of experimentally determined initial velocities; all plots were linear. The same initial velocities were fitted to the intersecting initial velocity equation of Cleland (7) by using Fortran program. The computer-derived best-fit reciprocal values of initial velocities are plotted versus reciprocal substrate concentrations at three different concentrations of PPi. The derived constants are (± standard deviation): v, 38.9 ± 0.48 mM⁻¹·min⁻¹; Kₘ(PPi), 0.11 ± 0.04 mM; Kₘ(F-6-P), 0.65 ± 0.15 mM; and Kₘ(A), 0.39 ± 0.11 mM. In the same companion series of experiments, also described in the text, the effect of PPi concentrations on the initial velocities of the PPi-PFP reaction at three concentrations of F-6-P was determined (data not shown). The combined data from these experiments were used to obtain the kinetic derived constants.

RESULTS

Purification of A. laidlawii B-PG9 PP₁-PFP. Only the PP₁-PFP from A. laidlawii was purified (Table 1). Crude lysate is
the supernatant after ultracentrifugation and overnight dialysis; it apparently contains an inhibitor of PP$_2$-PFP activity which is removed by chromatography on DEAE-Sephaloc, since the enzyme recovery is higher after passage through the matrix (Table 1). We therefore calculated two recovery patterns, one with the total activity in the crude lysate representing 100% recovery and the other with the total activity in the eluate of passage 1 through DEAE-Sephaloc representing 100% recovery. With this second alternative, we calculated an average recovery of ca. 3% with approximately 300-fold average purification.

In most experiments, we found that there were two peaks of PP$_2$-PFP activity elutable from columns of Blue Sepharose matrix. One peak was apparently not retarded by the matrix and was always found in the wash buffer. This nonretarded activity accounted for approximately 5 to 10% of the total activity recovered from Blue Sepharose. These two forms of PP$_2$-PFP activity were never resolved on other matrices. All our studies were conducted on the larger portion of PP$_2$-PFP activity which was eluted from the Blue Sepharose after application of 2 M KCl.

Estimation of enzyme purity. We detected two visible bands after slab gel electrophoresis and staining of 7.2 µg of protein from a 430-fold-purified enzyme preparation. The most prominent band had an R$_f$ of 0.63, corresponding to a molecular weight of 81,000, and the other band had an R$_f$ of 0.80, with a molecular weight of 37,000. Since we are able to detect bands containing 10 ng of protein, we calculated that at least 99% of our preparation was composed of only these two protein species. However, in other radiographic studies of this preparation with $^{32}$P$_2$PP (see Table 3), we found spots identified as triose $^{32}$P$_2$P$_2$phosphates derived from $^{32}$P-label F-1,6-P$_2$ (data not shown). Therefore, our 430-fold-purified preparation was apparently contaminated with aldolase and perhaps with other enzymes, and our preparations are only partially purified.

**TABLE 1. Purification of PP$_2$-PFP activity from A. laidlawii B-PG9**

| Step                  | Total vol (ml) | Total U | Protein (mg ml$^{-1}$) | Sp act | Recovery (%) compared with purification (fold) |
|-----------------------|----------------|---------|------------------------|--------|-----------------------------------------------|
| Crude lysate          | 120            | 14      | 1.8                    | 0.066  | Crude lysate = 100 | DEAE-Sephaloc (I) = 1                        |
| DEAE-Sephaloc (I)     | 260            | 200     | 0.43                   | 1.8    | 1,400                                        | 100                                         | 100                                         | 27                                           |
| DEAE-Sephaloc (II)    | 100            | 80      | 0.47                   | 1.7    | 570                                          | 40                                          | 26                                           |
| Phenyl Sepharose      | 54             | 26      | 0.051                  | 9.3    | 190                                          | 13                                          | 140                                          |
| Blue Sepharose        | 15             | 12      | 0.067                  | 12.0   | 86                                           | 6                                           | 180$^a$                                      |

$^a$ Cells were harvested from 2 liters of growth medium (approximately $2 \times 10^{11}$ cells).

$^b$ Data from one of four experiments performed for separate batches of crude lysate. The average recovery from all four experiments, relative to DEAE-Sephaloc (I), was 3 ± 2.2% (n = 4; range, 1 to 6%) (average recovery of 6% for this experiment).

$^c$ Micromoles of F-1,6-P$_2$ synthesized minute$^{-1}$ milligram of protein$^{-1}$ in standard assay.

$^d$ Purification was 180-fold, and the specific activity was 12 µmol of F-1,6-P$_2$ synthesized min$^{-1}$ mg$^{-1}$. The average purification was 302 ± 107-fold (n = 4; range, 180 to 430-fold), and the average specific activity was 13.2 ± 0.91 (n = 4; range, 13.7 to 15.5 µmol of F-1,6-P$_2$ synthesized min$^{-1}$ mg$^{-1}$).

**TABLE 2. Phosphate requirement of the partially purified PP$_2$-PFP activity from A. laidlawii B-PG9 in the absence or presence of tetrasodium PP$_2$**

| Phosphate donor added to complete standard reaction mixture (final concn [mM]) | Before addition of Na$_2$P$_2$O$_7$ | After addition of Na$_2$P$_2$O$_7$b |
|-----------------------------------------------------------------------------|-----------------------------------|-----------------------------------|
| None                                                                 | <0.07                            | 100                               |
| ATP                                                                 | 1.1                              | 110                               |
| dATP                                                                   | 1.4                              | 110                               |
| CTP                                                                   | 4.5                              | 110                               |
| dCTP                                                                  | 2.3                              | 110                               |
| GTP                                                                   | 1.4                              | 99                                |
| dGTP                                                                 | 2.3                              | 96                                |
| UTP                                                                   | 3.0                              | 98                                |
| dUTP                                                                  | 2.4                              | 100                               |
| ITP                                                                   | 2.3                              | 95                                |
| TTP                                                                   | 2.0                              | 100                               |
| ADP                                                                   | 0.67                             | 110                               |
| F$_i$ (17)                                                             | <0.07                            | 96                                |
| F$_i$ (33)                                                             | <0.07                            | 63                                |
| F$_i$ (50)                                                             | <0.07                            | 52                                |
| F$_i$ (66)                                                             | <0.07                            | 46                                |
| F$_i$ (130)                                                            | <0.07                            | 43                                |

$^a$ Percentages were calculated by comparison to the amount measured in the complete standard reaction mixture after addition of PP$_2$, as the only phosphate donor. The enzyme activity was assayed spectrophotometrically as described in the text. 100% = 230 pmol of F-1,6-P$_2$ synthesized min$^{-1}$ mg$^{-1}$.

$^b$ Na$_2$P$_2$O$_7$ and all nucleotides (pH 7.4) were added to the reaction mixture to a 2.06 mM final concentration.

**pH and metal requirements.** The optimum (maximum specific activity) was at a pH of 7.4. The response over the range of pH 3.49 to 10.3 was that of a smooth hyperbolic curve. Mg$^{2+}$ was required for activity although Co$^{2+}$ or Mn$^{2+}$ could substitute for Mg$^{2+}$. At a PP$_2$ concentration of 2.06 mM, the maximum nanomoles of F-1,6-P$_2$ synthesized minute$^{-1}$ milligram of protein$^{-1}$ were: 14.4 (for Mg$^{2+}$ at 6.1 mM), 9.0 (for Mn$^{2+}$ at 0.10 mM), and 5.7 (for Co$^{2+}$ at 0.20 mM). Higher concentrations of Mn$^{2+}$ or Co$^{2+}$ were inhibitory. Combinations of these metals at their respective concentrations were neither additive nor gave decreased responses, Ca$^{2+}$ or Zn$^{2+}$ could not substitute for Mg$^{2+}$, Co$^{2+}$, or Mn$^{2+}$. Incorporation of EDTA (50 µM) into reaction mixtures did not significantly change any recovered value (<5% change).

**Specificity of the tetrasodium PP$_2$ requirement.** The PFP activity of A. laidlawii B-PG9 required tetrasodium PP$_2$, and various deoxyribo- and ribonucleotides could not be substituted (Table 2). Furthermore, these nucleotides did not inhibit or enhance the PP$_2$-dependent activity since in the presence of equimolar concentrations of any nucleotide and PP$_2$, there was no appreciable change in activity compared with data from reaction mixtures without nucleotides. Also,

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of interest interfered with the PP₁-dependent reaction (Table 2). In studies designed to test the purity of the CTP and ATP used, we found (data not shown) by using PP₁-PFK from Propionibacterium freudenreichii (Sigma) that our CTP and ATP were contaminated with PP₁ (approximately 4 and 0.1 µg of PP₁ per mg of nucleotide, respectively) (data not shown). We assumed that the low level of activity we detected with CTP and perhaps also with ATP was due to this contaminating PP₁. By using the 430-fold-purified preparation, we found that citrate, AMP, GDP, phosphoenolpyruvate, or (NH₄)₂SO₄ did not affect the specific activity, but that F-2,6-P₂ (200 µM) did, in some experiments, stimulate the reaction by about 5%. Increasing the F-2,6-P₂ concentration to 2 mM did not alter our findings. We did not test the effect of F-2,6-P₂ on PP₁-PFK activity from cells in different growth stages, as was done by Wu et al. with peas (39).

Identification of ³²P-labeled product F-1,6-P₂. We found that the synthesis of putative F-1,6-P₂ product from F-6-P and tetraysodium [³²P]PP₁ was diminished when nonradioactive tetraysodium PP₁ was added to the reaction mixture (Table 3). We also found that after reaction with aldolase, triose phosphate isomerase, and α-glycerophosphate dehydrogenase, the amount of synthesized ³²P-radioactive material that had comigrated with the F-1,6-P₂ standard was reduced and that there was a concomitant appearance of ³²P-radioactive compounds more mobile than F-1,6-P₂. These compounds were not conclusively identified but were apparently phosphorylated trioses (29). The data were compatible with the synthesis and utilization of F-1,6-P₂; therefore, we concluded that F-1,6-P₂ was the product of the PP₁- and F-6-P-dependent PFK activity isolated from A. laidlavii B-PG9.

Kinetic data. The double-reciprocal plot of the computer-derived best-fit initial velocities at three concentrations of PP₁ was examined (Fig. 1). The computer-derived kinetic constants (± standard deviation) were \( V_{\text{max}} = 38.9 ± 0.48 \text{ mM min}^{-1} \); \( K_{\text{MPP}} = 0.11 ± 0.04 \text{ mM} \); \( K_{\text{MPP}} = 0.65 ± 0.15 \text{ mM} \); and \( K_{\text{IppP}} = 0.39 ± 0.11 \text{ mM} \).

Molecular weight determination. From our sucrose density gradient studies, we determined that the enzyme which eluted from Blue Sepharose in 2 M KCl has an approximate molecular weight of 74,000 (Fig. 2). The sedimentation coefficient of this material is about 6.7. From our slab gel electrophoresis studies, we determined that there were two proteins in our preparations. The most abundant form had a molecular weight of 81,000, and the other form had a molecular weight of 37,000 (data not shown). Although we did no enzyme studies with these electrophoretically separated proteins, we assumed that the band at 81,000 molecular weight was the 74,000-molecular-weight PP₁-PFK active peak observed in our sucrose density gradient studies and that the 37,000-molecular-weight band was the PP₁-PFK active material that did not stick to Blue Sepharose. These results suggested the presence of two forms of the enzyme, presumably a monomer and dimer. Probably the 74,000-molecular-weight material, which was the value we used as the most probable approximate molecular weight, was the dimer.

Stability. The partially (430-fold) purified enzyme lost approximately 25% of its specific activity when it was kept in kapp buffer with 10% glycerol (vol/vol) at −22°C for about 6 months. The specific activity of this stored preparation was reduced by about 60% after exposure to 70°C for 2 min and was completely lost after 10 min.

PP₁-PFK activity in acholplasmas. We also found PP₁-PFK activity in the crude lysates from three different batches of A. florum L1T: 16.4 ± 3.4 nmol of F-1,6-P₂ synthesized min⁻¹ mg⁻¹. There was slight activity when equimolar ATP (2.0 mM) was substituted for PP₁; 0.21 ± 0.13 nmol of F-1,6-P₂ synthesized min⁻¹ mg⁻¹. We found no PP₁-PFK activity in washed membrane fractions of A. laidlavii B-PG9 or in A. florum L1T (<0.16 nmol of F-1,6-P₂ synthesized min⁻¹ mg⁻¹).

**DISCUSSION**

All ATP samples, whether already possessed or newly purchased, had traces of PP₁, as determined by a PP₁ assay (Sigma kit) which uses the PP₁-dependent PFK from P. freudenreichii. By using ATP contaminated with PP₁, as a
phosphorus donor, we found low levels of PFP activity. By using PP (Fisher) or the ATP contaminated with PP, we compared PP-PFP activity in both crude and partially purified preparations. With either enzyme preparation, the specific activity was about 33.5-fold higher with PP than with ATP samples. With propionibacteria, Wood and Goss (37) have shown that the enzymes of phosphorylation, aside from glucokinase, are specific for only one phosphate donor or acceptor. Others have shown that either ATP or PP could serve as phosphorus donors in this reaction (24). Because of the relatively slight activity with ATP, we cannot be absolutely sure that PP is the only permissible phosphorus donor in the PP-PFP reaction of A. laidlawii B-P9.

The absence of any effect, positive or negative, on PP-PFP activity of low levels of ADP, AMP, GDP, phosphoenolpyruvate, citrate, or ammonium ion, and the absence of any appreciable effect of F-2,6-P$_2$, suggest that either the PP-PFP activity in A. laidlawii B-P9 cannot be allosterically modified by them or that it is fully activated. Other PP-PFP or PP-PFKs, but not all, are generally known to be modified by these compounds (27, 32, 39). F-2,6-P$_2$ has little or no effect on the PP-PFK from propionibacteria or from various other cells (6, 9, 37). Furthermore, the presence of 2 mM of any one of a variety of ribo- or deoxyribonucleotides and ADP did not affect the velocity of the reaction in the presence of 2 mM PP$_i$. This finding supports our view of both the dependence of the reaction on PP$_i$ and its essential unresponsiveness to other ribo- or deoxyribonucleotides.

P$_i$ was inhibited to the PP-dependent reaction (Table 2); this was expected, since P$_i$ is a product. However, the role of P$_i$ is more complicated, because we found in preliminary experiments that the PP-PFP reaction was reversible (D. DeSantis and J. D. Pollack, unpublished data). The reverse reaction; (F-1,6-P$_2$ to F-6-P) requires P$_i$, and PP, ADP, ATP, or other ribo- or deoxyribonucleotides tested could not substitute. Furthermore, the reverse reaction of the partially purified PP$_i$-PP$_i$ from A. laidlawii B-P9, like the reverse reaction of the PP$_i$-PP$_i$ in E. histolytica, can use arsenate instead of P$_i$ (23). This reverse reaction is detected by the reduction of NADP in the presence of rabbit muscle phosphoglucone isomerase and yeast glucose 6-phosphate dehydrogenase. Although our partially purified PP$_i$-PP$_i$ had no phosphoglucone isomerase activity and the reverse reaction was conducted with excess aldolase, triose phosphate isomerase, a-glycerophosphate dehydrogenase, and NADH (thereby favoring the oxidation of NADH), it is still possible that the forward reaction (F-6-P to F-1,6-P$_2$) was impeded. Therefore, the kinetic data we have presented should be viewed with some reservation, since the effects of any competition for F-1,6-P$_2$ by the reverse reaction on the kinetics we present for the forward reaction velocities are underestimated. What we have attempted to establish is that the presumed rate-limiting step of glycolysis in A. laidlawii B-P9 is essentially or totally PP$_i$ dependent.

Kruger and Dennis (13) report that the auxiliary enzymes commonly used in the assay for PFP or PFK may be contaminated with UDP-glucose pyrophosphorylase and that such contamination can account for apparent activity of PP$_i$-PP$_i$. That is, in the presence of UDP-glucose and PP$_i$, the enzyme catalyzes the synthesis of UTP and glucose 1-phosphate. PFK from rabbit muscle can accept UTP, as well as ATP, as a donor in the phosphorylation of F-6-P to F-1,6-P$_2$. In this case, the requirement for PP$_i$ falsely appears to be due to a PP$_i$-dependent phosphofructokinase, when in fact, PP$_i$ is required for the synthesis of UTP which drives a nucleotide-dependent phosphofructokinase. In our studies, there is essentially no activity with UTP or dUTP as phosphorus donors with our partially purified PFP (Table 2). Also, we started the reactions with F-6-P, not with PP$_i$, and then only after we had first determined the rate in the absence of F-6-P. Therefore, even if PP$_i$, UDP-glucose, and UDP-glucose-pyrophosphorylase were all present after our purification steps, our assay procedure would have detected their combined activity. Any oxidation of NADH in the complete reaction mixture minus F-6-P was only observed in crude fractions, and then only in traces. We corrected for this oxidation whenever it was detected. This activity was attributed to contamination of crude fractions with membrane-bound NADH oxidase activity. This activity is localized in the membrane fraction of the nonsterol mollicutes. In the sterol-requiring mollicutes, NADH oxidase activity is localized in the cytoplasm (19). Also, in the presumed absence of UDP-glucose, the conversion of ATP-PFK to PP$_i$-PP$_i$ reported in spinach leaf cytosol and rabbit muscle was not considered by us to be applicable (2, 13, 35).

We searched for PP$_i$-PP$_i$ activity in other mollicutes to establish its general presence in this group of microorganisms. We found PP$_i$-PP$_i$ activity in crude cytoplasmic fractions of A. floreatum, but none in Spiroplasma floridica or Mycoplasma gallisepticum (23, 36-38). We have also shown that phosphogluconate kinase (23, 36-38) and PEP carboxykinase (23, 36-38) are present in cell extracts of these two mollicutes, which contain NADH oxidase activity, would inhibit or nullify the test. To determine whether these two mollicutes and other sterol-requiring species have any PP$_i$-PP$_i$ activity, a different technique that does not rely on NADH oxidation must be used. The earlier report of ATP-PFK activity in M. gallicpticum (M. E. Tourtellotte, Ph.D. thesis) cannot be verified by our procedure.

The presence of a PP$_i$-PP$_i$ implies that in A. laidlawii B-P9, and possibly in other acholopathia, glycolysis is dependent on PP$_i$. This may be related to the observation that during growth in log-phase PFP activity is very low in mollicutes in log-phase growth (by viable cell count) have a lower than expected EC$_A$ (ranging from 0.69 to 0.76) (3). Karl (10) indicates that biosynthesis and growth are possible only at EC$_A$ ≥ 0.8. If PP$_i$ spares ATP, this action may permit log-phase growth with an apparently less than optimal EC$_A$ status and may result in relatively high yields of cells (3). In A. laidlawii B-P9, levels of cellular PP$_i$ may be dependent on the PP$_i$-synthesizing activities of dUTPase (33, 34), adenine, and the hypoxanthine-guanine phosphoribosyltransferases (30). PP$_i$ may also arise from the activities of polymerase (11), phosphoenolpyruvate-carboxytransphosphorylase, or pyruvate diphosphate dikinase (23, 36-38). These last two enzymes are generally found in organisms with PP$_i$-PP$_i$ activity (36, 38). We have detected low levels of phosphoenolpyruvate-carboxytransphosphorylase activity in A. laidlawii B-P9 (K. D. Beanman and J. D. Pollack, Yale J. Biol. Med. 57:897, 1984), but have not determined the levels of cellular PP$_i$ during all growth phases.

The fact that PP$_i$ has an important role in A. laidlawii B-P9 metabolism is supported by the additional observation that the purine nucleoside kinase activities in this and
other species of acholeplasmas tested is PP, dependent, a requirement which is unknown in any other organism (30, 31). We did not find inorganic pyrophosphatase (EC 3.6.1.1) in extracts of A. laidlawii B-P9g by using tetrasodium (52P2P)PP (V. V. Tryon and J. D. Pollack, unpublished data). Also, O’Brien et al. did not find any inorganic pyrophosphatase activity in A. laidlawii B-P9g (17). Although all our studies were with cell extracts, the ability to use PP, rather than ATP may offer some advantage to growing cells. Furthermore, the absence of cytochrome pigments in this group of organisms (21) infers some possible shortage of ATP, perhaps compensated by a sparing action of PP, not only in the P-O-P level of glycolysis but also in the phosphorylation of some purine nucleosides by the action of purine nucleoside kinases. The requirement for PP, at these loci may represent a conserved remnant of earlier development processes, as PP, has been proposed in a study of Desulfotomaculum species to be an evolutionary precursor of ATP (14).

ACKNOWLEDGMENTS

We thank J. Williams, J. O. Alben, F. Framingo, and P. Karki, Ohio State University, for their valuable help with the statistical analysis of our data, and the Ohio State University Instruction and Research Computer Center for computer time furnished without charge. We also thank Nancy W. Teders and G. A. Tejwani for their help in preparing and critically reviewing this manuscript.

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