Biochemistry

 Volume 34, Number 24 June 20, 1995

Articles

Acid–Base Catalytic Mechanism and pH Dependence of Fructose 2,6-Bisphosphate Activation of the *Ascaris suum* Phosphofructokinase†

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Received February 20, 1995; Revised Manuscript Received March 30, 1995®

ABSTRACT: A form of phosphofructokinase (PFK) from *Ascaris suum* desensitized to hysteresis in the reaction time course and ATP allosteric inhibition has been used to study the activation by fructose 2,6-bisphosphate ($F26P_2$) at varied pH in both reaction directions. In the direction of phosphorylation of F6P, V and V/K_{MgATP} are constant over the pH range 6–9, while V/K_{F6P} decreases at low pH, giving a pK value of 7.0, and at high pH, giving a pK of 8.9. V and V/K_{MgATP} are insensitive to the presence of $F26P_2$, but V/K_{F6P} is increased by a constant amount in the presence of saturating $F26P_2$ over the entire pH range studied. The concentration of $F26P_2$ that gives half the change in V/K_{F6P}, K_{act}, increases as the pH decreases, giving a pK of 7.4, reflecting an enzyme group that must be unprotonated for optimum binding of $F26P_2$. In the direction of phosphorylation of MgADP, V and V/K_{MgADP} are pH-independent, and both are insensitive to the presence of $F26P_2$. V/K_{FBP} decreases at high pH, giving a pK of about 7.3, and is increased by a constant amount in the presence of $F26P_2$ over the entire pH range studied. A mechanism consistent with the data requires an enzymic general base with a pK of 7.0 to accept a proton from the 1-hydroxyl of F6P concomitant with nucleophilic attack of the hydroxyl on the γ-phosphate of MgATP, while a second enzyme group with a pK of 8.9 must be protonated and is postulated either to neutralize the negative charge on the γ-phosphate of MgATP to facilitate the nucleophilic attack or to bind the 6-phosphate of F6P. A group with a pK of 7.4 in the $F26P_2$ binding site must be unprotonated for optimum binding of the effector and likely hydrogen-bonds to the hydroxyl group at C1, C2, or C3 of $F26P_2$. The effect of $F26P_2$ is the pH-independent decrease of the off rate for F6P and FBP from binary and ternary enzyme–reactant complexes.

Phosphofructokinase (PFK)[1] catalyzes the formation of FBP by transfer of the γ-phosphate of MgATP to the C1 hydroxyl of F6P. The PFK from the parasitic helminth *Ascaris suum* has been studied with respect to its mechanism using a combination of kinetic and spectroscopic approaches. The native enzyme from *Ascaris* exhibits hysteretic changes in its reaction time courses at neutral pH that are overcome by positive homotropic (F6P) and heterotropic ($F26P_2$ and AMP) effectors (Cook et al., 1987). It has also been shown

† This study was supported by grants from the National Institutes of Health to B.G.H. (AI 24155) and P.F.C. (GM 36799) and from the Robert A. Welch Foundation to P.F.C. (B-1031).

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® Abstract published in *Advance ACS Abstracts,* June 1, 1995.

[1] Abbreviations: cAPK, catalytic subunit of cyclic AMP-dependent protein kinase; BME, β-mercaptoethanol; DEPC, diethyl pyrocarbonate; F6P, fructose 6-phosphate; $F26P_2$, fructose 2,6-bisphosphate; FBP, fructose 1,6-bisphosphate; GDP, guanosine 5′-diphosphate; PFK, phosphofructokinase, with the leading consonants n, d, and pd indicating the native, desensitized, and phosphorylated-desensitized forms, respectively; TCA, trichloroacetic acid.

0006-2960/95/0434-7781$09.00/0

that the *Ascaris* PFK, like its mammalian counterparts, is strongly inhibited by ATP binding at an allosteric site and that this inhibition can be overcome by positive homotropic and heterotropic effectors (Hofer et al., 1982a,b; Cook et al., 1987). The above data led to the conclusion that positive effectors functioned by overcoming the allosteric inhibition by ATP. However, a form of the *Ascaris* PFK (d-PFK) desensitized to time-course hysteresis and ATP allosteric inhibition by modification of the ATP inhibitory site with diethyl pyrocarbonate (Rao et al., 1987a) is still activated by the heterotropic effectors $F26P_2$ and AMP (Rao et al., 1991). The native enzyme at pH 8.0 behaves similarly to the d-PFK; that is, both exhibit Michaelis−Menten saturation curves but are still activated by $F26P_2$ and AMP. Thus, activation of PFK can occur by increasing the pH to 8 or by desensitization, and in both cases further activation is realized in the presence of the heterotropic effectors. The activation by the heterotropic effectors has been studied and a kinetic mechanism suggested (Payne et al., 1991). Nothing is known of the mechanism of pH activation of the *Ascaris* PFK or its acid−base chemistry. The latter have been difficult to sort out since the kinetics of the native enzyme change from cooperative to noncooperative as the pH is increased.

In this study, the pH dependence of the kinetic parameters for d-PFK have been measured in both reaction directions and used to propose a chemical mechanism for the enzyme. In addition, kinetic parameters have been measured in the absence and presence of varied concentrations of the positive effector $F26P_2$. Results clearly demonstrate that the maximum activation by $F26P_2$ is pH-independent, but the dissociation constant for $F26P_2$ changes with pH. Data are interpreted in terms of the interactions that occur between enzyme and the effector at the allosteric site.

MATERIALS AND METHODS

Enzyme Purification. Phosphofructokinase was purified from 200 g of *Ascaris suum* according to Starling et al. (1982) with the exception that a cocktail of protease inhibitors containing aprotinin, trypsin inhibitor, and phenylmethanesulfonyl fluoride was added to the crude extract and DEAE-Sepharose eluate.

Desensitization. The desensitized form of PFK (d-PFK) was prepared by the method of Rao et al. (1987a). Following desensitization, the d-PFK was dialyzed into storage buffer containing 50 mM potassium phosphate, pH 7.4, 3 mM dithiothreitol (DTT), and 15% (v/v) glycerol and stored in 1-mL aliquots at −20 °C. The d-PFK stored in this manner is stable to repeated freeze−thaw cycles. For assays in the direction of F6P phosphorylation, the enzyme was diluted (1:8 to 1:12) before use into the same buffer but with 2% (v/v) glycerol. For assays of the reverse reaction, the enzyme was passed through a Centricon-30 three times to remove traces of F6P left from the desensitization and used without dilution.

Protein Concentration. Protein concentration was determined spectrophotometrically using the $E^{1\%}$ at 280 nm of 6.5 reported by Starling et al. (1982). Briefly, d-PFK was precipitated with 10% TCA, the precipitate was centrifuged, and the pellet was washed three times with 1 mL of 10% TCA, followed by three washes with 1 mL of diethyl ether. The pellet was finally dissolved in 88% formic acid and the absorbance was measured at 280 nm. The concentration of d-PFK in the stock solution was determined to be 0.55 mg/mL.

Enzyme Assays. All assays in the direction of F6P phosphorylation were carried out by coupling the production of FBP to the aldolase/triosephosphate isomerase/α-glycerolphosphate dehydrogenase reactions. Assays were carried out in a volume of 1 mL in 100 mM bis,tris-propane hydrochloride at the appropriate pH, 8 mM $MgCl_2$, 0.2 mM NADH, 2.1 units of aldolase, 34 units of triosephosphate isomerase, 3.3 units of α-glycerolphosphate dehydrogenase, and variable concentrations of ATP, F6P, and $F26P_2$. Typically, 4.8−7.2 nM d-PFK was used for the experiments. Aldolase was obtained as an ammonium sulfate suspension, extensively dialyzed against 50 mM imidazole hydrochloride, pH 6.8, 3 mM DTT, and 10% (v/v) glycerol prior to use. Aliquots of 3 μL/assay triosephosphate isomerase and α-glycerolphosphate dehydrogenase were added directly from an ammonium sulfate suspension. A final concentration of 7 mM ammonium sulfate was present in the assay mix. The concentrations of coupling enzymes used were sufficient to support the highest rate observed without an appreciable lag in the time course except at the pH extremes, and then steady state was established in 1 min. Assays in the direction of MgADP phosphorylation were carried out by coupling the production of F6P to the phosphoglucose isomerase/glucose-6-phosphate dehydrogenase (PGI/G6PDH) reactions or the production of MgATP to the hexokinase/glucose-6-phosphate dehydrogenase (HK/G6PDH) reactions. Assays making use of PGI/G6PDH contained in a 1-mL volume 1 mM NADP, appropriate concentrations of FBP, MgADP, and $F26P_2$ (when appropriate), and lyophilized yeast PGI and G6PDH reconstituted with water before use. Assays making use of HK/G6PDH contained in a 1-mL volume 1 mM NADP and 10 mM glucose, appropriate concentrations of FBP, MgADP, and $F26P_2$ (when appropriate), and a lyophilized mixture of yeast HK/G6PDH reconstituted with water before use. The assay amount of d-PFK in the direction of MgADP phosphorylation was 50 nM. Assays that gave the highest rates were periodically checked for linearity with d-PFK concentration. When $F26P_2$ was used as an effector, it was added to each cuvette immediately prior to addition of enzyme to minimize potential degradation of $F26P_2$. All reactions were started with d-PFK. The rate of disappearance of NADH was monitored at 340 nm with a Beckman Kintrac monochromator upgraded with a Gilford light source, sample compartment, and detector, with the output connected to a strip chart recorder. For all assays in the direction of MgADP phosphorylation, the appearance of NADPH was monitored at 340 nm. Data were collected with the output of the spectrophotometer connected in parallel to a strip-chart recorder and a computerized data acquisition system (Payne, unpublished work). The temperature was maintained at 30 °C by using a circulating water bath to heat and cool the thermospacers of the cell compartment and a cuvette heating block to maintain the temperature of the assay mixtures prior to assay. The pH of each assay was checked immediately following the assay with a Radiometer PHM 83 pH meter equipped with a small-bore glass Ag/AgCl with calomel reference electrode.

Data Processing. Reciprocal initial velocities were plotted against reciprocal substrate concentrations, and all plots and replots were linear. Appropriate rate equations were fitted to the data using FORTRAN programs developed by Cleland

(1979). Equation 1 was used for substrate saturation curves, while eq 2 was used for initial velocity patterns in which both substrates were varied. Data for the dependence of K_{F6P} on the concentration of $F26P_2$ were fitted using eq 3, which describes a hyperbolic function. Data obtained for the pH dependence of $1/K_{act}$ for $F26P_2$ were fitted using eq 4, which describes a plot that decreases at low pH with a limiting slope of 1, while the pH dependence of V/K_{F6P} and V/K_{FBP} were fitted using eq 5, which describes a plot that decreases at low pH with a limiting slope of 1 and at high pH with a limiting slope of -1, and eq 6, which describes a plot that decreases at high pH with a limiting slope of -1, respectively.

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

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

$$Y = A(1 + X/K_{IN})/(1 + X/K_{ID}) \quad (3)$$

$$\log Y = \log [C/(1 + H/K_1)] \quad (4)$$

$$\log Y = \log [C/(1 + H/K_1 + K_2/H)] \quad (5)$$

$$\log Y = \log [C/(1 + K_2/H)] \quad (6)$$

In eqs 1 and 2, v and V are the initial and maximal velocities of the reaction, A and B are reactant concentrations, K_a and K_b are Michaelis constants for reactants A and B, respectively, and K_{ia} is the inhibition constant for A. In eq 3, Y is the observed value of K_{F6P} at any $F26P_2$ concentration, A is the value of K_{F6P} at zero $F_{26}P_2$, X is the $F26P_2$ concentration, K_{ID} is the dissociation constant for $F26P_2$ (K_{act}), K_{IN} is a mechanism-dependent term that causes Y to plateau at a finite value (in the present case it is equal to the value K_{F6P} at zero $F26P_2$, i.e., A), and $A(K_{ID}/K_{IN})$ is the value of K_{F6P} at infinite $F26P_2$ concentration. The ratio of A to $A(K_{ID}/K_{IN})$, (K_{IN}/K_{ID}), is the maximum fold activation. Values of Y obtained at 200 μM $F26P_2$ were weighted by a factor of 10. In eqs 4–6, Y is $1/K_{act}$, V/K_{F6P}, or V/K_{FBP}, respectively, C is the pH-independent value of Y, H is the hydrogen ion concentration, and K_1 and K_2 are acid dissociation constants for groups on enzyme.

RESULTS

Experimental Strategy. The kinetic theory applied to the study of effectors at a given pH (Payne et al., 1991) should apply at all pH values provided the mechanism does not change over the pH range studied. Changes in kinetic mechanism as the pH changes have been observed in other systems including creatine kinase (Cook et al., 1981) and dopamine β-hydroxylase (Ahn & Klinman, 1982) and most recently for the catalytic subunit of the cyclic AMP-dependent protein kinase (Qamar & Cook, 1993). Thus, one must know what changes, if any, occur as the pH changes. In addition to the kinetic parameters V and V/K, the effects of the allosteric modifier as a function of pH on the kinetic parameters generates values of K_{act} and the maximum fold activation (Cook, 1982).

In this study, initial velocity patterns were obtained at the pH extremes and in the presence and absence of $F26P_2$. At zero $F26P_2$, initial velocity data (not shown) in the absence and presence of inhibitors are consistent with a steady-state ordered kinetic mechanism as shown by Rao et al. (1987b). Specifically, the inhibition pattern by arabinose 5-phosphate, a dead-end analog of F6P, is uncompetitive vs MgATP at all pH values tested. In the presence of $F26P_2$, patterns are nearly parallel, and the pattern by arabinose 5-phosphate is noncompetitive vs MgATP (unpublished results of G. Gibson in this lab). Thus, the mechanism has become random with synergism in the binding of MgATP and F6P. The randomness likely results from an increase in the affinity of F6P for free enzyme.[2]

To obtain the parameters of interest, an activation study such as that shown in Figures 1 and 2 of Payne et al. (1991) must be performed at each pH. General theory for competitive activation by two effectors was developed by these authors based on the activation of PFK by $F26P_2$ and AMP. Activation is observed as a decrease in K_{F6P} and synergistic activation by the two effectors is indicated. A plot of K_{F6P} against activator concentration gives a decrease in K_{F6P} to a lower constant value at infinite effector concentration with K_{act} equal to the concentration of effector that gives half the change in K_{F6P}. Plots of $\log(1/K_{act})$, that is, pK_{act}, and fold activation versus pH can be used to obtain pKs for ionizable groups responsible for binding of the effector, possible pH-dependent conformational changes brought about by binding of the effector, and changes in the pK values of catalytic groups at the active site.

Effects of pH on the Kinetic Parameters of d-PFK in the Direction of F6P Phosphorylation. The values of V_f and V/K_{MgATP} do not change with pH in the absence and presence of saturating $F26P_2$. It was thus possible to simplify experiments by fixing the concentration of MgATP at saturation and examining the variation of V/K_{F6P} at varied pH and with varied concentrations of $F26P_2$. Data were collected and treated as previously described at pH 6.8 (Payne et al., 1991) from about pH 6–9. The relevant kinetic parameters were then plotted against pH to obtain pKs for essential ionizable groups.

Figure 1A shows the pH dependence of V_f in the absence and presence of 200 μM $F26P_2$ (known to be saturating across the range of pH studied); V_f/E_t values may show a slight pH dependency but for all intents and purposes are pH-independent with an average value of 45 ± 6 s^{-1}, about 2-fold higher than the value of 19 s^{-1} reported previously (Rao et al., 1987b). The difference is ascribed to the higher quality of the preparation used in these studies. The $V/K_{MgATP}E_t$ is also pH- and $F26P_2$-independent (data not shown) with an average value of $(1.4 \pm 0.3) \times 10^6$ M^{-1} s^{-1}, again about 2-fold greater than the value of 8.8×10^5 M^{-1} s^{-1} reported previously (Rao et al., 1987b). Figure 1B shows the pH dependence of V/K_{F6P} in the absence and presence of 200 μM $F26P_2$. For data obtained at zero, $(V/K_{F6P})^0$, and saturating, $(V/K_{F6P})^\infty$, concentrations of $F26P_2$, the log (V/K) decreases at low pH with a limiting slope of 1 and at high pH with a limiting slope of -1. The pKs obtained from a fit using eq 5 in the absence of $F26P_2$ are 7.0 ± 0.1 on the acid side and 8.9 ± 0.1 on the basic side, while in the presence of $F26P_2$ the pK on the acid side is 6.9 ± 0.1 and the pK on the basic side is 9.1 ± 0.2. The pH-independent values of $V/K_{F6P}E_t$ in the absence and

[2] Even in the absence of $F26P_2$, the kinetic mechanism is random but the dissociation constant for E·F6P is estimated at 50 mM, and thus the mechanism is predominantly ordered (Rao et al., 1987b).

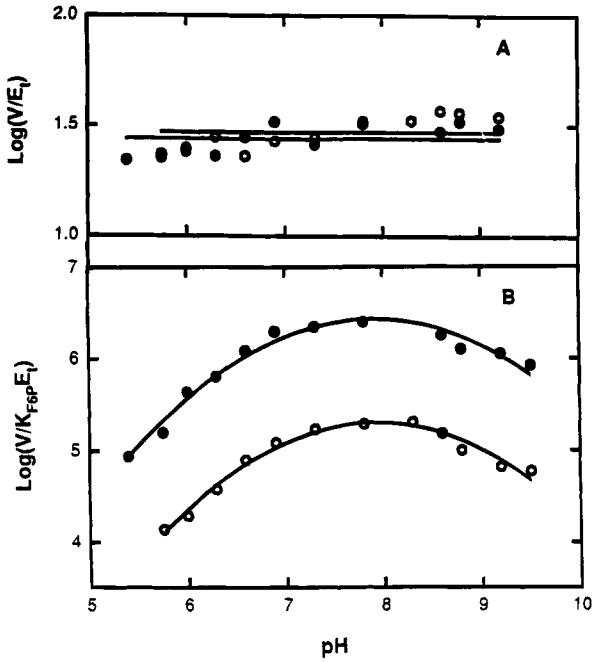


FIGURE 1: pH dependence of V and V/K_{F6P} in the absence and presence of 200 μM $F26P_2$. Filled circles represent values obtained with 200 μM $F26P_2$, while open circles represent values obtained in the absence of effector. The lines in panel A are from linear regression analysis of the data, while the curves in panel B are theoretical from a fit of eq 5 to the data. In all cases the points are experimental.

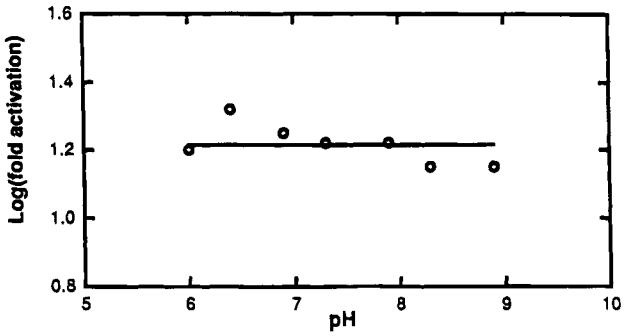


FIGURE 2: pH dependence of the maximum activation by $F26P_2$. The log (fold activation) is obtained as the difference in the curves shown in Figure 1A and reflect the ratio of the V/K_{F6P} in the presence of 200 μM $F26P_2$ to the V/K_{F6P} with $F26P_2$ absent. The line is from a linear regression analysis of the data.

presence of $F26P_2$ are $(1.8 \pm 0.3) \times 10^5$ M^{-1} s^{-1} and $(2.4 \pm 0.3) \times 10^6$ M^{-1} s^{-1}, respectively. The ratio of the pH-independent values of $(V/K_{F6P})^{\infty}/(V/K_{F6P})^0$ in the presence and absence of $F26P_2$ is 14 ± 1, the maximum fold activation by $F26P_2$. There is no significant difference in the pKs obtained in the absence and presence of $F26P_2$. Since V is independent of pH, the observed pKs are intrinsic values for the groups involved and all groups must be in their correct protonation state for optimum binding and catalysis to occur (Cleland, 1977). Thus, in the direction of F6P phosphorylation, the action of F26P is manifest in an increase in V/K_{F6P} (or a decrease in K_{F6P}). Figure 2 gives the pH dependence of the ratio $(V/K_{F6P})^{\infty}/(V/K_{F6P})^0$, the maximum fold activation, which has an average pH-independent value of 16 ± 4. Both values of the maximum fold activation are in excellent agreement with the value of 15 reported previously (Payne et al., 1991). The above ratio, $(V/K_{F6P})^{\infty}/(V/K_{F6P})^0$, is also the allosteric parameter (or interaction coefficient) Q_{AX} as

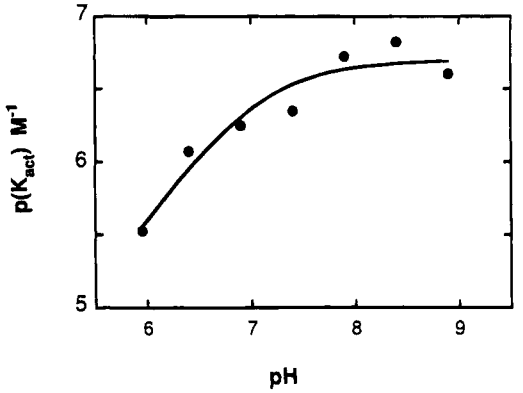


FIGURE 3: pH dependence of $1/K_{act}$ for $F26P_2$. The value of K_{act} is obtained as K_{ID} from a fit of eq 3 to the dependence of V/K_{F6P} on the concentration of $F26P_2$. The curve is theoretical from a fit of eq 4 to the data, and the points are experimental.

defined by the thermodynamic linkage analysis of Reinhart (Symcox & Reinhart, 1992; Reinhart, 1983), where A represents F6P and X represents $F26P_2$.

At each pH, the dependence of K_{F6P} on the concentration of $F26P_2$ was obtained. Data are described (not shown) by a hyperbolic function, and the concentration of $F26P_2$ that gives half the change in K_{F6P} is the activation constant for $F26P_2$. K_{act} is also the dissociation constant for $F26P_2$ from enzyme when MgATP is saturating. Values of $(K_{F6P})^0$ and $(K_{F6P})^{\infty}$ are also estimated from the titration of K_{F6P} with $F26P_2$ and in all cases the calculated values and their ratio (maximum fold activation) agree with the measurements made at zero and saturating $F26P_2$ given above. Figure 3 shows the pH dependence of K_{act}. Log $(1/K_{act})$ decreases at low pH with a limiting slope of 1 and giving a pK of 7.4 ± 0.1, suggesting that the group being titrated must be deprotonated for optimum binding of $F26P_2$ to occur. The pH-independent value of K_{act} is 0.20 ± 0.02 μM.

Effects of pH on the Kinetic Parameters of the d-PFK Reaction in the Direction of MgADP Phosphorylation. To assist in clarifying the effects of $F26P_2$ on specific steps along the reaction pathway and to aid in the elucidation of the acid–base catalytic mechanism, kinetic parameters were also determined for the reverse reaction direction. As found for the forward reaction direction, V_r and V/K_{MgADP} are independent of pH and $F26P_2$ concentration with average values of about 1.1 ± 0.2 s^{-1} and $(2.0 \pm 0.2) \times 10^5$ M^{-1} s^{-1}, respectively, for V_r/E_t and $V/K_{MgADP}E_t$. The only kinetic parameter influenced by pH and/or the presence of $F26P_2$ is V/K_{FBP}. Thus, V/K_{FBP} was obtained at saturating MgADP in the absence and presence of 200 μM $F26P_2$. The V/K_{FBP}, Figure 4, decreases at high pH with a limiting slope of -1 and gives pK values of 7.4 ± 0.2 and 7.3 ± 0.1 in the absence and presence of saturating $F26P_2$, respectively. Thus, again the maximum fold activation is pH-independent. The pH-independent values of $V/K_{FBP}E_t$ are $(1.1 \pm 0.1) \times 10^3$ M^{-1} s^{-1} and $(8 \pm 5) \times 10^3$ M^{-1} s^{-1}, respectively, in the absence and presence of $F26P_2$. The maximum fold activation is again pH-independent and has a value of 8 ± 5. The latter is apparently lower than that obtained in the opposite reaction direction but has a large associated error. The ratio of the maximum fold activations in the forward and reverse reaction directions is 2 ± 1.

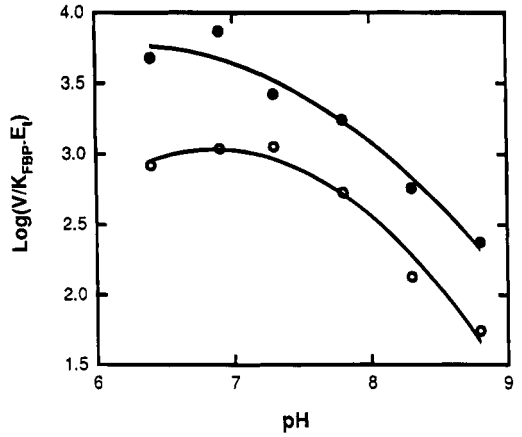


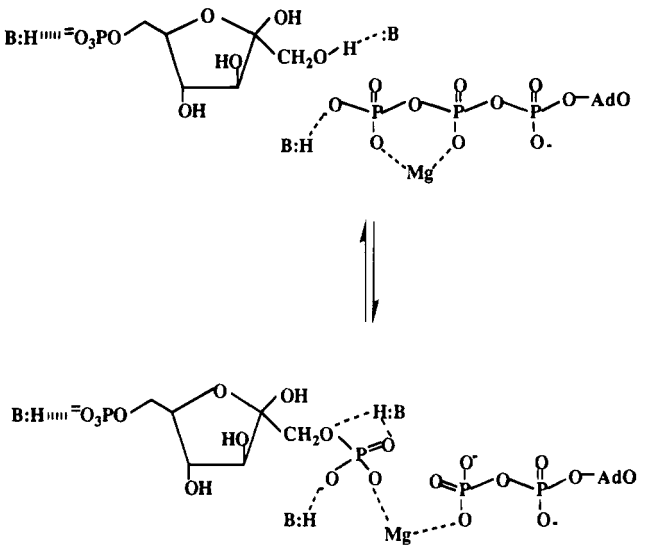
FIGURE 4: pH dependence of V/K_{FBP} in the absence and presence of 200 μM $F26P_2$. pH profiles are obtained in the absence (open circles) and the presence (filled circles) of $F26P_2$. The curves are theoretical form a fit of eq 6 to the data, while points are experimental.

DISCUSSION

Interpretation of pH Profiles in the Absence of F26P$_2$. The V_{max} in both reaction directions is independent of pH. These data indicate either that reactants and enzyme must be in the correct protonation state for optimum binding and catalysis and that the observed pK values are intrinsic (Cleland, 1977) or that a step outside the catalytic pathway limits the overall reaction. It is likely that the former is correct, since evidence from initial velocity studies suggest rapid release of both FBP and MgADP (Rao et al., 1987b). The V/K_{MgATP} and V/K_{MgADP} values are also pH-independent. The kinetic mechanism is for all intents steady-state ordered, with the nucleotides bound prior to the sugar phosphate at zero $F26P_2$, and the V/K profiles thus reflect free enzyme and free nucleotide as the predominant enzyme and substrate forms. Thus, only groups on enzyme and substrate that affect the binding of the nucleotides will be observed in the V/K pH profiles. The pK for the γ-phosphate of MgATP and the β-phosphate of MgADP is about 4.6 at 0.1 ionic strength (Martell & Smith, 1982), and thus neither of these will be observed in the V/K profiles since they are outside the pH range studied. As will be discussed below, it is possible that an enzyme group with a pK of about 8.6 interacts with the γ-phosphate of MgATP to neutralize the negative charge, facilitating nucleophilic attack. The pK for this group is not observed in the V/K_{MgATP} profile, even though data were collected to a pH of 8.8, where V/K_{MgATP} would be about 2-fold lower than the pH-independent value observed from pH 6 to 8. Thus, either the pK of the group that interacts with the γ-phosphate is higher in free enzyme than in the E·MgATP complex or it does not interact with the γ-phosphate and the group with a pK of 8.6 until F6P is bound. In the presence of saturating $F26P_2$, the kinetic mechanism of PFK is random, and the V/K_{MgATP} reflects the E·F6P complex and free MgATP. Since F6P is bound in this case and a pK is still not observed at pH 8.6, the former explanation above—that is, the pK of the group that interacts with the γ-phosphate is higher in free enzyme than in the E·MgATP complex—is likely correct if the group with a pK of 8.6 functions in this capacity.

Most of the information concerning acid–base chemistry and binding comes from the pH dependence of the V/K for the sugar phosphate substrates. V/K_{F6P}, which reflects

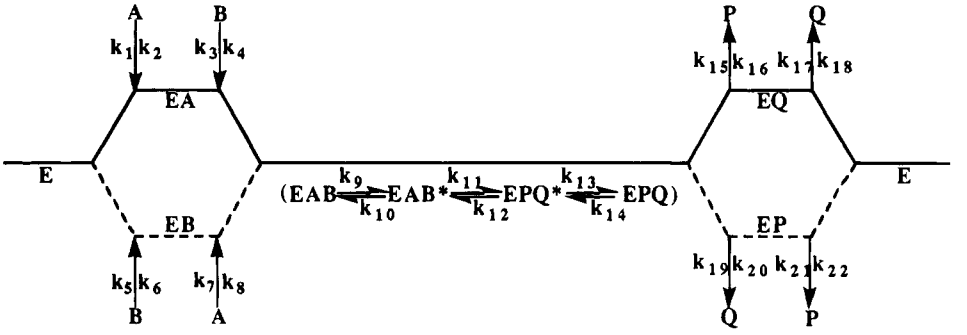
Scheme 1: Proposed Acid–Base Catalytic Mechanism for *Ascaris suum* PFK



E·MgATP and F6P, decreases at low pH with a pK of 7.0 and at high pH with a pK of 8.9, while V/K_{FBP}, which reflects E·MgADP and FBP, decreases at high pH with a pK of about 7.4. The group with a pK of 7.0 in the V/K_{F6P} profile likely reflects that of a general base catalyst that is required to accept a proton from the 1-hydroxyl of F6P to facilitate nucleophilic attack of the hydroxyl on the γ-phosphate of MgATP. This same group should also be required in the opposite reaction direction to donate a proton to the 1-hydroxyl of F6P as it is formed upon attack of the β-phosphate of MgADP on the 1-phosphate of FBP. The V/K_{FBP} pH profile exhibits the requirement for a group that must be protonated with a pK of 7.4, which is likely the same group but acting as a general acid in the direction of phosphorylation of MgADP. The difference in the value of the pK in the V/K_{F6P} and V/K_{FBP} profiles can likely be attributed to the fact that different enzyme complexes, E·MgATP and E·MgADP, respectively, are titrated. The remaining ionization observed in the V/K_{F6P} pH profile is that of a group with a pK of 8.9 that must be protonated for optimum binding of F6P and/or catalysis. There is a need to decrease the electron density around the γ-phosphate of MgATP to make the group more susceptible to nucleophilic attack by the 1-hydroxyl of F6P. The group with a pK of 8.9 may fulfill this function, or it may be involved in the binding of the 6-phosphate of F6P. Although the pK is not observed in the V/K_{FBP} pH profile, its pK could be perturbed to higher pH, as observed for the group with a pK of 7.0.

Chemical Mechanism. A number of lines of evidence can be combined to propose a mechanism for the phosphoryl transfer catalyzed by PFK. The crystal structure of the PFK from *Escherichia coli* has been resolved in the presence of its reaction products (Shirakihara & Evans, 1988) and provides a good starting point for the regiochemistry of the *Ascaris* PFK, although the functional groups and specifics of the acid–base mechanism may certainly differ. In addition, the stereochemistry of phosphoryl transfer in most phosphotransferases (Cullis, 1987), including the PFKs from *Bacillus stearothermophilus* and rabbit skeletal muscle (Jarvest et al., 1981), has been observed to occur with inversion of configuration of the transferred phosphoryl group, suggesting an in-line S_N2 or S_N1 mechanism. These

Scheme 2



observations along with the pH studies presented above suggest Scheme 1 as a working model.

In Scheme 1, the reactants are shown immediately prior to reaction and the products are shown immediately after reaction. Dianionic F6P [fructose 6-sulfate is a very poor substrate for PFK (Martensen & Mansour, 1976)] binds to the E·MgATP complex with the 1-hydroxyl likely hydrogen-bonded to an enzyme group with a pK of about 7.0. The γ-phosphate is in close proximity either to an enzyme group with a pK of 8.9, which is protonated and likely positively charged, or to backbone NHs and side-chain hydroxyls. The group with a pK of 7.0 then acts as a general base, accepting a proton as the hydroxyl oxygen attacks the γ-phosphate of MgATP, while electron density around the γ-phosphate is reduced by enzyme side chain and/or backbone interactions. The identity of the above enzyme residues is at present unknown. Rao et al. (1987a) have shown that F6P protects against inactivation of an active-site histidine by diethyl pyrocarbonate and that this group has a pK of 6.4 in free enzyme, so that the histidine may then be the general base. Assignment will have to await at least the completion of the primary sequence, to determine its alignment with other PFKs, or the determination of the crystal structure.

Once the products are formed, the general base is now protonated and can act as a general acid in the reverse reaction direction. The Mg is coordinated to ATP in the Δ-β,γ-bidentate complex (Dunaway-Mariano & Cleland, 1980) and after phosphoryl transfer will still be coordinated to these phosphates, and thus it will undergo a ligand displacement reaction in which the α-phosphate displaces the 1-phosphate of the newly formed FBP.

Effects of pH and F26P$_2$ on the d-PFK-Catalyzed Phosphorylation of MgADP. Activation of an enzyme-catalyzed reaction can occur at a number of discrete steps along the reaction pathway, and the effect of the kinetic parameters provides information on which steps might be affected (Cook, 1982). The only kinetic parameters affected by the presence of the allosteric activator F26P$_2$ are the V/K values for the sugar phosphate substrates in both reaction directions. The kinetic mechanism of the *Ascaris suum* PFK is predominantly steady-state ordered in the direction of F6P phosphorylation, but with rapid release of both products FBP and MgADP. A depiction of the kinetic mechanism as proposed by Rao et al. (1987b) is provided in Scheme 2. The dotted line indicates the mechanism is actually random, but the operational pathway requires binding of MgATP prior to F6P. In Scheme 2, the letters A, B, P, and Q represent MgATP, F6P, FBP, and MgADP; k_1–k_8 and k_{15}–k_{22} reflect binding and release of reactants; k_9, k_{10}, k_{13}, and k_{14} reflect conformation changes that almost certainly exist to close and reopen the enzyme active site to exclude water and properly align binding and catalytic groups, and k_{11} and k_{12} represent the catalytic rate constants in the directions of F6P and MgADP phosphorylation, respectively. Since only V/K_{F6P}, and V/K_{FBP} are increased in the presence of F26P$_2$, the rate constant(s) increased must be present in the expressions for these V/Ks and not in V or V/K for the nucleotides. The expressions for V/K_{MgATP} and V/K_{MgADP} in the mechanism shown in Scheme 2 are k_1 and k_{18}, respectively, in the absence of F26P$_2$, rate constants that are not contained in the expressions for V or the V/Ks for F6P and FBP. In the presence of F26P$_2$, however, the expressions for V/K_{MgATP} and V/K_{MgADP} in the mechanism shown in Scheme 2 contain all steps from addition of the substrate to release of the first product. Since no effect is observed on the V or V/K for the nucleotides in the absence or presence of F26P$_2$, the only effect of the allosteric activator is to decrease the off rates for F6P and FBP. Consistent with this notion is the observed increase in importance of the alternate pathway with F6P binding prior to MgATP when F26P$_2$ is present that is, the switch to a random kinetic mechanism. However, if k_{15} (or k_{21}) is decreased and V_f is unaffected, the ratio k_{11}/k_{15} (or k_{11}/k_{21}) must be very close to zero; that is, the off rate for FBP must be much faster than the catalytic step so that it does not limit the overall rate in the direction of F6P phosphorylation. The same logic must also be applied to the reverse reaction; that is, the off rate for F6P cannot limit the overall rate of MgADP phosphorylation. In order for this to be true, the equilibrium constant for the catalytic steps on enzyme must be very close to the solution equilibrium constant, which is far in the direction of FBP at most pH values. A value of 3×10^{-5} M has been estimated by Rao et al. (1987b) for the solution equilibrium constant, that is about 300 at pH 7. To be consistent with the above interpretation, $(V/K_{F6P})/(V/K_{FBP})$ must be the same in the absence and presence of F26P$_2$ as required by the Haldane relationship (Cleland, 1982):

$$K_{eq} = V_f K_p K_{iq} / V_r K_b K_{ia} \quad (6)$$

where V_f is the forward maximum velocity, V_r is the reverse maximum velocity, K_p is K_{FBP}, K_b is K_{F6P}, K_{ia} is K_{iMgATP}, and K_{iq} is K_{iMgADP}. If the other kinetic parameters are constant, it follows that K_p and K_b must vary together. Although the results presented are consistent with the above interpretation, there may be a difference of up to 2-fold in the maximum fold activation in the two reaction directions (see Results). If the difference is real (there is a 50% standard error associated with the value of 2), there must also be a 2-fold increase in the ratio of $(K_{iMgATP})/(K_{iMgADP})$

in the presence vs the absence of $F26P_2$ to compensate for the difference. There is no evidence at present for the latter suggestion.

The pH dependence of the $F26P_2$ activation constant reflects the titration of a group on enzyme (since the effector has no titrable groups with a pK of 7) in the $F26P_2$ site that is required to be unprotonated for optimum binding of the allosteric effector. The enzyme residue probably hydrogen-bonds to one of the free hydroxyls of $F26P_2$.

The pH independence of the maximum activation by $F26P_2$ in the forward reaction direction reveals important information about the action of the effector. First, the observation that the maximum activation (which is related to the free energy of binding) is constant with varied pH implies that the allosteric transition induced by the effector is pH-insensitive. That the pK_s observed for the binding of F6P are not perturbed by the presence of the effector further suggests that the allosteric transition acts in a way only to facilitate the binding of F6P and not MgATP. In addition, the pH independence of the transition suggests that the effect is only observed in that portion of the enzyme pool that is correctly protonated for catalysis and binding of the sugar phosphate substrates at a given pH.

Circular dichroism spectra have been obtained for n-PFK at pH 6 and 8 in the absence and presence of saturating $F26P_2$ (Rao et al., unpublished work). Increasing the pH from 6 to 8 causes a shift in ellipticity opposite to that observed upon addition of $F26P_2$. In contrast, CD spectra of d-PFK are the same at pH 6 and 8 and are identical to the spectrum of n-PFK at pH 8. Apparently d-PFK is locked in a pH activated conformation. It is evident that changes in pH and the presence/absence of effectors have different modes of action in activating PFK and consequently induce (or stabilize) different conformations of the enzyme. It will be of interest to determine the nature of the conformational change induced by $F26P_2$ which subtly perturbs the secondary structure yet releases a reasonable amount of free energy and is pH-insensitive.

Comparison to E. coli PFK. The PFK from *E. coli* has also been studied with respect to the pH dependence of its kinetic parameters (Deville-Bonne et al., 1991; Auzat & Garel, 1992). The pH dependence of the forward reaction kinetic parameters show notable differences compared with the *Ascaris* PFK. The apparent affinity for F6P as measured by $S_{0.5}$ is invariant across the pH range 6−9 in the absence and the presence of the activator, GDP, although the $S_{0.5}$ is about 4-fold lower in the presence of GDP. The Hill coefficient (n_H) is also decreased by GDP to a value close to 1 across the entire pH range, while n_H increases from about 2.5 at pH 6 to about 5.5 at pH 9 in the absence of GDP. The maximum rate increases over the pH range 6−9 with the titration of one group with a pK of about 7 in the absence and 6.6 in the presence of GDP; V is also 15−20% in the presence of GDP. The presence of the inhibitor PEP reduces V but does not change the pK of 7. Thus, the activation of V by GDP can be accounted for by the shift in a critical pK, leading to an increase in the relative amount of active enzyme at lower pH. Clearly the effects of pH and effectors on the regulation of *E. coli* PFK are distinct from those on *Ascaris* PFK and, by analogy, mammalian PFKs.

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BI950372Y