Isotope Partitioning with *Ascaris suum* Phosphofructokinase Is Consistent with an Ordered Kinetic Mechanism†

Grant E. Gibson, Ben G. Harris, and Paul F. Cook*

Department of Biochemistry and Molecular Biology, University of North Texas Health Science Center at Fort Worth, 3500 Camp Bowie Boulevard, Fort Worth, Texas 76107

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ABSTRACT: Isotope partitioning and initial velocity studies have been used to study the kinetic mechanism of *Ascaris suum* phosphofructokinase (PFK) at pH 8.0 for the native enzyme (nPFK), and at pH 6.8 for a form of enzyme desensitized (dPFK) to hysteresis in the reaction time course, to ATP allosteric inhibition, and to F6P homotropic cooperativity. Complete trapping ($P^*_{max} \approx 100\%$) of the E:MgATP* complex as fructose (1-^{32}P)-1,6-bisophosphate for both enzyme forms is consistent with the previously proposed steady-state ordered mechanism [Rao, G. S. J., Harris, B. G., & Cook, P. F. (1987) *J. Biol. Chem. 262*, 14074−14079] with MgATP binding before fructose 6-phosphate (F6P). K'_{F6P} values for trapping of MgATP* of 0.54 ± 0.09 mM for nPFK and 0.85 ± 0.15 mM for dPFK were obtained. Saturating amounts of the heterotropic activator fructose 2,6-bisphosphate ($F26P_2$) gives no change in the trapping parameters for nPFK with a P^*_{max} of 100% and a K'_{F6P} of 0.40 ± 0.06 mM. For dPFK, however, $F26P_2$ causes a decrease in both parameters, giving a P^*_{max} of 54% and a K'_{F6P} of 0.26 ± 0.07 mM. The partial trapping of E:MgATP* in the presence of $F26P_2$ for dPFK suggests that the activator changes the kinetic mechanism from an ordered to a random binding of substrates. Initial velocity studies confirm the change in mechanism. Uncompetitive inhibition by arabinose 5-phosphate (Ara5P), a dead-end inhibitory analog of F6P, versus MgATP for nPFK in the absence and presence of $F26P_2$ is consistent with an ordered mechanism with MgATP adding to enzyme prior to F6P. An uncompetitive pattern is also obtained with dPFK for Ara5P versus MgATP in the absence of $F26P_2$, but the pattern becomes noncompetitive in the presence of $F26P_2$, consistent with a change to a random mechanism. No trapping of the E:[^{14}C]F6P complex could be detected, indicating either that the E:[^{14}C]F6P complex does not form in a significant amount under the conditions used or that the off-rate for F6P from enzyme is much faster than the net rate constant for formation of the first product, FBP. The data are consistent with a predominantly ordered mechanism with MgATP binding prior to F6P. The minor pathway with MgATP dissociating from the E:F6P:MgATP ternary complex becomes apparent for the dPFK in the presence of $F26P_2$.

Phosphofructokinase (PFK)[1] catalyzes the transfer of the γ-phosphate of MgATP to F6P to form FBP. Phosphofructokinase from the parasitic helminth *Ascaris suum* is a key regulatory enzyme in the overall metabolism of this organism. The *Ascaris* PFK is activated by a number of small molecule effectors including $F26P_2$, AMP, and small inorganic ions such as NH_4^+, K^+, and phosphate. The enzyme also exhibits homotropic cooperativity with F6P and allosteric substrate inhibition by MgATP. The fact that PFK exhibits such allosteric behavior has complicated kinetic studies of the enzyme, leading most researchers to carry out experiments at pH 8.0, where allosteric behavior is minimized.

A diethylpyrocarbonate-modified form of the *Ascaris* enzyme (dPFK) which is desensitized to hysteresis, to homotropic cooperativity, and to MgATP inhibition has allowed kinetic studies to be carried out at neutral pH and below (Rao *et al*., 1987b). The kinetic mechanism of both nPFK (pH 8.0) and dPFK (pH 6.8) has been shown to be steady-state ordered, with MgATP bound before F6P and FBP released before MgADP (Rao *et al*., 1987a). A minor alternative pathway in which F6P binds to enzyme prior to MgATP has also been suggested based on protection by F6P of the active site from diethylpyrocarbonate modification. However, the dissociation constant for the E:F6P complex was estimated to be 50−60 mM, which effectively eliminates the pathway in which F6P binds prior to MgATP at physiological levels of F6P.

The established ordered mechanism for *Ascaris* PFK is unique for a phosphofructokinase. Both rabbit muscle PFK (Merry & Britton, 1985) and *Escherichia coli* PFK (Deville-Bonne *et al*., 1991) have a random kinetic mechanism. The pyrophosphate-dependent enzymes (PP_i-PFK) from *Propionibacterium freudenreichii* (Cho *et al*., 1988), *Entamoeba histolytica* (Bertagnolli & Cook, 1984), and *Phaseoleus aureus* (Bertagnolli *et al*., 1986) all exhibit a rapid equilibrium random kinetic mechanism. The only case in which an ordered mechanism is observed is for the nonallosteric PFK from *Lactobacillus plantarum*, but the order is reversed with F6P binding to enzyme before MgATP (Simon & Hofer, 1978).

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[1] Abbreviations: Ara5P, arabinose 5-phosphate; BME, β-mercaptoethanol; DEPC, diethyl pyrocarbonate; F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; $F26P_2$, fructose 2,6-bisphosphate; MgATP*, Mg[γ-^{32}P]ATP; PFK, phosphofructokinase with the leading consonants n and d indicating the native and desensitized forms, respectively.

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In the present study, the technique of isotope partitioning (Rose, 1980) has been used to study the kinetic mechanism of nPFK at pH 8.0 and dPFK at pH 6.8 in the presence and absence of $F26P_2$. The resulting partitioning data have been used to estimate some of the rate constants along the reaction pathway. Results for both enzymes (nPFK and dPFK) in the absence of effectors are consistent with a steady-state ordered mechanism in which MgATP binds to enzyme prior to F6P, while results in the presence of $F26P_2$ are consistent with an ordered mechanism for nPFK, but a random mechanism for dPFK.

MATERIALS AND METHODS

Chemicals and Enzymes. (γ-^{32}P)ATP and (U-^{14}C)FBP were purchased from ICN Radiochemicals. (U-^{14}C)F6P was prepared from (U-^{14}C)FBP in a 0.5 mL reaction mixture containing 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 unit of D-fructose 1,6-bisphosphatase, and 5 μCi of (U-^{14}C)FBP. After 30 min, greater than 95% of the radioactivity copurified with (U-^{14}C)F6P on anion exchange chromatography. The reaction mixture was passed through a Centricon-100 membrane to remove the bisphosphatase and was used without further purification.

Phosphofructokinase from *A. suum* was purified according to the method of Starling *et al*. (1982) with the exception of the addition of the protease inhibitors aprotinin (10 mg/L), trypsin inhibitor (20 mg/L), and phenylmethanesulfonyl fluoride (1 mM) to the crude extract and the DEAE-Sephacel eluate. The purified enzyme had a final specific activity of 43 units/mg. The dPFK was then prepared according to the method of Rao *et al*. (1987b), giving a final specific activity of 30 units/mg. The protein concentration was determined by the method of Bradford (1976) with reagents purchased from Bio-Rad and with bovine serum albumin as a standard. All other chemicals and enzymes were purchased from Sigma.

Enzyme Assays. All assays in the direction of phosphorylation of F6P were obtained by coupling product formation to the disappearance of NADH and monitoring the absorbance at 340 nm. The formation of FBP was coupled to the aldolase/triosephosphate isomerase/α-glycerolphosphate dehydrogenase reactions where a typical 1 mL reaction mixture contained 100 mM imidazole-HCl, pH 6.8 (or 100 mM Tris-HCl, pH 8.0), 8 mM $MgCl_2$, 14 units of aldolase, 34 units of triosephosphate isomerase, 4 units of α-glycerolphosphate dehydrogenase, 0.2 mM NADH, ATP and F6P in the amounts indicated, and 20 milliunits of PFK. MgADP formation was coupled to the pyruvate kinase/lactate dehydrogenase reactions where a typical 1 mL reaction mixture contained 100 mM imidazole-HCl, pH 6.8 (or 100 mM Tris-HCl, pH 8.0), 8 mM $MgCl_2$, 3 units of pyruvate kinase, 3 units of lactate dehydrogenase, 100 mM KCl, 0.2 mM NADH, 0.2 mM PEP, ATP and F6P in the amounts indicated, and 20 milliunits of PFK. In the initial velocity and inhibition experiments, a double coupled assay was used which monitors the production of both FBP and MgADP in the same 1 mL cuvette. This double coupled assay gives greater sensitivity than either assay alone, avoids product inhibition by removing both products, and gives longer linear time courses by recycling ATP. All assays were initiated with PFK.

Initial velocity patterns were determined using the double coupled assay by varying MgATP at several fixed concentrations of F6P. Inhibition patterns were also determined using the double coupled assay by fixing F6P equal to its K_m value and varying MgATP at several different concentrations of inhibitor. The individual K_m and V_{max} values which are used in the isotope partitioning calculations were determined using the aldolase/triosephosphate isomerase/α-glycerolphosphate dehydrogenase coupled assay by fixing one substrate at a saturating concentration and varying the other substrate. To avoid activation of PFK by $(NH_4)_2SO_4$, all coupling enzyme stock solutions were made either by desalting an ammonium sulfate suspension of the enzyme using a Centricon-30 centrifuge/filter apparatus or by dissolving a lyophilized powder of the enzyme.

Circular Dichroism Spectral Measurements. Circular dichroic spectra were recorded using an Aviv Model 62 DS CD spectrometer maintained at a constant temperature of 10 °C. Samples contained 0.2 mg/mL enzyme in 20 mM KH_2PO_4 plus 8 mM $MgCl_2$, at either pH 6.8 (for dPFK) or pH 8.0 (for nPFK), in 0.2-cm quartz cuvettes. Spectra were recorded from 250 to 200 nm at intervals of 1 nm and a dwell time of 3 s. Each spectrum was the average of two repetitions, and buffer blanks were substracted from each spectrum. Titrations were carried out in a single cuvette by adding 2-μL increments of stock ATP so that final MgATP concentrations ranged from 0.4 to 10.1 μM and the total volume increased by <3%. The increase in ellipticity at 222 nm for each concentration of MgATP was then fitted using eq 1 to obtain the calculated dissociation constant for the E:MgATP complex.

Isotope Partitioning. Isotope partitioning experiments were performed at 30 °C according to the method of Rose (1980) for the dPFK:MgATP*, nPFK:MgATP*, and dPFK:[U-^{14}C]F6P complexes in the absence or presence of saturating $F26P_2$.

For the dPFK:MgATP* experiment in the absence of $F26P_2$, the pulse consisted of 130 μM dPFK (based on a MW of 90 000 per subunit) and 0.24 mM MgATP* (9000 cpm/nmol) in a total volume of 50 μL. The chase consisted of 100 mM imidazole-HCl (pH 6.8), 2.0 mM MgATP, and 0.5, 1.0, 4.2, or 10.0 mM F6P in a total volume of 5 mL. Using a K_D value of 2.0 μM for the dPFK:MgATP* complex, $[dPFK:MgATP^*]_0$ is 129 μM. For experiments in the presence of saturating $F26P_2$, the pulse consisted of 60 μM dPFK, 0.24 mM MgATP* (9000 cpm/nmol), and 0.2 mM $F26P_2$ in a total volume of 50 μL, and the chase consisted of 100 mM imidazole-HCl (pH 6.8), 2.0 mM MgATP, and 0.2, 0.5, 1.0, or 10.0 mM F6P plus 0.2 mM $F26P_2$ in a total volume of 5 mL. Using a K_D value of 2.3 μM for the dPFK:MgATP* complex, $[dPFK:MgATP^*]_0$ is 59 μM.

For the nPFK:MgATP* experiments in the absence or presence of saturating $F26P_2$, the pulse consisted of 55 μM nPFK and 0.2 mM MgATP* (10 000 cpm/nmol), plus and minus 0.2 mM $F26P_2$, and the chase consisted of 100 mM Tris-HCl (pH 8.0), 2.0 mM MgATP, and 0.1, 0.2, 2.0, or 10.0 mM F6P, plus and minus 0.2 mM $F26P_2$. Using a K_D of 2.0 μM for the nPFK:MgATP* complex in the absence of $F26P_2$, $[nPFK:MgATP^*]_0$ is 54 μM. Using a K_D of 6 μM for the nPFK:MgATP* complex in the presence of $F26P_2$, $[nPFK:MgATP^*]_0$ is 53 μM.

For the dPFK:[U-^{14}C]F6P experiments in the presence of saturating $F26P_2$, the pulse consisted of 90 μM dPFK, 1 mM [U-^{14}C]F6P (2000 cpm/nmol), and 0.2 mM $F26P_2$ in a total volume of 50 μL, and the chase consisted of 100 mM

imidazole (pH 6.8), 1 mM MgATP, 10 mM F6P, and 0.2 mM $F26P_2$ in a total volume of 5 mL. The K_D for the E:F6P complex has been estimated to be 50−60 mM (Rao *et al*., 1987b). Since $F26P_2$ has been shown by Payne *et al*. (1991) to activate PFK by decreasing K_{F6P} by 15-fold without affecting V_{max}, it is assumed that $F26P_2$ decreases the off-rate for F6P. This 15-fold decrease in the off-rate for F6P in the presence of $F26P_2$ gives an upper limit of around 4 mM for the K_D for the E:F6P complex. The pulse solution for the [^{14}C]F6P trapping experiment in the presence of $F26P_2$ should therefore have a lower limit of approximately 20 μM of [^{14}C]F6P (2000 cpm) initially bound to enzyme. Under these conditions, partial trapping of as low as 25% (500 cpm) should be detectable by scintillation counting.

For the ^{14}C-F6P trapping experiments, it is also assumed that if no significant trapping occurs in the presence of $F26P_2$, then no trapping should occur in the absence of $F26P_2$, since the initial binary complex is even more difficult to form. It is not feasible to study [^{14}C]F6P trapping in the absence of $F26P_2$ due to the high concentrations of ^{14}C-F6P necessary to form the initial E:[^{14}C]F6P complex in the pulse solution and the large dilution of unlabeled F6P that is necessary in the chase solution.

For all isotope partitioning experiments, the pulse was added to the rapidly stirring chase solution, and the reaction was quenched after 3 s by adding 200 μmol of EDTA. A 1 mL aliquot of the quenched reaction mix was then injected onto a Whatman Partisil 10-SAX column with 250 mM phosphate as the running buffer, a gradient from 0 to 1.5 M KCl, and a flow rate of 2 mL/min. Fractions of 2 mL were collected, and the amounts of MgATP*/[1-^{32}P]FBP and [U-^{14}C]F6P/[U-^{14}C]FBP were determined by scintillation counting. For each experiment two controls were done. Control 1 was a zero point for the substrate varied in the chase, thus accounting for any ATPase reaction in the pulse solution. This control was necessary because P_i and FBP coelute from the anion exchange column under these conditions. Control 2 was carried out for each concentration of varied chase substrate by placing the radiolabeled substrate only in the chase solution, thus accounting for any steady-state production of radiolabeled product in the pulse/chase mixture. All data points and controls were the average of duplicate experiments.

Data Analysis. Steady-state kinetic data were fitted using the appropriate rate equations and computer programs developed by Cleland (1979). Equation 1 was used for substrate saturation curves. Equation 2 was used for initial velocity patterns in the absence of inhibitors. Equations 3 and 4 were used for uncompetitive and noncompetitive inhibition patterns, respectively, where one substrate was fixed and the other substrate was varied at several different concentrations of inhibitor. Partitioning data were fitted

$$v = VA/(K_a + A) \quad (1)$$

$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB) \quad (2)$$

$$v = VA/\{K_a + A(1 + I/K_{is})\} \quad (3)$$

$$v = VA/\{K_a(1 + I/K_{is}) + A(1 + I/K_{ii})\} \quad (4)$$

using eq 1 with P^* and P^*_{max} substituted for v and V_{max}, and K'_a (K_m for trapping) substituted for K_a. In eqs 1−4, v and V are initial and maximum velocities, A, B, and I are reactant and inhibition concentrations, K_a and K_b are Michaelis constants for A and B, and K_{ia}, K_{is}, and K_{ii} are inhibition constants for A, slope, and intercept, respectively.

Table 1: Steady-State Kinetic Parameters for *Ascaris* PFK

	nPFK		dPFK	
	−$F26P_2$	+$F26P_2$	−$F26P_2$	+$F26P_2$
V_{max}/E_t (s^{-1})	65 ± 3	65 ± 3	42 ± 1	42 ± 1
K_{F6P} (mM)[a]	1.0 ± 0.1	0.07 ± 0.02	0.86 ± 0.06	0.10 ± 0.01
K_D (μM)[b]	2.0 ± 0.6	6 ± 3	2.0 ± 0.5	2.3 ± 0.7

[a] All K_{F6P} values were determined using the single aldolase/triosephosphate isomerase/α-glycerolphosphate dehydrogenase coupled assay with coupling enzyme solutions made fresh from lyophilized powder to avoid the effects of $(NH_4)_2SO_4$ on K_{F6P}. [b] Calculated from the CD studies.

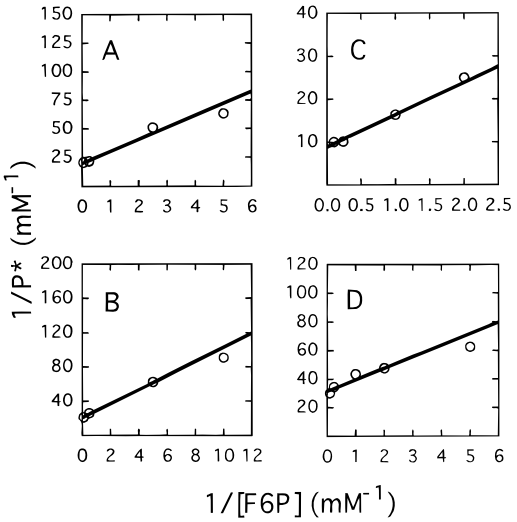


FIGURE 1: Isotope trapping of the E:MgATP* complex with *Ascaris* PFK. The amount of [1-^{32}P]FBP formed at different concentrations of F6P in the chase solution was determined as described in the text. (A) nPFK trapping in the absence of $F26P_2$ (pH 8.0). (B) nPFK trapping in the presence of 0.2 mM $F26P_2$ (pH 8.0). (C) dPFK trapping in the absence of $F26P_2$ (pH 6.8). (D) dPFK trapping in the presence of 0.2 mM $F26P_2$ (pH 6.8). The lines are the best fit of the data using eq 1, while the points are experimental values.

RESULTS

K_D Determination. The increase in ellipticity at 222 nm for nPFK and dPFK in the absence or presence of $F26P_2$ as a function of MgATP concentration is hyperbolic, and a fit of the data to eq 1 gives the dissociation constant for the E:MgATP complex (data not shown). For nPFK, the K_D for the E:MgATP complex is 2.0 ± 0.6 μM in the absence of $F26P_2$ and 6 ± 3 μM in the presence of $F26P_2$. For dPFK, the K_D is 2.0 ± 0.5 μM in the absence of $F26P_2$ and 2.3 ± 0.7 μM in the presence of $F26P_2$. Data are summarized in Table 1.

Isotope Partitioning of nPFK:MgATP in the Absence or Presence of F26P$_2$*. The amount of the nPFK:MgATP* trapped as a function of the F6P concentration in the chase solution is shown in Figure 1. Panel A shows the trapping in the absence of $F26P_2$ with a P^*_{max} of 0.051 ± 0.002 mM estimated, representing 94% of the 54 μM MgATP* present in the initial binary complex ([nPFK:MgATP*]$_0$), calculated using a K_D of 2.0 μM. A K'_{F6P} of 0.54 ± 0.09 mM is estimated. Panel B shows the partitioning data in the

Table 2: Summary of Data from Isotope Partitioning of E:MgATP* with *Ascaris* PFK

	nPFK		dPFK	
	$-F26P_2$	$+F26P_2$	$-F26P_2$	$+F26P_2$
P^*_{max} (μM)	51 ± 2 (94%)	48 ± 2 (89%)	110 ± 10 (85%)	32 ± 2 (54%)[a]
K'_{F6P} (mM)	0.54 ± 0.09	0.40 ± 0.06	0.85 ± 0.15	0.26 ± 0.07
$[E:A]_0/P^*_{max}$	1.06 ± 0.11	1.12 ± 0.11	1.18 ± 0.11	1.85 ± 0.11
k_{off} (s^{-1})	34 ± 7	370 ± 120	42 ± 8	110 ± 30 to 200 ± 45
k_7/k_5				0.85 ± 0.05
k_7 (s^{-1})				36 ± 2
k_{on} (M^{-1} s^{-1})	(1.7 ± 0.7) × 10^7	(6.2 ± 3.2) × 10^7	(2.1 ± 0.7) × 10^7	(4.8 ± 2.9) × 10^7 to (8.7 ± 3.7) × 10^7

[a] The dPFK used in the experiments for isotope partitioning in the presence of $F26P_2$ was from a separate preparation than that used for experiments in the absence of $F26P_2$. The specific activity of both preparations was 30 U/mg, but the final concentration of enzyme used in the presence of $F26P_2$ was lower than that of the enzyme used in the absence of $F26P_2$, giving a lower concentration of the initial binary complex. The 54% trapping is calculated based on the lower concentration of binary complex and is not directly comparable to the absolute trapping in the absence of $F26P_2$. As a control, the $-F26P_2$ experiment was repeated with this enzyme preparation, and approximately 85% trapping was obtained. However, limited amounts of enzyme from this preparation allowed only 3 data points to be measured, so data obtained using the previous preparation (with 4 data points, Figure 1C) is shown.

presence of 0.2 mM $F26P_2$, giving a P^*_{max} of 0.048 ± 0.002 mM, within error equal to the P^*_{max} obtained in the absence of $F26P_2$. The K'_{F6P} value decreases only slightly to 0.40 ± 0.06 mM in the presence of $F26P_2$. Data are summarized in Table 2.

Isotope Partitioning of dPFK:MgATP in the Absence or Presence of F26P₂.* The amount of the dPFK:MgATP* trapped as a function of the F6P concentration in the chase solution is shown in Figure 1. Panel C shows that trapping in the absence of $F26P_2$ gives a P^*_{max} of 0.11 ± 0.01 mM, or 85% trapping of the 129 μM MgATP* initially present in the pulse solution binary complex, estimated using a K_D of 2.0 μM. The K'_{F6P} for the pulse/chase reaction in the absence of $F26P_2$ is 0.85 ± 0.15 mM. Panel D shows the trapping of dPFK:MgATP* in the presence of 0.2 mM $F26P_2$. A P^*_{max} of 0.032 ± 0.002 mM is obtained, estimated as 54% trapping of the 59 μM MgATP* initially present in the pulse solution binary complex, calculated using a K_D of 2.3 μM. A K'_{F6P} for trapping in the presence of $F26P_2$ of 0.26 ± 0.07 mM is obtained, a value 3.3-fold lower than the value in the absence of $F26P_2$. Data are again summarized in Table 2.

Isotope Partitioning of dPFK:[^{14}C]F6P in the Presence of F26P₂. No trapping of the dPFK:[^{14}C]F6P complex as [^{14}C]FBP was detected in the presence of $F26P_2$ (see Materials and Methods section).

Initial Velocity Studies. Initial velocity patterns in the absence or presence of 0.2 mM $F26P_2$ for both nPFK and dPFK were measured using the double coupled assay described in the Materials and Methods section, and all give similar results. A typical initial velocity pattern is shown in Figure 2. In all cases the patterns exhibit near-parallel lines, suggesting a very low K_D of MgATP relative to the K_m for MgATP. The fit of the nPFK pattern in the presence of $F26P_2$ gives a K_{MgATP} value of 9 ± 1 μM, a K_{F6P} value of 130 ± 15 μM, and a K_D for MgATP that is undefined (the associated error is larger than the calculated value). The fit of the dPFK pattern in the presence of $F26P_2$ gives a K_{MgATP} value of 17 ± 4 μM, a K_{F6P} value of 72 ± 21 μM, and a K_D for MgATP that is undefined.

The arabinose 5-phosphate (Ara5P) inhibition patterns in the presence of 0.2 mM $F26P_2$ were also measured using the double coupled assay and are shown in Figure 3. Data for nPFK, Figure 3A, adhere to uncompetitive inhibition, while data for dPFK, Figure 3B, adhere to noncompetitive

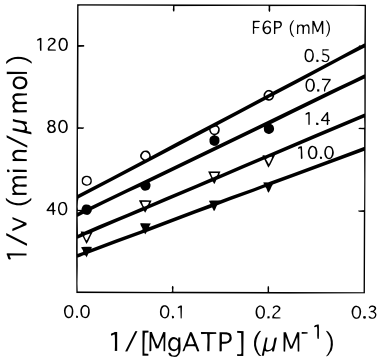


FIGURE 2: A typical initial velocity pattern for *Ascaris* dPFK at pH 6.8, in 100 mM imidazole-HCl in the absence of $F26P_2$. The lines are the best fit of the data using eq 2, while the points are experimental values. Estimated kinetic parameters for all initial velocity patterns are discussed in the Results section.

inhibition. The uncompetitive inhibition pattern for nPFK yields a K_{MgATP} of 7.0 ± 0.4 μM, and a K_{ii} of 12.0 ± 0.5 mM for Ara5P, while the noncompetitive pattern for dPFK gives a K_{MgATP} of 10 ± 1 μM, and K_{is} and K_{ii} values for Ara5P of 12 ± 3 mM and 10 ± 1 mM, respectively.

While the initial velocity patterns give estimates of dissociation constants for both substrates, the estimated K_m and V_{max} values from these patterns can have relatively large standard errors. For this reason, the K_m and V_{max}/E_t values used in the isotope partitioning calculations were determined from single saturation curves as described in the Materials and Methods section. The results are shown in Table 1. V_{max}/E_t values are 65 ± 3 s^{-1} and 42 ± 1 s^{-1} for nPFK and dPFK, respectively. K_{F6P} values in the absence of $F26P_2$ are 1.0 ± 0.1 mM for nPFK and 0.86 ± 0.06 mM for dPFK. In the presence of $F26P_2$, the K_{F6P} value for nPFK decreases to 0.07 ± 0.02 mM, while that of dPFK decreases to 0.10 ± 0.01 mM.

DISCUSSION

Isotope Partitioning in the Absence of F26P₂. With nPFK, 94% of the estimated E:MgATP* complex was trapped and converted to product. The specific activity of the nPFK used in these experiments was 43 U/mg, or only 86% of the specific activity reported by Starling *et al.* (1982) for *Ascaris* PFK. The lower specific activity for the nPFK used in these experiments is likely the result of the presence of contami-

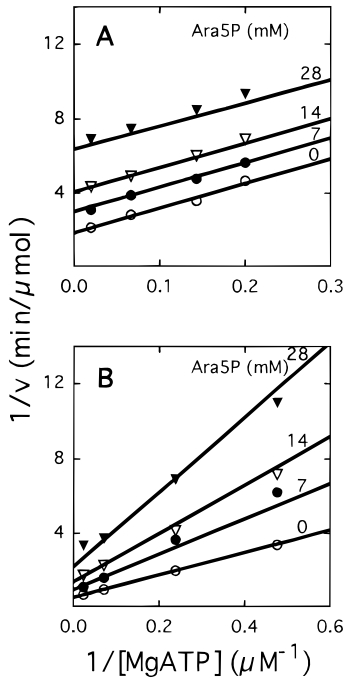


FIGURE 3: Dead-end inhibition by arabinose 5-phosphate of (A) nPFK, pH 8.0, and (B) dPFK, pH 6.8, both in the presence of 0.2 mM $F26P_2$. The lines in panels A and B are the best fits of the nPFK data using eqs 3 and 4, respectively. Points are experimental values.

nating impurities and/or an inactive fraction of the nPFK itself. About 85% of the calculated E:MgATP* complex is trapped with dPFK. The dPFK is prepared by diethyl pyrocarbonate (DEPC) modification of the ATP allosteric site with the enzyme active site protected from modification by bound F6P (Rao *et al*., 1987b). The decrease in the specific activity of dPFK compared to nPFK from which it was prepared suggests inactivation of some of the enzyme during preparation. This inactivation is expected since the concentration of F6P is not maintained high enough to give complete saturation at the active site. An estimate of 80% is obtained for protection at the active site (Rao *et al*., 1987b). Thus, for both nPFK and dPFK the actual concentration of the initial E:MgATP* complex is less than the estimated value based on the assumption that all protein is active enzyme. Taking into account the inactive enzyme in both the nPFK and the dPFK preparations, both enzyme forms trap very close to 100% of the E:MgATP* initially present. The data for both nPFK and dPFK are consistent with a predominantly steady-state ordered kinetic mechanism, in agreement with the initial velocity data obtained previously (Rao *et al*., 1987a). However, even 100% trapping of the E:MgATP complex does not rule out the possibility of a random mechanism, but only says that catalysis is much faster than MgATP dissociation. Indeed, the fact that F6P alone protects the active site against DEPC modification suggests that a minor pathway in which F6P binds to enzyme prior to MgATP probably does exist. However, the high K_D (50−60 mM) for the E:F6P complex compared to the low K_D (2 μM) for E:MgATP and the rate constants for dissociation of E:MgATP:F6P gives an essentially ordered mechanism.

The K'_{F6P} values of 0.54 ± 0.09 mM and 0.85 ± 0.15 mM for nPFK and dPFK, respectively, allow the estimation of the off-rate of MgATP from the binary complex using the following equation (Rose, 1980):

$$(K'_{F6P}/K_{F6P})(V_{max}/E_t) < k_{off} < (K'_{F6P}/K_{F6P})(V_{max}/E_t)([E{:}MgATP^*]_0/P^*_{max}) \quad (5)$$

Assuming 100% trapping, for nPFK the estimated k_{off} is about 34 ± 7 s^{-1}, and for dPFK k_{off} is about 42 ± 8 s^{-1} (Table 2). Using the dissociation constant value of 2 μM for both enzyme forms, the net on-rate for MgATP to enzyme is on the order of 10^7 M^{-1} s^{-1}, which is about 10- to 100-fold less than the diffusion rate of combination of a small molecule and a macromolecule (Fersht, 1977). This difference suggests the possibility of a structural isomerization occurring after MgATP binds to enzyme.

Isotope Partitioning in the Presence of F26P₂. In the presence of 0.2 mM $F26P_2$, no change in the P^*_{max} or K'_{F6P} values for nPFK is observed; that is, essentially 100% of the E:MgATP* complex is trapped. Data are consistent with an ordered mechanism, and the off-rate for MgATP from the E:MgATP complex is 370 ± 120 s^{-1}, about 10-fold higher than that estimated in the absence of $F26P_2$. However, in the presence of 0.2 mM $F26P_2$ the dPFK gives a decrease in P^*_{max} to 54% of that obtained in the absence of effectors. The K'_{F6P} value for dPFK trapping in the presence of $F26P_2$ decreases by 3.3-fold from that obtained in the absence of effectors. The decrease in P^*_{max} in the presence of $F26P_2$ suggests that some of the MgATP is able to dissociate from the ternary complex prior to catalysis. Using eq 5, the data for dPFK in the presence of $F26P_2$ give a k_{off} range of 110 ± 30 to 200 ± 45 s^{-1}, 2.5- to 4-fold greater than the value in the absence of $F26P_2$. Assuming a dissociation constant of approximately 2−6 μM, the on-rates for both enzyme forms are again on the order of 10^7 M^{-1} s^{-1}.

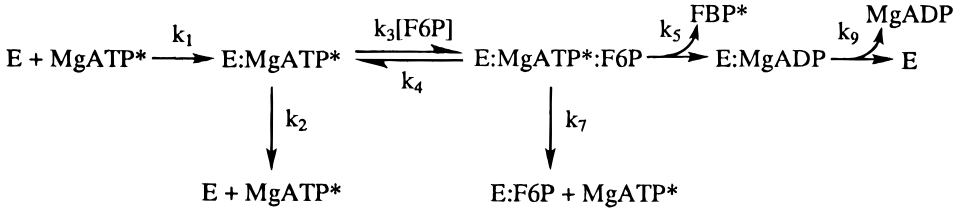
In the case of the dPFK where less than 100% trapping is estimated, the partition ratio, k_7/k_5 (Scheme 1) can be calculated using the following equation:

$$k_7/k_5 = ([E{:}MgATP^*]_0/P^*_{max}) - 1 \quad (6)$$

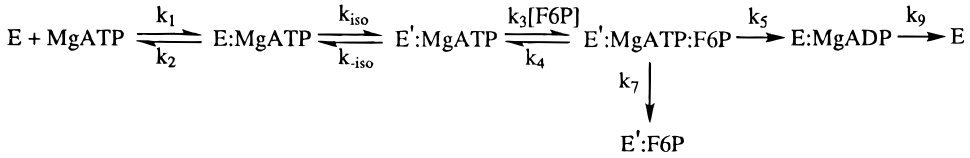
The partition ratio is zero when 100% trapping occurs because k_7 is much smaller than k_5. In order for only partial trapping to occur, k_7 must be of the same order as k_5, giving a partition ratio greater than zero. Thus, the increase in the partition ratio for dPFK in the presence of $F26P_2$ (see Table 2) must be caused either by nPFK having a much larger k_5 with respect to k_7 than dPFK, or by dPFK having a much larger k_7 than nPFK in the presence of $F26P_2$. Rao *et al*. (1987a) showed that the overall rate of the PFK reaction in the direction of F6P phosphorylation is not limited by product release, so that k_5 is the same as V_{max}/E_t.[2] Since the V_{max}/E_t values for nPFK and dPFK are similar (see Table 1), the high partition ratio for dPFK is likely not due to large differences in k_5 for nPFK and dPFK. So, the difference in the partition ratios between nPFK and dPFK in the presence of $F26P_2$ must be a result of dPFK having a much larger value for k_7 than nPFK. Such a "leaky" ternary complex is consistent with a random kinetic mechanism for dPFK in the presence of $F26P_2$. Using a k_5 value of 42 ± 1 s^{-1} for dPFK, k_7 is estimated to be 36 ± 2 s^{-1}.[3] It should be pointed

[2] k_5 is not necessarily a microscopic rate constant, and may contain rate processes for conformational changes in the enzyme required to close and reopen the active site prior to and after the catalytic steps. k_5 can be considered a net rate constant for catalysis.

Scheme 1: Steps in the Substrate Trapping of MgATP* with PFK



Scheme 2: Steps in Binding and Isomerization of MgATP with PFK



out that no trapping of the dPFK:[^{14}C]F6P complex occurred, which lends support to a strictly ordered mechanism. However, the lack of trapping of F6P does not rule out a partially random mechanism but only says that either the E:F6P complex does not form (or has a K_D much larger than the concentration of F6P used in the pulse solution), or the off-rate for F6P is much faster than catalysis.

Initial velocity studies also support the finding that $F26P_2$ shifts the kinetic mechanism for dPFK from ordered to random. The noncompetitive inhibition pattern (Figure 3B) by the F6P analog arabinose 5-phosphate versus MgATP for dPFK indicates binding of Ara5P to E and to E:MgATP, consistent with a random kinetic mechanism for dPFK in the presence of $F26P_2$. nPFK shows uncompetitive inhibition (Figure 3A) by Ara5P versus MgATP in the presence or absence of $F26P_2$, consistent with the isotope partitioning data and an ordered kinetic mechanism.

Initial velocity patterns for both nPFK and dPFK (Figure 2) give a series of near-parallel lines. A ping pong mechanism has previously been ruled out for *Ascaris* PFK (Rao *et al*., 1987a). The fact that radioactive product formation in the isotope partitioning experiments is dependent on the amount of chasing substrate (F6P) also rules out a ping pong mechanism. Any covalently bound enzyme intermediate that survives the brief pulse/chase time period would give a constant amount of trapping independent of the chasing substrate. The near-parallel lines in the initial velocity patterns are thus caused by the K_D for the E:MgATP complex being much smaller than the K_m for MgATP, and the fact that the concentration of MgATP was varied around the K_m. The same near-parallel initial velocity pattern is seen with rabbit muscle PFK (Uyeda, 1970). It has been postulated with the rabbit enzyme that the pattern is not due to a ping pong mechanism but to a low K_D/K_m ratio. The K_D's calculated from the present circular dichroism studies confirm a low K_D/K_m ratio for the *Ascaris* PFK.

The fact that earlier initial velocity studies with *Ascaris* PFK (Rao *et al*., 1987a) in the absence of $F26P_2$ showed initial velocity patterns that clearly intersected and gave estimates of K_D values similar to the K_{MgATP} values for both nPFK and dPFK is ascribed to error in the earlier assays due to rapid consumption of MgATP and to low sensitivity of the assay. (The slope effect was generated by the initial rates measured at the lowest concentration of MgATP.) The earlier initial velocity patterns were measured using the single aldolase/triosephosphate isomerase/α-glycerolphosphate dehydrogenase coupled assay. The present study has avoided the problems of rapid loss of substrate and low sensitivity by using the double coupled assay which recycles the consumed MgATP via the pyruvate kinase reaction and gives a 1.5-fold greater sensitivity than the aldolase coupled assay alone.

As mentioned, there is good evidence for an isomerization of E:MgATP. First, changes are observed in the far-UV CD spectrum of PFK upon binding of MgATP. Second, the $V_{max}/(K_{MgATP}E_t)$ is pH-independent and equal to 1.4×10^6 M^{-1} s^{-1} (Payne *et al*., 1995). The V/K for the first reactant in an ordered mechanism is the on-rate for that reactant binding to enzyme, yet the value of 10^6 M^{-1} s^{-1} is significantly lower than the diffusion limit, consistent with a multistep process for MgATP binding. Finally, the off-rate (k_{off}) for MgATP from enzyme that is estimated from the isotope partitioning studies increases in the presence of $F26P_2$ while the K_D for E:MgATP does not show any significant change, giving a net on-rate on the order of 10^7 M^{-1} s^{-1}. Since it is unlikely that the diffusion rate (k_1) of MgATP to E changes, other steps which limit the net on-rate must be involved in forming the E:MgATP active complex. The experimental k_{off} is therefore a net off-rate (expanding Scheme 1 to include the isomerization step, Scheme 2) which is described by eq 7. Expressions for the $V_{max}/(K_{MgATP}E_t)$ and the observed K_D for E′:MgATP are given by eqs 8 and 9. $V_{max}/(K_{MgATP}E_t)$

$$k_{off} = k_2k_{-iso}/(k_2 + k_{iso}) \quad (7)$$

$$V_{max}/(K_{MgATP}E_t) = k_1k_{iso}/(k_2 + k_{iso}) \quad (8)$$

$$K_D = (k_{-iso}/k_{iso})(k_2/k_1) \quad (9)$$

represents the net on-rate for MgATP to form the isomerized (active) complex. Under conditions for obtaining $V_{max}/(K_{MgATP}E_t)$, saturating F6P, the isomerization step is irreversible. K_D is the overall dissociation constant for the isomerized E′:MgATP complex. Limits can be placed on the isomerization rate constants. The constant k_{iso} must be greater than or equal to the turnover number for formation

[3] The isotope trapping of dPFK in the presence of $F26P_2$ is the only case where $[E:A]_0/P^*$ is clearly larger than unity. For this reason, Table 2 does not show the estimated values of the partition ratio, k_7/k_5, for the other three cases. However, if these other three $[E:A]_0/P^*_{max}$ values actually differ from unity, then the partition ratios are 0.06 ± 0.01 and 0.12 ± 0.01 for nPFK in the absence and presence of $F26P_2$, respectively, and 0.18 ± 0.02 for dPFK in the absence of $F26P_2$. The rate k_7 then is not zero but ranges from 4 to 8 s^{-1} for the three cases.

of FBP (42 s^{-1}), and k_{-iso} must be greater than or equal to the experimentally determined k_{off} (Table 2). Finally, for 100% trapping to occur, either k_{iso} must be greater than k_{-iso} so that all bound MgATP is in the active isomerized form, or k_{iso} must be greater than k_2 so that any nonisomerized E:MgATP will proceed toward product prior to dissociating. It is unlikely that k_{iso} will be much greater than k_2, the diffusion-limited off-rate for MgATP. More likely is that $k_{iso} > k_{-iso}$, so that enzyme is predominantly E′:MgATP once MgATP binds. Thus for all of the conditions studied, the presence of $F26P_2$ likely increases the rate of the isomerization step, so that if it was partially limiting in the absence of $F26P_2$, it no longer limits in the presence of the effector. The role of $F26P_2$ on the isomerization of the binary complex is consistent with the role proposed by Payne *et al*. (1991, 1995) for $F26P_2$ in F6P binding. These authors have suggested that binding of $F26P_2$ to its allosteric site decreases the off-rate for F6P, presumably by facilitating isomerization of the ternary complex to its catalytically optimal conformation.

Conclusions. The data confirm a predominantly steady-state ordered kinetic mechanism for both nPFK and dPFK in the absence of effectors due to the complete trapping of the E:MgATP* complex. It has previously been concluded that dPFK at pH 6.8 exhibits identical characteristics to the native enzyme at pH 8.0. The conformational and/or chemical change that occurs on enzyme upon treatment with DEPC in the presence of F6P is thought to lock the enzyme into an active or "high-pH" form (Rao *et al*., 1995). However, the current isotope partitioning and initial velocity data indicate a differential effect of $F26P_2$ on the two enzyme forms. The change from an ordered to a random mechanism in the presence of $F26P_2$ for dPFK and not for nPFK indicates that there must be subtle differences in the dPFK at neutral pH and nPFK at high pH. Finally, calculations from the isotope partitioning data as well as the changes seen in the circular dichroic spectra for both nPFK and dPFK indicate that an isomerization occurs upon binding MgATP.

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