Kinetic Mechanism of Pyrophosphate-Dependent Phosphofructokinase from *Propionibacterium freudenreichii*†

Byron L. Bertagnolli and Paul F. Cook*

ABSTRACT: Inorganic pyrophosphate dependent D-fructose-6-phosphate 1-phosphotransferase from *Propionibacterium freudenreichii* was purified to apparent homogeneity by the criterion of silver staining on sodium dodecyl sulfate (SDS) gels. In the direction of phosphorylation of fructose 6-phosphate (F6P), an intersecting initial velocity pattern is obtained when $MgPP_i$ is varied at several levels of F6P. In the reverse reaction direction, the reactants are Mg^{2+}, P_i, and fructose 1,6-bisphosphate (FDP). Variation of P_i at several levels of Mg^{2+} and a single level of FDP gives an intersecting pattern. When this pattern is repeated at several additional FDP levels, data are consistent with a fully random terreactant mechanism at pH 8.0 and 25 °C. The K_{eq} calculated from the Haldane relationship [$(5 \pm 1.5) \times 10^{-3}$ M] agrees with that determined directly from ^{31}P NMR of the equilibrium mixture [$(7 \pm 2) \times 10^{-3}$ M]. Product inhibition by P_i is competitive vs. either $MgPP_i$ or F6P with the other reactant saturating but changes to noncompetitive inhibition when the fixed reactant is decreased to K_m levels. Product inhibition by $MgPP_i$ is competitive vs. either P_i or FDP with the other reactant saturating but changes to noncompetitive when the fixed reactant is decreased to K_m levels. Tagatose 6-phosphate is competitive vs. F6P and noncompetitive vs. $MgPP_i$. Methylenediphosphonate is competitive vs. $MgPP_i$ and noncompetitive vs. F6P. Sulfate is competitive vs. P_i and noncompetitive vs. FDP, while 2,5-anhydro-D-mannitol 1,6-bisphosphate is competitive vs. FDP and noncompetitive vs. P_i. These data are consistent with a rapid equilibrium random bi-ter mechanism.

Pyrophosphate-dependent phosphofructokinase (pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.90) catalyzes the reaction

$$PP_i + F6P \overset{M^{2+}}{\rightleftharpoons} FDP + P_i \quad (1)$$

Following the initial discovery of PP_i-PFK[1] activity in an extract of anaerobically grown *Entamoeba histolytica* by Reeves et al. (1974), the enzyme was found in the facultative anaerobic bacterium *Propionibacterium shermanii* (O'Brien et al., 1975). The stoichiometry of the reaction catalyzed by the PP_i-PFK is given above, and the substrate saturation curves are all hyperbolic. With regard to reactant specificity, data have also been presented to show that no other mono- or diphosphorylated sugar can substitute for F6P or FDP. Also, no nucleotide triphosphate or polyphosphate can replace PP_i as donor of the phosphoryl group in the forward reaction, although AsO_4^{2-} can replace P_i in the reverse reaction direction (Reeves et al., 1974, 1976; O'Brien et al., 1975). For the amoebal enzyme, Mn^{2+} and Co^{2+} stimulate the rate of phosphorylation of F6P in the presence of Mg^{2+}, while Ca^{2+}, Ni^{2+}, and Zn^{2+} inhibit (Reeves et al., 1976). By use of ^{31}P NMR (Gray, 1971) and ^{13}C NMR (Koerner et al., 1973), it has been shown that the predominant form of F6P and FDP in solution at pH values near neutrality are the β-anomers of the furanose ring. More specifically, Koerner et al. (1974, 1976, 1980) present evidence that rabbit muscle ATP-PFK utilizes the $^4T_3$ twist conformation of the β-D-fructofuranoses.

Following the initial discovery of PP_i-PFK in anaerobes, it has been reported to occur in aerobic marine bacteria of the genus *Alcaligenes* and in *Pseudomonas marina* by Sawyer et al. (1977) as well as in the photosynthetic bacteria *Rhodospirillum rubrum* and *Rhodopseudomonas gelatinosa* by Pfleiderer & Klemme (1980). There have also been reports of PP_i-PFK activity in photosynthetic plant tissues including pineapple leaves (Carnal & Black, 1979) and in the cytoplasm, but not the chloroplasts of spinach leaves (Csëke et al., 1982). For nonphotosynthetic plant tissues, it has been reported in mung bean hypocotyl (Sabularse & Anderson, 1981), potato tubers (Van Schaftingen et al., 1982), and castor bean seedlings (Kruger et al., 1983). The K_m values for PP_i and F6P are larger for the plant enzymes even though the substrate saturation curves remain hyperbolic. These sources also contain significant amounts of an allosteric ATP-PFK (Carnal & Black, 1979; Sabularse & Anderson, 1981; Kruger et al., 1983). Csëke et al. (1982) and Van Schaftingen et al. (1982) have reported allosteric PP_i-PFKs from spinach leaves and potato tubers, respectively, in which the substrate saturation curve for F6P is sigmoidal, while that for P_i is hyperbolic. There has been no report of a PP_i-PFK from animal tissues.

Initial velocity studies in the absence and presence of products were used by Reeves et al. (1976) to suggest a random mechanism for the enzyme from *Entamoeba histolytica*. In these studies, however, attention was not paid to the potential metal–reactant chelate complexes which form, and metal was varied as a pseudoreactant in both reaction directions.

On the basis of initial velocity patterns, ^{31}P NMR, product inhibition, and dead-end inhibition studies in both reaction directions, we report here a detailed steady-state kinetic study of the overall reaction mechanism for PP_i-PFK from *Propionibacterium freudenreichii*. Data suggest a rapid equi-

† From the Department of Biochemistry, North Texas State University/Texas College of Osteopathic Medicine, Denton, Texas 76203. *Received January 6, 1984.* This research was supported by National Institutes of Health Grant GM 31686 (P.F.C.) and Grant BRSG S07 RR07195-03 (P.F.C.) awarded by the Biomedical Research Grant Program, Division of Research Resources, National Institutes of Health. P.F.C. is the recipient of a Research Career Development Award from NIH (AM-01155).

[1] Abbreviations: PP_i-PFK, pyrophosphate-dependent phosphofructokinase; PP_i, inorganic pyrophosphate; P_i, inorganic phosphate; F6P, fructose 6-phosphate; FDP, fructose 1,6-bisphosphate; PCP, methylenediphosphonate; PNP, imidodiphosphate; T6P, tagatose 6-phosphate; F2,6P, fructose 2,6-bisphosphate; F1,2-cyclic-6-P, fructose 1,2:6-bisphosphate; PPP_i and $PPPP_i$, inorganic tripolyphosphate and tetrapolyphosphate, respectively; Pipes, 1,4-piperazinediethanesulfonic acid; Taps, 3-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-1-propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

0006-2960/84/0423-4101$01.50/0

librium random mechanism with several dead-end complexes. These findings will eventually allow a comparison of the kinetic mechanisms for PP_i-PFK with both the allosteric and nonallosteric ATP-PFKs.

Materials and Methods

Chemicals. Sodium pyrophosphate was obtained from Mallinckrodt. Sodium tripolyphosphate, hexaammonium tetrapolyphosphate, trisodium methylenediphosphonate, F 1,2-cyclic-6-P (sodium salt), fructose 2,6-bisphosphate (sodium salt), and D-glyceraldehyde-3-P (diethyl acetal, dicyclohexylammonium salt) were from Sigma. The D-glyceraldehyde-3-P was prepared by mixing 50 mg of the powder with 2 mL of distilled water and then adding 2 mL of Dowex 50W-X8 (200–400 mesh) in the H^+ form. The mixture was then filtered (filtrate $\simeq$ pH 2), hydrolyzed for 15 h at 35 °C, and used immediately. Tagatose-6-P (barium salt) was obtained from Research Plus, Inc. The 2,5-anhydro-D-mannitol 1,6-bisphosphate was a gift from Dr. R. E. Reeves (Department of Biochemistry, LSU Medical Center). Deuterium oxide (100 atom % deuterium) was obtained from Aldrich Chemical Co. All other chemicals and reagents were obtained from commercial sources and were of the highest purity available.

Enzymes. Fructose-bisphosphate aldolase (EC 4.1.2.13) from rabbit muscle, triosephosphate isomerase (EC 5.3.1.1) from rabbit muscle, α-glycerol-phosphate dehydrogenase (EC 1.1.1.8) from rabbit muscle, 3-phosphoglycerate kinase (EC 2.7.2.3) from yeast, and glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) from rabbit muscle were obtained from Boehringer-Mannheim and dialyzed against 20 mM Pipes, pH 7.3 ($\simeq$5 °C), before use. Phosphoglucoisomerase (EC 5.3.1.9) from yeast (sulfate-free powder) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) from bakers' yeast (sulfate-free powder) were obtained from Sigma and dissolved in 100 mM Taps, pH 7.8, prior to use.

In order to be sure that coupling enzymes were devoid of competing activities, all enzymes were tested for fructose-1,6-bisphosphatase, inorganic pyrophosphatase, and phosphoglucoisomerase activities. The first two of these tests made use of time courses obtained by using ^{31}P NMR with FDP or $MgPP_i$, respectively, in the presence of each of the individual coupling enzymes, monitoring appearance of P_i. No fructose-1,6-bisphosphatase was detected in any case, but pyrophosphatase activity was present in both phosphoglucoisomerase and glucose-6-phosphate dehydrogenase. Phosphoglucoisomerase activity of each of the coupling enzymes was monitored by using 5 units of glucose-6-phosphate dehydrogenase and 1 mM NADP and 100 mM Taps, pH 8. None of the coupling enzymes had a significant amount of isomerase. In order to allow product inhibition studies by $MgPP_i$, phosphoglucoisomerase and glucose-6-phosphate dehydrogenase preparations were purified of contaminating pyrophosphatase as described below. The PP_i-PFK preparation obtained from Sigma was also tested for the above competing activities and was found to contain phosphoglucoisomerase and aldolase. Therefore, it was also necessary to purify this enzyme.

Phosphoglucoisomerase (Sigma P9010) was partially purified by using cellulose phosphate chromatography (enzyme eluted with 10 mM F6P) to a final specific activity of 866 μmol min^{-1} (mg of protein)$^{-1}$. Glucose-6-phosphate dehydrogenase (Sigma G6378) was partially purified by chromatography using a Amicon Dyematrex Blue A column (eluting with 10 mM NADP) to a final specific activity of 732 μmol min^{-1} (mg of protein)$^{-1}$. Both of these partially purified preparations were free of pyrophosphatase activity as judged by a lack of change in the signal intensity of $MgPP_i$ when ^{31}P NMR was used with 2 mM $MgPP_i$ in 100 mM Taps, pH 8.0, and 30% D_2O, at 25 °C. The incubation mixture was scanned before addition of enzyme and after a 2-h incubation with excess enzyme.

The pyrophosphate-dependent fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.90) obtained from Sigma (F8381) has a specific activity of 10 μmol min^{-1} (mg of protein)$^{-1}$, when assayed as described below. This preparation shows approximately 25 major bands on SDS–PAGE run according to the method of O'Farrel (1975) as modified by Atkins et al. (1975). This crude commercial preparation contained significant amounts of phosphoglucoisomerase and fructose-bisphosphate aldolase. The enzyme obtained from Sigma was purified by using the method of O'Brien et al. (1975), beginning with the final phosphocellulose column. Purified enzyme was stored in 100 mM Pipes, pH 7.2, with 20% glycerol at −20 °C. For several preparations, specific activities have ranged from 325 to 369 μmol min^{-1} (mg of protein)$^{-1}$ at pH 8 and 25 °C. Purity and molecular weight were determined on 7.5–12.5% gradient SDS slab gels (1 mm), stained with Coomassie Brilliant Blue. Standards were included ranging in molecular weight from 29 000 to 250 000. A single band with molecular weight of approximately 48 000 was obtained with both Coomassie and silver stain (Bio-Rad). This monomer molecular weight agrees very well with the value of M_r 95 000 reported for the dimer by O'Brien et al. (1975) for PP_i-PFK from the same source. Our stock concentration of the PP_i-PFK ranged from 4×10^{-7} to 10×10^{-7} M.

The PP_i-PFK from *Entamoeba histolytica* was also partially purified. Lyophilized cells were hypoosmotically disrupted with 20 mL of cold 20 mM potassium phosphate, pH 7.0, containing 1 mM EDTA. After centrifugation at 5200*g* for 20 min to eliminate cell debris, a 55–80% $(NH_4)_2SO_4$ fractionation was carried out. The resuspended and dialyzed enzyme was then further purified by the method of O'Brien et al. (1975) starting with the cellulose phosphate columns. The overall purification was 65-fold with a final specific activity of 58 μmol min^{-1} (mg of protein)$^{-1}$. This partially purified preparation of PP_i-PFK from *Entamoeba* gave approximately eight bands on SDS slab gels stained with Coomassie Brilliant Blue. The final preparation contained considerable aldolase but no pyrophosphatase or fructose-1,6-bisphosphatase activities.

Enzyme Assays. In the direction of phosphorylation of F6P, coupled assays were used to measure production of both P_i and FDP by the appearance and disappearance of NADH, respectively. The appearance of FDP was coupled to the aldolase, triosephosphate isomerase, and α-glycerol-phosphate dehydrogenase reactions. A typical assay contained, in a 1 mL volume, 100 mM Taps, pH 8, 2 units of aldolase, 3 units of triosephosphate isomerase, 3 units of α-glycerol-phosphate dehydrogenase, 0.2 mM $NADH_f$, 2 mM $F6P_f$, 1 mM $MgPP_i$, and 0.01 unit of PP_i-PFK.[2] Reaction was initiated by the addition of PP_i-PFK, and all time courses were obtained by monitoring the disappearance of NADH at 340 nm. This coupled assay was optimized for a 30-s lag and cost according to the method of Cleland (1979a). All other coupled assays

[2] The chelate complexes MgP_i, MgF6P, and MgFDP are not substrates for this enzyme as will be shown. Instead, the uncomplexed forms of these reactants and the metal are required. We suggest using Mg_f^{2+}, P_{if}, $F6P_f$, and FDP_f to represent the concentrations of Mg^{2+}, P_i, F6P, and FDP, respectively, which have been corrected for the concentration of the chelate complexes.

were optimized for a 3-s lag by using the same method. The velocity vs. enzyme curve was obtained by using the optimum assay described and repeated at the highest concentration of selected inhibitors when inhibition studies were carried out to ensure that PP_i-PFK was limiting. Under all conditions used a linear velocity vs. enzyme curve was obtained.

The appearance of P_i was coupled to the glyceraldehyde-3-phosphate dehydrogenase reaction in the presence of phosphoglycerate kinase to ensure the dehydrogenase was maintained away from equilibrium. A typical assay contained, in a 1 mL volume, 100 mM Taps, pH 8, 5 units of glyceraldehyde-3-phosphate dehydrogenase, 2 units of phosphoglycerate kinase, 1 mM glyceraldehyde 3-phosphate, 1 mM NAD_f, 1 mM MgADP, 2 mM $F6P_f$, 1 mM $MgPP_i$, and 0.01 unit of PP_i-PFK. Reaction was initiated by the addition of PP_i-PFK, and all time courses were obtained by monitoring the appearance of NADH at 340 nm. Under all conditions used, a linear velocity vs. PP_i- PFK curve was obtained.

All assays were obtained on a Cary 210 spectrophotometer with cells of 1-cm light path. Temperature was maintained at 25 ± 0.1 °C by using a water bath with capacity to heat and cool the cell holder. Temperature was routinely monitored by using a YSI Tele thermometer.

Correction for Metal Chelate Complexes. Since reactants are charged and enzyme requires a divalent metal, it is necessary to be able to calculate complexed and uncomplexed concentrations of reactants. The following dissociation constants were used: MgP_i, 3.2 mM; $MgPP_i$, 0.002 mM; MgFDP, 2 mM; MgF6P, 25.7 mM; $MgPPP_i$, 0.0017 mM; $MgPPPP_i$, 0.001 mM; MgPCP, 0.002 mM; (Martell & Smith, 1977); those for $NADP^+$, 19.1 mM, and NADH, 18.6 mM, were taken from Apps (1973). For Mg–anhydromannitol 1,6-bisphosphate, Mg–fructose 2,6-bisphosphate, and Mg–fructose 1,2:6-bisphosphate the value for MgFDP was used, while for Mg–tagatose 6-phosphate, the Mg–F6P value was used. The concentration of total ligand was calculated from the following equation:

$$[L_t] = [L_f] + \frac{[L_f][Mg_f]}{K_{MgL}} \quad (2)$$

while the total Mg^{2+} concentration was calculated according to the following equation:

$$[Mg_t] = [Mg_f] + \sum_i \frac{[(Mg_f)(L_{if})]}{K_{MgL_i}} \quad (3)$$

where $[L_t]$, $[L_f]$, $[Mg_t]$, and $[Mg_f]$ are total and free concentrations of ligand and Mg^{2+}, respectively. K_{MgL} is the dissociation constant for the metal–ligand complex while K_{MgL_i} is the dissociation constant for the metal–ligand complex with the ith ligand.

In the case of PP_i, PCP, PPP_i, and $PPPP_i$, the dissociation constants are essentially stoichiometric for the Mg^{2+} concentration used in these studies. Some ionizations for the above are around pH 8; e.g., the highest pK for PP_i is 8.37 (Martell & Smith, 1977). However, the dissociation constant for the protonated form is still quite low, e.g., 0.7 mM for $MgHPP_i$ (Martell & Smith, 1977), and the pK for the $MgHPP_i$ complex is 6.1. The end result is that all PP_i exists as $MgPP_i$.

^{31}P NMR Spectra. The ^{31}P spectra were recorded at 36.3 MHz on a JEOL FX-90Q spectrometer with HOD as the locking signal. Spectra were obtained by using a 90° pulse of 25-μs duration, a pulse delay of 3 s, and a pulse acquisition of 3.4 s. Typically, 120 scans were accumulated and the sweep width was 1200 Hz. Chemical shifts were referenced to 85% H_3PO_4 as external standard. All measurements were made by using 1-cm tubes at a probe temperature of 28 °C. A typical ^{31}P NMR spectrum for the equilibrium mixture of reactants is shown in Figure 2.

Data Processing. Reciprocal initial velocities were plotted vs. reciprocal substrate concentrations, and all plots were linear. All data were fitted to the appropriate rate equations by using the Fortran programs of Cleland (1979b). Data for velocity vs. enzyme concentration were fitted to the equation for a straight line. Data for initial velocity studies in the direction of fructose 6-phosphate phosphorylation were fitted to eq 4,

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \quad (4)$$

while initial velocity data obtained in the reverse direction were fitted to eq 5. Data for competitive and noncompetitive

$$v = VABC/[\text{constant} + (\text{coef A})A + (\text{coef B})B + (\text{coef C})C + K_aBC + K_bAC + K_cAB + ABC] \quad (5)$$

inhibition were fitted to eq 6 and 7, respectively. In eq 4–7,

$$v = \frac{VA}{K_a(1 + I/K_{is}) + A} \quad (6)$$

$$v = \frac{VA}{K_a(1 + I/K_{is}) + A(1 + I/K_{ii})} \quad (7)$$

V is the maximum velocity, A, B, C, and I are concentrations of reactants and inhibitor, respectively, K_a, K_b, and K_c are Michaelis constants for the respective reactants, K_{ia} is the dissociation constant for A, K_{is} and K_{ii} are slope and intercept inhibition constants, and constant, coef A, coef B, and coef C are products of constants which are mechanism dependent and will be defined later.

Results

Reactants for PP_i-PFK. The stoichiometry of the PP_i-PFK-catalyzed reaction from *Entamoeba histolytica* (Reeves et al., 1974) and *Propionibacterium shermanii* (O'Brien et al., 1975) is

$$F6P + PP_i \overset{Mg^{2+}}{\rightleftharpoons} FDP + P_i \quad (8)$$

No attempt was made in previous studies, however, to determine whether the magnesium chelate complexes of the above reactants or the uncomplexed reactants are the actual substrates. The following experiments were used to determine whether complexed or uncomplexed P_i and PP_i are reactants.

First, the total concentration of reactant was maintained at a low level, and Mg^{2+} concentration (corrected for MgFDP and MgF6P, respectively) was increased from a concentration where very little of the reactant was complexed to a value where a large amount was complexed. If the uncomplexed form is the reactant, the rate would be expected to increase at first since the reaction requires metal and reactant but will then decrease as the concentration of the uncomplexed form decreases. This will mimic substrate inhibition by Mg^{2+}. This experiment is then repeated by maintaining the Mg^{2+} concentration (again corrected for MgFDP and MgF6P) at a low level and increasing reactant (P_i or PP_i). Again, if uncomplexed Mg^{2+} is a pseudoreactant, the rate will first increase since both Mg^{2+} and reactant are required but will then decrease as uncomplexed Mg^{2+} decreases. This will mimic substrate inhibition by reactant. In the case of PP_i, both curves increased to a finite maximum velocity and remained there until either Mg^{2+} (or PP_i) became high enough to result in precipitation. In the case of P_i, apparent substrate inhibition was obtained for both experiments (Figure 1). There are two

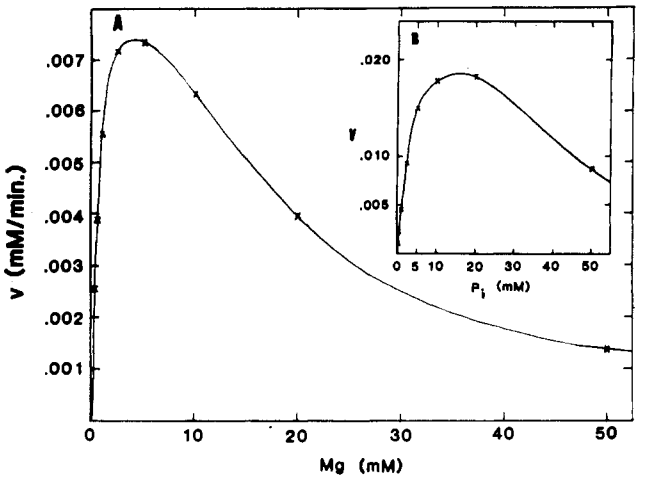


FIGURE 1: Dependence of velocity on the concentration of Mg^{2+} (A) and P_i (B). The concentrations of Mg^{2+} and FDP (maintained at 0.3 mM) were corrected for MgFDP as described under Materials and Methods. (A) The total phosphate concentration was maintained at 400 μM at all Mg^{2+} concentrations indicated. (B) The Mg_f^{2+} concentration (uncorrected for MgP_i) was maintained at 200 μM at all P_i concentrations indicated. All assays were carried out at pH 8, 100 mM Taps, and 25 °C.

possibilities for these results. First, the complex is the substrate in both cases, that is, MgP_i and $MgPP_i$. In the case of P_i, both free P_i and Mg^{2+} inhibit, while in the case of PP_i, neither Mg^{2+} nor PP_i inhibits significantly. The other most likely possibility is that $MgPP_i$ is the reactant while in the opposite direction P_i and Mg^{2+} are reactants. It would otherwise be difficult to rationalize the substrate inhibition by Mg^{2+} in the P_i direction while no substrate inhibition is obtained in the opposite direction. These possibilites are further treated below.

Second, an initial velocity pattern was obtained by varying Mg_f^{2+} at several different fixed levels of P_{if} (the subscript f indicates correction for chelate complex); all reciprocal plots were linear. For each of the 16 points obtained, a different MgP_i concentration was calculated. If MgP_i were the reactant, a reciprocal plot of velocity vs. MgP_i should give a single straight line with 16 points. This is not the case.

Third, if the concentrations of Mg_f^{2+} and P_{if} (corrected for MgP_i) are increased to high levels, e.g., 50–60 mM, no substrate inhibition is obtained with any fixed FDP concentration. This is consistent with no inhibition by either of the uncomplexed forms of Mg^{2+} or P_i.

The experiments described above were also carried out with F6P and FDP (correcting the Mg concentration for $MgPP_i$ and MgP_i as needed) as a test. By all criteria discussed above, the uncomplexed forms of F6P and FDP are reactants. Thus, the reaction for PP_i-PFK can be written as

$$F6P + MgPP_i \rightleftharpoons FDP + P_i + Mg^{2+} \quad (9)$$

Initial Velocity Studies in the Absence of Products. When the velocity is determined at varying F6P concentrations and several fixed levels of $MgPP_i$, an intersecting initial velocity pattern is obtained. A fit of the data to eq 4 for a sequential mechanism gives the kinetic parameters in Table I.

In the reverse direction for PP_i-PFK, Mg_f^{2+} is a pseudo-reactant since the velocity will vary with Mg^{2+} concentration even though Mg^{2+} will not be chemically changed. Thus, the systematic variation of the concentration of all three components, Mg_f^{2+}, P_{if}, and FDP_f (Cook, 1982; Viola & Cleland, 1982), will provide information on the kinetic mechanism. When the velocity is determined at varying P_{if} concentrations, several fixed levels of Mg_f^{2+}, and a single fixed level of FDP_f, a sequential initial velocity pattern is obtained. This pattern

Table I: Kinetic Parameters for the Pyrophosphate-Dependent Phosphofructokinase Reaction at pH 8.0 and 25 °C

forward reaction direction	reverse reaction direction[a]
$V = 0.075 \pm 0.0012$ mM/min	$V = 0.087 \pm 0.003$ mM/min
$K_{MgPP_i} = 0.0046 \pm 0.0006$ mM	$K_{FDP} = 0.030 \pm 0.004$ mM
$K_{F6P} = 0.127 \pm 0.004$ mM	$K_{Mg} = 0.100 \pm 0.066$ mM[b]
$K_{i,F6P} = 0.62 \pm 0.11$ mM	$K_{P_i} = 0.560 \pm 0.086$ mM
$K_{i,MgPP_i} = 0.023 \pm 0.002$ mM	$K_{i,Mg} = 6.1 \pm 2.0$ mM
$V/E_T = 890$ s^{-1}	$K_{i,FDP} = 0.086 \pm 0.013$ mM
$V/[(K_{MgPP_i})(E_T)] = 1.9 \times 10^5$ M^{-1} s^{-1}	$K_{i,P_i} = 10.3 \pm 4.8$ mM
	$V/E_T = 1035$ s^{-1}
$V/[(K_{F6P})(E_T)] = 7 \times 10^3$ M^{-1} s^{-1}	$V/[(K_{FDP})(E_T)] = 3.5 \times 10^4$ M^{-1} s^{-1}
	$V/[(K_{P_i})(E_T)] = 1.8 \times 10^3$ M^{-1} s^{-1}

[a] The initial velocity rate equation in this direction, once constant and coefficient terms have been defined by using the method of Cha (1968), is $v = VPQB/(K_{iq}K_{ip}K_b + K_{iq}K_bP + K_{ip}K_{iq}B + K_{ip}K_bQ + K_bPQ + K_pBQ + K_qBP + PQB)$ where P, B, and Q are defined for mechanism 11. [b] The K_{Mg} value was confirmed by varying Mg^{2+} at saturating concentrations of FDP (0.6 mM) and P_i (6 mM). A value of 0.167 ± 0.005 mM was obtained from a fit of the data to $v = (V/A)(K + A)$.

was then repeated at several additional FDP_f concentrations to generate three additional initial velocity patterns. Graphical analysis suggested that all terms in the equation for a random mechanism were present, and data were fitted to eq 5. Kinetic parameters are listed in Table I.

Product Inhibition. Inhibition by P_{if} was obtained by using the aldolase/glycerol-phosphate dehydrogenase couple. Inhibition patterns were obtained by varying $F6P_f$ at saturating $MgPP_i$ or with $MgPP_i = K_{MgPP_i}$ and by varying $MgPP_i$ at saturating $F6P_f$ or with $F6P_f = K_{F6P}$. When the fixed reactant was maintained at a saturating level, P_{if} was competitive vs. the variable substrate while decreasing the concentration of the fixed reactant to K_m resulted in noncompetitive inhibition vs. the variable substrate.

Inhibition by FDP_f was obtained by using the glyceraldehyde-3-phosphate dehydrogenase/phosphoglycerate kinase couple. With $F6P_f$ as the variable substrate, FDP_f was competitive whether $MgPP_i$ was maintained saturating or equal to its K_m. Saturation by $F6P_f$ eliminated the inhibition by FDP_f.

In the reverse direction, inhibition by $MgPP_i$ was obtained by using the phosphoglucoisomerase/glucose-6-phosphate dehydrogenase couple. The Mg_f^{2+} concentration was maintained saturating for all patterns. With FDP_f or P_{if} saturating, $MgPP_i$ was competitive vs. P_{if} and FDP_f, respectively. If FDP_f and P_{if} were maintained equal to their respective K_m values, $MgPP_i$ was noncompetitive vs. P_{if} and FDP_f, respectively. Results are summarized in Table II.

Dead-End Inhibition. In order to further define the kinetic mechanism, dead-end analogues of all reactants were used as inhibitors. In these studies, the fixed reactant was maintained equal to its K_m. In the direction of phosphorylation of F6P, D-tagatose 6-phosphate (T6P) was used as an analogue of F6P. No phosphorylation of T6P was observed when saturating levels of $MgPP_i$ were used. Inhibition is competitive vs. F6P and noncompetitive vs. $MgPP_i$. Methylenediphosphonate (PCP) was used as a nonhydrolyzable analogue of PP_i. Inhibition is competitive vs. $MgPP_i$ and noncompetitive vs. F6P.

In the direction of phosphorylation of P_i, sulfate was used as an analogue of P_i while 2,5-anhydro-D-mannitol 1,6-bisphosphate was used as an analogue of FDP. Sulfate is competitive vs. P_i and noncompetitive vs. FDP, while 2,5-anhydro-D-mannitol 1,6-bisphosphate is competitive vs. FDP and noncompetitive vs. P_i.

Tripolyphosphate and tetrapolyphosphate are competitive vs. FDP but gave noncompetitive inhibition vs. $MgPP_i$. Fructose 2,6-bisphosphate (F2,6P) also gives a competitive

Table II: Product Inhibition Patterns[a]

variable substrate	fixed substrate	product	pattern[e]	K_{is} ± SE (mM)	K_{ii} ± SE (mM)
		Forward Reaction			
F6P	$MgPP_i$[b]	P_i	C	10.1 ● 0.7	
F6P	$MgPP_i$[d]	P_i	NC	5.1 ± 1.2	ca. 10
$MgPP_i$	F6P[b]	P_i	C	8.8 ± 0.3	
$MgPP_i$	F6P[d]	P_i	NC	4.4 ± 1.1	17.2 ± 5.1
F6P	$MgPP_i$[b]	FDP	C	0.65 ± 0.31	
F6P	$MgPP_i$[d]	FDP	C	0.12 ± 0.015	
$MgPP_i$	F6P[b]	FDP	none		
		Reverse Reaction			
P_i	Mg,[c] FDP[b]	$MgPP_i$	C	0.65 ± 0.07	
P_i	Mg, FDP[d]	$MgPP_i$	NC	0.085 ± 0.006	0.34 ± 0.038
FDP	Mg, P_i[b]	$MgPP_i$	C	0.204 ± 0.015	
FDP	Mg, P_i[d]	$MgPP_i$	NC	0.055 ± 0.008	3.25 ± 0.90

[a] All data were obtained at pH 8, 100 mM Taps, 25 °C. The concentrations of Mg^{2+}, F6P, FDP, and P_i were corrected for the Mg^{2+} chelate complexes by using dissociation constants given under Materials and Methods. [b] The concentration of the fixed reactant was maintained at a saturating level ($20K_m$). [c] The concentration of Mg_f^{2+} was saturating (2.5 mM) for all $MgPP_i$ product inhibition patterns. [d] The fixed reactant was maintained at a value equal to its K_m. [e] Data adhering to competitive and noncompetitive inhibition were fit to eq 6 and 7, respectively.

pattern vs. FDP and is noncompetitive vs. $MgPP_i$. The FDP analogue in which phosphate is present as the 1,2-cyclic phosphodiester gave no inhibition. Results are summarized in Table III.

Initial Velocity Studies with PP_i-PFK from E. histolytica. When the velocity of the pyrophosphate-dependent PFK from *Entamoeba histolytica* was obtained by varying the concentration of F6P at several fixed levels of $MgPP_i$, an intersecting initial velocity pattern is observed. Kinetic parameters are summarized in Table IV.

To further define the mechanism for this enzyme so that comparison could be made with the enzyme from *Propionibacterium*, product inhibition by phosphate was obtained by varying either $MgPP_i$ or F6P at saturating concentrations of the other reactant. Inhibition was also obtained by $MgPP_i$ by varying either P_i or FDP with Mg_f^{2+} and the other reactant saturating. All patterns are competitive. Results are summarized in Table IV.

Haldane Relationship. In order to be certain kinetic data are correctly determined, kinetic parameters must adhere to the Haldane relationship. One of the kinetic Haldane relationships for a bi-ter mechanism (Cleland, 1982) is given as follows:

$$K_{eq} = \frac{(V_f)(K_{Mg})(K_{i,FDP})(K_{i,P_i})}{(V_r)(K_{F6P})(K_{i,MgPP_i})} \quad (10)$$

Substitution of values from Table I and II into the above equation gives a value of $(5 \pm 1.5) \times 10^{-3}$ M for K_{eq}.

The equilibrium constant was also determined by using ^{31}P NMR. A reaction mixture was prepared as close to equilibrium as possible and the spectrum recorded prior to the addition of enzyme and as a function of time after the addition of enzyme to ensure that equilibrium had been attained. These data are shown in Figure 2. The percent increase and percent decrease in given phosphate resonances were used to adjust the reactant concentrations. The concentration of PP_i was assumed equal to $MgPP_i$, and the concentrations of Mg_f, FDP_f, P_{if}, MgP_i, and MgFDP were recalculated. The total Mg^{2+} concentration used for these calculations was the difference between total added and total PP_i at equilibrium. The concentrations of free and complexed species were adjusted until the free Mg^{2+} concentration could be used to satisfy the expressions for both K_{MgP_i} and K_{MgFDP}. The amount of MgF6P was assumed insignificant. The range of equilibrium constants calculated in this manner was $(7 \pm 2) \times 10^{-3}$ M. This is in agreement with the value obtained from the Haldane relationship.

Discussion

Initial Velocity Studies in the Absence of Products. The systematic variation of Mg^{2+} and reactant concentrations

Table III: Dead-End Inhibition Patterns[a]

variable substrate	fixed substrate[b]	inhibitor	pattern[c]	K_{is} ± SE (mM)	K_{ii} ± SE (mM)
		Forward Reaction			
F6P	$MgPP_i$	D-tagatose-6-P	C	1.5 ± 0.1[d]	
F6P	$MgPP_i$	MgPCP	NC	1.97 ± 0.09	7.5 ± 0.5
$MgPP_i$	F6P	D-tagatose-6-P	NC	4.6 ± 0.7	3.4 ± 0.2
$MgPP_i$	F6P	MgPCP	C	0.91 ± 0.07	
$MgPP_i$	F6P	PPP_i	NC	0.69 ± 0.33	3.75 ± 0.12
$MgPP_i$	F6P	$PPPP_i$	NC	0.24 ± 0.04	2.22 ± 0.30
$MgPP_i$	F6P	F2,6P	NC	3.84 ± 2.37	5.86 ± 1.46
$MgPP_i$	F6P	F1,2-cyclic-6-P	none		
		Reverse Reaction			
FDP	Mg, P_i	2,5-anhydro-D-mannitol 1,6-bisphosphate	C	0.10 ± 0.005	
FDP	Mg, P_i	SO_4^{2-}	NC	31 ± 5	72 ± 4
P_i	Mg, FDP	2,5-anhydro-D-mannitol 1,6-bisphosphate	NC	0.45 ± 0.03	0.66 ± 0.06
P_i	Mg, FDP	SO_4^{2-}	C	19.4 ± 1.0	
FDP	Mg, P_i	PPP_i	C	0.90 ± 0.13	
FDP	Mg, P_i	$PPPP_i$	C	1.12 ± 0.12	
FDP	Mg, P_i	F2,6P	C	3.93 ± 0.20	
FDP	Mg, P_i	F1,2-cyclic-6-P	none		

[a] Experimental conditions and fitting procedures are as given for product inhibition; all inhibitor concentrations were corrected for chelate complexes. [b] In all cases, the fixed substrate was maintained at a concentration equal to its K_m, except Mg_f^{2+} which was saturating for patterns obtained in the reverse reaction direction. [c] Data for competitive and noncompetitive inhibition were fit to eq 6 and 7, respectively. [d] $MgPP_i = 10K_{MgPP_i}$.

Table IV: Kinetic Parameters for PP_i-PFK from *E. histolytica*[a]

Absence of Products	
forward reaction direction[b]	reverse reaction direction[c]
V = 0.0230 ± 0.0002 mM/min	V = 0.036 ± 0.001 mM/min
K_{MgPP_i} = 0.0220 ± 0.0007 mM	K_{P_i} = 0.86 ± 0.10 mM
K_{F6P} = 0.203 ± 0.012 mM	K_{FDP} = 0.028 ± 0.003 mM
$K_{i,F6P}$ = 0.41 ± 0.02 mM	

Presence of Products				
variable substrate	fixed substrate[d]	products	pattern	K_{is} ± SE[e] (mM)
$MgPP_i$	F6P	P_i	C	5.9 ± 0.2
F6P	$MgPP_i$	P_i	C	3.1 ± 0.2
FDP	Mg, P_i	$MgPP_i$	C	0.16 ± 0.01
P_i	Mg, FDP	$MgPP_i$	C	0.43 ± 0.06

[a] All assays were carried out at pH 8, 100 mM Taps, 25 °C. In both reaction directions, under all conditions velocity vs. PP_i-PFK was linear and passed through the origin. [b] Data obtained from an initial velocity pattern varying F6P at several different levels of $MgPP_i$. Data were fitted to eq 4. [c] V is the average of two values, one obtained at saturating Mg_f^{2+} and P_i with FDP varied and the other at saturating Mg_f^{2+} and FDP with P_i varied. Both K_m values were obtained from the above experiment and were the control lines for product inhibition by $MgPP_i$. [d] The fixed reactant was maintained at saturating levels ($20K$ for Mg_f^{2+} and $18K$ for FDP_f) except for P_i which was fixed at $3K$. [e] Data were fitted to eq 6.

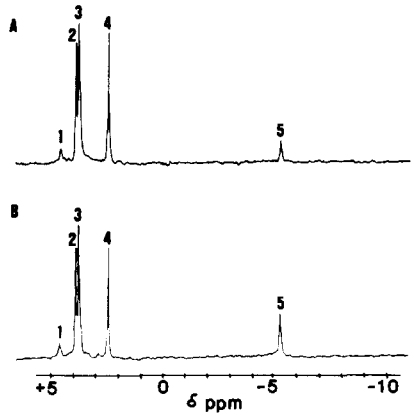


FIGURE 2: Fourier transform ^{31}P NMR of the PP_i-PFK equilibrium mixture. (A) Reaction mixture minus enzyme. The initial total concentrations of reactants are as follows: Mg^{2+}, 10.7 mM; FDP, 15.2 mM; P_i, 13.3 mM; F6P, 2.6 mM; PP_i, 1.4 mM; Taps, 100 mM, pH 8.0; 30% D_2O. (B) Reaction mixture 2 h after the addition of 0.1 unit of enzyme. Probe temperature was 28 °C. Enzyme activity was redetermined after the spectrum was taken with no change in activity. Both spectra are the result of 100 scans with proton decoupling. The resonances indicated are the following (1) 1-phosphate of α-FDP; (2) 1-phosphate of β-FDP; (3) 6-phosphate of α-FDP, β-FDP, and F6P; (4) P_i; (5) PP_i.

suggests that the reactants for PP_i-PFK are $MgPP_i$ and F6P in the direction of F6P phosphorylation and Mg^{2+}, P_i, and FDP in the reverse direction. This is based on a hyperbolic titration of PP_i by Mg^{2+} with the maximum rate attained when virtually no uncomplexed PP_i exists in solution. All other reactants produce first an increase in rate, followed by a decrease as Mg^{2+} concentration is increased (apparent substrate inhibition), but yield normal hyperbolic saturation curves if the concentration of reactant is corrected for chelate complex formation.

Intersecting initial velocity patterns were attained in both reaction directions in agreement with Reeves et al. (1974, 1976) and O'Brien et al. (1975). However, unlike previous studies, Mg^{2+} was also varied as a pseudoreactant in the direction of P_i phosphorylation. This systematic variation of all three reaction components indicated that all terms are present in the denominator of the general rate equation for a random mechanism (Viola & Cleland, 1982). This mechanism was then used as the working hypothesis. Kinetic parameters shown in Table I agree very well with those of previous investigators with the exception of the K_m for PP_i reported by O'Brien et al. (1975), which is an order of magnitude larger than that reported here or by Reeves et al. (1974, 1976). With this one exception, parameters are remarkably similar, not only between this study and previous work but also between the two organisms from which this enzyme has been obtained, i.e., *Entamoeba histolytica* (Reeves et al., 1974; 1976) and *Propionibacterium shermanii* (O'Brien et al., 1975).

Data in this study were obtained at pH 8 to ensure that all reactants were ionized. This is not true for PP_i, but with the levels of Mg^{2+} used in this study all PP_i is $MgPP_i$, and the pK for this species is 6.1 as compared to 8.3 for uncomplexed PP_i. Data obtained by the above authors were collected at pH 7, and thus there is essentially no difference in kinetic parameters when the pH is increased 1 unit. Also, in agreement with the previous work on both bacterial and amoebal enzymes cited above, the ratio of the maximum velocities for both reaction directions is close to 1.

Interpretation of Product Inhibition Patterns. Inhibition by P_i is competitive vs. F6P and $MgPP_i$ when the other reactant is saturating. These patterns become noncompetitive when the fixed reactant becomes equal to its K_m. Thus, P_i combines to E, E–$MgPP_i$ and E–F6P and behaves as a dead-end inhibitor. Quantitatively, the data are internally consistent with K_i values of 5 mM and 4.0 mM, respectively, for release of P_i from E–P_i, obtained from variation of F6P and $MgPP_i$, respectively, with the other reactant at K_m. A value of 10 mM is obtained for dissociation of P_i from E–$MgPP_i$–P_i (saturating $MgPP_i$, varying F6P). The value of 17 mM for K_{ii} obtained when $MgPP_i$ is varied with F6P equal to K_{F6P} is equal to the true K_i for dissociation of P_i from E–$MgPP_i$–P_i multiplied by $(1 + F6P/K_{F6P})$. Since F6P = K_{F6P}, the true K_i is calculated as 8.5 mM in good agreement with the value of 10 mM discussed above. Only one of the values pertaining to the E–F6P–P_i complex was well defined, but qualitatively again both patterns support formation of this complex.

Inhibition by FDP is competitive vs. F6P whether $MgPP_i$ is saturating or equal to its K_m, while (as a check) no inhibition was obtained at saturating F6P. Thus, FDP combines to E and E–$MgPP_i$ and like P_i combines only as a dead-end analogue. These data support rapid equilibrium random release of P_i and FDP and random addition of F6P and $MgPP_i$.

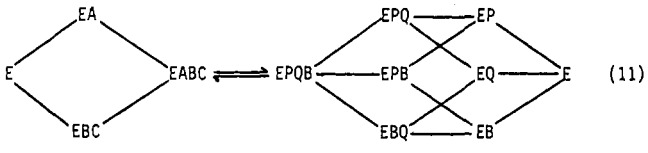
In the reverse direction, inhibition patterns using $MgPP_i$ are qualitatively identical with those obtained by using P_i. Competitive inhibition is obtained when the fixed reactant is saturating, and these patterns become noncompetitive when the fixed reactant is maintained at its respective K_m value. Thus, $MgPP_i$ combines to E–Mg, E–Mg–P_i, and E–Mg–FDP (Mg^{2+} was maintained saturating for all of these patterns), and this reactant also combines only as a dead-end analogue. Values of 83 μM and 55 μM are obtained for release of $MgPP_i$ from E–Mg–$MgPP_i$ when P_i and FDP are varied, respectively. A value of 200 μM is obtained for dissociation of $MgPP_i$ from E–Mg–P_i–$MgPP_i$ (varying FDP with Mg^{2+}, P_i saturating), while a value of 170 μM is calculated from the K_{ii} value when P_i is varied (FDP = K_{FDP}). The K_i for dissociation of $MgPP_i$ from E–Mg–FDP–$MgPP_i$ is 640 μM (varying P_i with Mg^{2+}, FDP saturating), while a value of 1.6 mM is calculated from the K_{ii} by varying FDP with P_i at its K_m. The agreement is not as good in this case, but the K_{ii} value does have a large standard error. Inhibition by $MgPP_i$ indicates that, except for dead-end complexes, $MgPP_i$ combines to free enzyme

alone. This eliminates all but an ordered mechanism in which $MgPP_i$ combines first or a rapid equilibrium random mechanism. Patterns obtained in the other direction, however, rule out the ordered mechanism, and one is left with a rapid equilibrium random mechanism. These data agree qualitatively with the assignment of Reeves et al. (1976) based on somewhat limited data. There is not exact quantitative agreement, but this is not surprising since there was no attempt to correct for chelate complex formation in the previous studies.

The dissociation constant for P_i from E–P_i is about half the value obtained for either E–$MgPP_i$–P_i or E–F6P–P_i. The latter complex is expected in a random mechanism since neither reactant has the group to be transferred. However, P_i appears to bind equally well to E–$MgPP_i$ and E–F6P which is not predicted since the P_1 site should be filled by $MgPP_i$. The inhibitor may bind to the 6-phosphate binding site in the latter case. Even more surprising is the formation of an E–$MgPP_i$–FDP complex which is observed in both reaction directions. In this case, however, the binding of FDP is 5-fold less favorable when $MgPP_i$ is bound, while the binding of $MgPP_i$ is 7-fold less favorable when FDP is bound.

Interpretation of Dead-End Inhibition Patterns. Tagatose 6-phosphate, a dead-end analogue of F6P, is competitive vs. F6P, whether $MgPP_i$ is saturating or equal to its K_m, while noncompetitive inhibition is obtained vs. $MgPP_i$. The analogue of $MgPP_i$, MgPCP, is competitive vs. $MgPP_i$ and noncompetitive vs. F6P. The diphosphonates were also found to be competitive vs. $MgPP_i$ by Eubank & Reeves (1982). These patterns support the random mechanism with E–T6P, E–$MgPP_i$–T6P, E–MgPCP, and E–F6P–MgPCP complexes allowed. The quantitative agreement of K_i values is again very good. For example, a K_i of 1.5 mM is obtained for T6P dissociating from E–T6P–$MgPP_i$, while a value of 1.7 mM is calculated from the K_{ii} when $MgPP_i$ was varied with F6P equal to its K_m.

In the reverse direction, a qualitatively indentical pattern is obtained. The analogue of FDP (2,5-anhydro-D-mannitol 1,6-bisphosphate) is competitive vs. FDP and noncompetitive vs. P_i, while the analogue of P_i (sulfate) is competitive vs. P_i and noncompetitive vs. FDP. Quantitative agreement of K_i values is again very good. Thus, the mechanism for the PP_i-PFK catalyzed reaction is rapid equilibrium random and can be depicted as follows:



(11)

where A, B, C, P, and Q are F6P, Mg^{2+}, PP_i, P_i, and FDP, respectively. In addition, the E–$MgPP_i$–P_i, E–F6P–P_i, and E–FDP–$MgPP_i$ dead-end complexes form.

Four additional compounds were tested as inhibitors. Tripolyphosphate and tetrapolyphosphate compete with both FDP and $MgPP_i$ for free enzyme. Surprisingly, they also bind, albeit much more poorly, to the E–$MgPP_i$ complex but not to E–FDP. Fructose 2,6-bisphosphate also competes with FDP and $MgPP_i$ for free enzyme and will bind to E–$MgPP_i$. In all cases, the latter compound binds rather poorly. The cyclic phosphodiester fructose 1,2:6-bisphosphate apparently does not bind at all up to 2 mM concentrations. Whether the polyphosphates act as P_i analogues and can occupy the 6-phosphate site or bind somewhere close to the site and sterically affect FDP binding is unclear. The E–$MgPP_i$–F2,6P complex does offer some corroborative data in support of the E–$MgPP_i$–FDP complex.

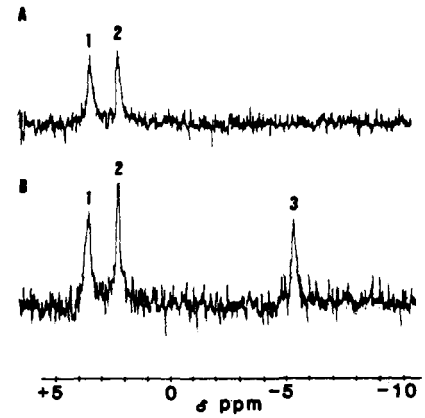


FIGURE 3: Fourier transform ^{31}P NMR of the PP_i-PFK reaction with 2,5-anhydro-D-mannitol 1,6-bisphosphate as a reactant. Initial concentration of reactants are as follows: Mg^{2+}, 4.5 mM; 2,5-anhydro-D-mannitol 1,6-bisphosphate, 2.5 mM; P_i, 4.5 mM; Taps, 100 mM, pH 8.0; 30% D_2O. (A) Reaction mixture minus enzyme (NGAIN = 7). (B) Reaction mixture 24 h after the addition of 0.6 unit of enzyme (NGAIN = 8). Probe temperature was 28 °C. Both spectra are the result of 300 scans with proton decoupling. The resonances indicated are the following: (1) both phosphates of 2,5-anhydro-D-mannitol 1,6-bisphosphate; (2) P_i; (3) PP_i.

Using the coupled spectrophotometric assays described under Materials and Methods, we determined that of the dead-end inhibitors listed in Table III, none could serve as a reactant. The FDP analogue 2,5-anhydro-D-mannitol 1,6-bisphosphate was a reactant in the reverse reaction direction in the presence of Mg^{2+} and P_i. This is shown in Figure 3. Thus, like the rabbit muscle enzyme (Koerner et al., 1974), the *Propionibacterium* enzyme also most likely requires the β-anomer of F6P and FDP. Imidodiphosphate (PNP) was also tested as a reactant by using the coupled assays, with identical results. Thus, it appears that PP_i-PFK, unlike apyrase (Harmony et al., 1983), does not protonate the bridge oxygen of pyrophosphate during catalysis. The possibility remains, however, that some of these analogues are utilized at very low rates beyond the limits of detection used in the assays. However, when the possible utilization of the dead-end inhibitors (those containing phosphorus) as reactants was tested by ^{31}P NMR analysis over a 2-h period, the results were the same as those obtained by the spectrophotometric assays with the exception of 2,5-anhydro-D-mannitol 1,6-bisphosphate as discussed above. We also have no evidence that any of the dead-end inhibitors utilized stimulated the activity of PP_i-PFK. Reeves et al. (1976) also stated that arsenate substitutes for phosphate, giving the same relative V_{max} but a K_m 4-fold higher than that of P_i.

Comparison of the Enzymes from Entamoeba and Propionibacterium. The product inhibition patterns by P_i and $MgPP_i$ vs. both reactants with the fixed reactant saturating are competitive for both enzymes. Thus, qualitatively, both enzymes have identical mechanisms, that is, rapid equilibrium random with three dead-end complexes, E–$MgPP_i$–P_i, E–F6P–P_i and E–FDP–$MgPP_i$. These patterns are fairly diagnostic since, irrespective of which reactant is varied, the competitive pattern indicates that the varied reactant and the inhibitor combine to the same enzyme form.

Quantitatively, there are also very few differences. The K_m values for the sugar substrates obtained with the amoebal enzyme are essentially identical with those obtained with the bacterial enzyme. There are some differences in the values of kinetic parameters for P_i and $MgPP_i$. The K_m value for $MgPP_i$ is 5-fold higher for the amoebal than for the bacterial enzyme, while that for P_i is about 2-fold higher. The K_i values

for product inhibition by $MgPP_i$ vs. FDP or P_i are essentially identical for both enzymes. For the bacterial enzyme, P_i binds equally well to E–F6P (K_i = 8.8 mM) and E–$MgPP_i$ (K_i = 10 mM). For the amoebal enzyme, however, P_i binds twice as well to E–$MgPP_i$ (K_i = 3 mM) than it does to E–F6P (K_i = 6 mM). Even with these quantitative differences, the trends for binding of P_i and $MgPP_i$ are similar for both enzymes. Pyrophosphate binds about 8–40-fold better to E–F6P than it does to E–Mg–P_i and about 10-fold better to E–Mg–P_i than to E–Mg–FDP. On the other hand, P_i binds roughly equally well to E–$MgPP_i$ and E–F6P but about 10-fold better to E–Mg–FDP.

In summary, On the basis of kinetic analyses of initial velocity, product inhibition, and dead-end inhibition patterns in both reaction directions at pH 8 and 25 °C, we propose that the reaction mechanism for PP_i-PFK purified from *Propionibacterium freudenreichii* is rapid equilibrium random, with E–$MgPP_i$–P_i, E–F6P–P_i, and E–$MgPP_i$–FDP dead-end complexes. Work is currently in progress on isotope exchange at equilibrium to test the proposed mechanism.

Acknowledgments

We thank Dr. Richard E. Reeves of the Department of Biochemistry at LSU Medical Center for the 2,5-anhydro-D-mannitol 1,6-bisphosphate and the lyophilized *Entamoeba histolytica* (DKB stain) cells and Charles Pittman of the Chemistry Department at NTSU for operation of the Fourier transform ^{31}P NMR. We also thank Joye Wesley and Rita Huber for typing the manuscript.

Registry No. PP_i–PFK, 55326-40-4; $MgPP_i$, 13446-24-7; F6P, 643-13-0; FDP, 488-69-7; P_i, 14265-44-2; MgPCP, 91112-32-2; PPP_i, 7758-29-4; $PPPP_i$, 16751-28-3; F2,6P, 77164-51-3; T6P, 73544-42-0; Mg, 7439-95-4; SO_4^{2-}, 14808-79-8; 2,5-anhydro-D-mannitol 1,6-bisphosphate, 671-08-9.

References

Apps, D. K. (1973) *Biochim. Biophys. Acta 320*, 379.
Atkins, J. F., Lewis, J. B., Anderson, C. W., & Gesteland, R. E. (1975) *J. Biol. Chem. 250*, 5688.
Carnal, N. W., & Black, C. C. (1979) *Biochem. Biophys. Res. Commun. 86*, 20.
Cha, S. (1968) *J. Biol. Chem. 243*, 820.
Cleland, W. W. (1979a) *Anal. Biochem. 99*, 142.
Cleland, W. W. (1979b) *Methods Enzymol. 63*, 103.
Cleland, W. W. (1982) *Methods Enzymol. 87*, 366.
Cook, P. F. (1982) *Biochemistry 21*, 113.
Csêke, C., Weeden, N. F., Buchanan, B. B., & Uyeda, K. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4322.
Eubank, W. B., & Reeves, R. E. (1982) *J. Parasitol. 68*, 599.
Gray, G. R. (1971) *Biochemistry 10*, 4705.
Harmony, T. P., Knight, W. B., Dunaway-Mariano, D., & Sundaralingam, M. (1983) *Biochemistry 22*, 5015.
Koerner, T. A. W., Jr., Cary, L. W., Bhacca, N. S., & Younathan, E. S. (1973) *Biochem. Biophys. Res. Commun. 51*, 543.
Koerner, T. A. W., Jr., Younathan, E. S., Ashour, A. E., & Voll, R. J. (1974) *J. Biol. Chem. 249*, 5749.
Koerner, T. A. W., Jr., Voll, R. J., Ashour, A. E., & Younathan, E. S. (1976) *J. Biol. Chem. 251*, 2983.
Koerner, T. A. W., Jr., Voll, R. J., Cary, L. W., & Younathan, E. S. (1980) *Biochemistry 19*, 2795.
Kruger, N. J., Kombrink, E., & Beevers, H. (1983) *FEBS Lett. 153*, 409.
Martell, A. E., & Smith, R. M., Eds. (1977) *Critical Stability Constants*, Vol. 3–4, Plenum Press, New York.
O'Brien, W. E., Bowien, S., & Wood, H. G. (1975) *J. Biol. Chem. 250*, 8690.
O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007.
Pfleiderer, C. and Klemme, J. H. (1980) *Z. Naturforsch. C: Biosci. 35C*, 229.
Reeves, R. E., South, D. J., Blytt, H. J., & Warren, L. G. (1974) *J. Biol. Chem. 249*, 7737.
Reeves, R. E., Serrano, R., & Soth, D. J. (1976) *J. Biol. Chem. 251*, 2958.
Sabularse, D. C., & Anderson, R. L. (1981) *Biochem. Biophys. Res. Commun. 100*, 1423.
Sawyer, M. H., Baumann, P., & Baumann, L. (1977) *Arch. Microbiol. 112*, 169.
Van Schaftingen, E., Lederer, B., Bartrons, R., & Hers, H. G. (1982) *Eur. J. Biochem. 129*, 191.
Viola, R. E., & Cleland, W. W. (1982) *Methods Enzymol. 87*, 353.