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pH Dependence of the Kinetic Parameters for the Pyrophosphate-Dependent Phosphofructokinase Reaction Supports a Proton-Shuttle Mechanism†

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Received October 3, 1988; Revised Manuscript Received December 14, 1988

ABSTRACT: The pH dependence of kinetic parameters for the pyrophosphate-dependent phosphofructokinase from *Propionibacterium freudenreichii* suggests that the enzyme catalyzes its reaction via general acid–base catalysis with the use of a proton shuttle. The base is required unprotonated in both reaction directions. In the direction of fructose 6-phosphate phosphorylation the base accepts a proton from the hydroxyl at C-1 of F6P and then donates it to protonate the leaving phosphate. Whether this occurs in one or two steps cannot be deduced from the present data. The maximum velocity of the reaction is pH independent in both reaction directions while V/K profiles exhibit pKs for binding groups (including enzyme and reactant functional groups) as well as pKs for enzyme catalytic groups. These data suggest that reactants bind only when correctly protonated and only to the correctly protonated form of the enzyme. Specifically, the requirement for two enzyme ϵ-amino groups in the protonated form for reactant binding was detected as was the requirement for the ionized phosphates of fructose 6-phosphate, fructose 1,6-bisphosphate, $MgPP_i$ and HPO_4^{2-}. The protonation state of enzyme and reactant binding groups is in agreement with data obtained previously [Cho, Y.-K., & Cook, P. F. (1988) *J. Biol. Chem. 263*, 5135].

The pyrophosphate-dependent phosphofructokinase from *Propionibacterium freudenreichii* catalyzes the phosphorylation of the hydroxyl at C-1 of β-D-fructose 6-phosphate to produce β-D-fructose 1,6-biphosphate (Reeves et al., 1974). This enzyme is dimeric and is not regulated by either substrates or allosteric effectors (O'Brien et al., 1975).

It has been shown by initial velocity studies (Bertagnolli & Cook, 1984) that the enzyme has a rapid equilibrium random kinetic mechanism. More recently, Cho et al. (1988) have shown by using isotope exchange techniques that the phosphoryl-transfer step is likely the rate-determining step in the overall mechanism.

Little is known of the chemical interconversion of reactants and products for the pyrophosphate-dependent enzyme. The pH dependence of the enzyme–reactant dissociation constants has been obtained (Cho & Cook, 1988) by making use of protection by reactants against the modification of two active-site lysine residues by pyridoxal 5′-phosphate. One of the lysines is apparently in the vicinity of the 6-phosphate of the sugar reactants, while the second is in the vicinity of the phosphate to be transferred from $MgPP_i$[1] to the C-1 hydroxyl of F6P. For optimum binding of reactants, both lysines must be ionized as well as $MgPP_i$ and the phosphates at C-1 and C-6 of the sugar phosphates. Inorganic phosphate apparently bind as HPO_4^{2-}.

With the above background on the optimum protonation state of active-site lysines and reactants available, it is an easier task to determine the acid–base chemistry catalyzed by the enzyme via pH–rate profiles. There is no evidence for nucleophilic catalysis by enzyme, and thus two possible mechanisms are suggested. The first involves general base–general acid catalysis in which the general base accepts a proton from the hydroxyl of C-1 concomitant with nucleophilic attack of the hydroxyl oxygen on pyrophosphate while the general acid would protonate the leaving group phosphate (Scheme IA). A second possibility is general acid–base catalysis in which a proton shuttle accepts a proton from the hydroxyl at C-1 and donates it to the leaving group phosphate either in the same step (Scheme IB) or in a subsequent step (Scheme IC). In Scheme IB, the general base could be pictured as, for example, an imidazole, accepting a proton on one imidazole nitrogen and donating the proton via the other in the same step. The mechanism depicted in Scheme IA sug-

† This work was supported by grants to P.F.C. from NIH (GM 36799) and the Robert A. Welch Foundation (B-1031) and by Grant BRSG SO7 RR07195 awarded by the Biomedical Research Grant Program, Division of Research Resources, National Institutes of Health, and a Faculty Research Grant from the University of North Texas.

[1] Abbreviations: Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid); Taps, 3-[[tris(hydroxymethyl)methyl]amino]-propanesulfonic acid; PP_i-PFK, pyrophosphate-dependent phosphofructokinase; F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; PP_i, inorganic pyrophosphate; P_i, inorganic phosphate; PCP, methylene diphosphonate; PLP, pyridoxal 5′-phosphate; HBP, 1,6-hexanediol bisphosphate; T6P, tagatose 6-phosphate.

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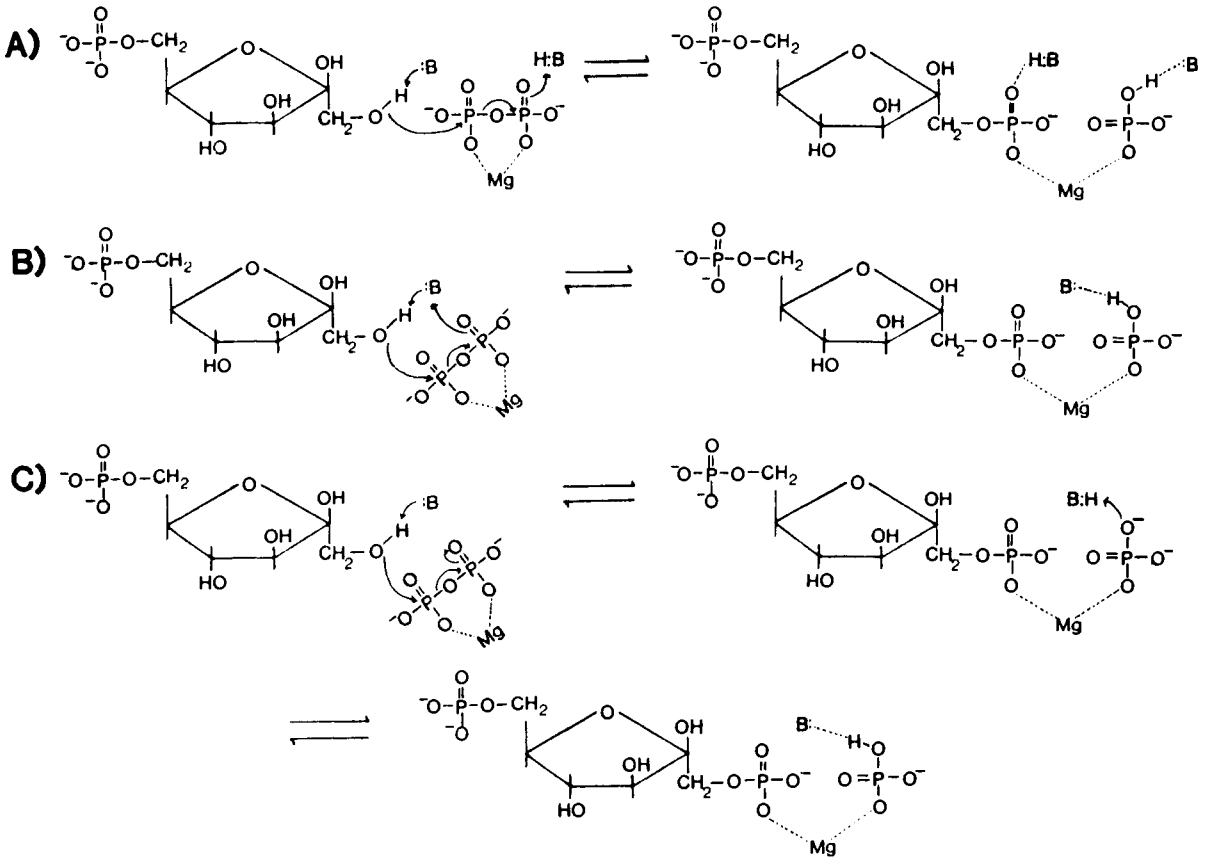
Scheme I: Possible Acid–Base Catalytic Mechanisms for PP_i-PFK: (A) General Base–General Acid Mechanism; (B) Proton-Shuttle Mechanism in Which Catalysis Occurs in a Single Step; (C) Two-Step Proton-Shuttle Mechanism



gests that activity obtained with substrate limiting should decrease at both low and high pH in both reaction directions, while the mechanism depicted in Scheme IB,C suggests that activity should decrease only at low pH at limiting substrate concentrations in both reaction directions. In the present study, the pH–rate profiles have been obtained in both reaction directions. Data are consistent with the mechanism given in Scheme IB,C.

Materials and Methods

Chemicals. Fructose 6-phosphate, fructose 1,6-bisphosphate, NADH, and NADP were from Sigma. Tagatose 6-phosphate was obtained from Research Plus, Inc. Hexanediol 1,6-bisphosphate was prepared according to the method of Hartman and Barker (1965). All other substrates, inhibitors, and buffers were obtained from commercially available sources and were of the highest quality available.

Enzymes. The pyrophosphate-dependent phosphofructokinase from *P. freudenreichii* was obtained from Sigma and purified by the method of Bertagnolli and Cook (1984) based on a procedure developed by O'Brien et al. (1975). Fructose bisphosphate aldolase, triosephosphate isomerase, α-glycerol-3-phosphate dehydrogenase from rabbit muscle and phosphoglucoisomerase and glucose-6-phosphate dehydrogenase from yeast were obtained from Sigma. Enzymes were tested for contaminating activities and purified if required according to procedures of Bertagnolli and Cook (1984).

pH Studies. Initial velocities were obtained by using the coupled spectrophotometric assays developed by Bertagnolli and Cook (1984). The velocity was a linear function of the PP_i-PFK concentration for all assay conditions used in these studies. The pH dependence of V and the V/K for a given reactant were obtained by varying the levels of the desired substrate at saturating concentrations of the other substrate. Saturating reactant concentrations are dependent on pH. Saturating F6P was 38 mM at pH 4.7 and 45 mM at pH 9.8; $MgPP_i$ was 4.8 mM at pH 4.7 and 4.2 mM at pH 9.8; P_i was 318 mM at pH 4.7 and 118 mM at pH 9.8; and FBP was 42 mM at pH 4.7 and 125 mM at pH 9.8. The K_i values for competitive inhibitors were determined by varying the concentration of the desired substrate at several different inhibitor concentrations including zero and a saturating concentration of the fixed substrate. Saturation by coupling enzymes was tested by monitoring velocity vs PP_i-PFK at the extremes of pH in both reaction directions. In addition, for competitive inhibition patterns, saturation by the coupling system was tested at all pH values at the lowest concentration of the variable substrate and the highest concentration of competitive inhibitor. In all cases the coupling enzymes were found to be saturating. All substrate and inhibitor concentrations were corrected for the concentration of the chelate complexes, MgF6P, MgFBP, and MgP_i as described by Bertagnolli and Cook (1984). The above experiments were carried out as a function of pH. Buffers at 100 mM final concentration were used over the following pH ranges: *N*,*N*′-bis(2-hydroxyethyl)piperazine, 4.7–5.0; Mes, 5.0–6.5; Pipes, 6.5–7.5; Taps, 7.5–9.0; Ches, 9.0–9.8. All buffers were titrated to pH with KOH. In all cases, overlaps were obtained when buffers were changed so that correction could be made for spurious buffer effects. The pH of the reaction mixture was measured with a Radiometer PHM82 pH meter with a combined microelectrode before and after sufficient data were collected for determination of initial velocities. Negligible pH changes were observed before and after reaction.

Data Processing. Reciprocal initial velocities were plotted vs reciprocal substrate concentrations, and all plots were linear. All data were fitted by using the appropriate rate equations

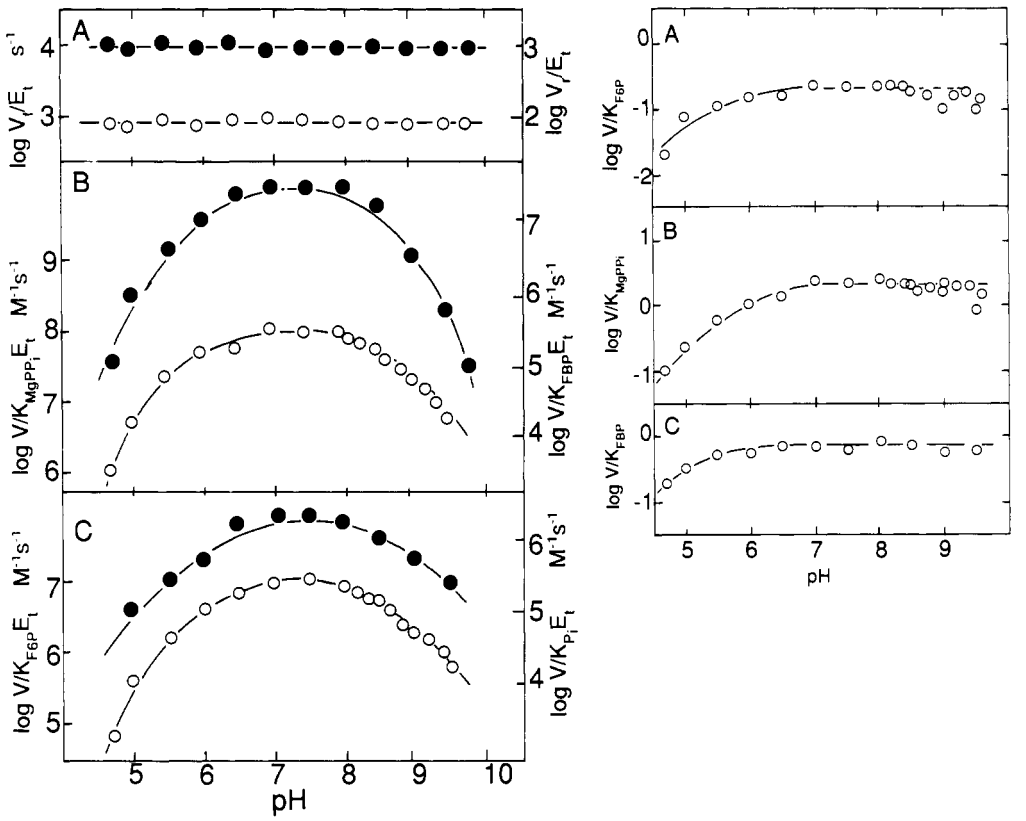


FIGURE 1: pH dependence of the kinetic parameters for the PP_i-PFK reaction. Parameters obtained in the direction of F6P phosphorylation are denoted by open circles, while those obtained in the direction of P_i phosphorylation are denoted by filled circles. The V/K for $MgPP_i$ was obtained at saturating concentrations of F6P and Mg^{2+} with the concentration of $MgPP_i$ varied around its K_m, while the V/K for F6P was obtained at saturating concentrations of $MgPP_i$ and Mg^{2+} with the concentration of F6P varied around its K_m. The V/K for FBP was obtained at saturating concentrations of P_i and Mg^{2+} with the concentration of FBP varied around its K_m, while the V/K for P_i was obtained at saturating concentrations of FBP and Mg^{2+} with the concentration of P_i varied around its K_m. All substrate concentrations were corrected for the amount of metal chelate complex as described under Materials and Methods. The points shown are the experimentally determined values, while the curves are theoretical from a fit of the data as discussed under Results. (Inset) Corrected pH profiles for V/K_{F6P}, V/K_{MgPP_i}, and V/K_{FBP}. Each of the pH profiles above was corrected from the data shown for binding pKs as discussed under Results. The points are the corrected experimental values, while the curves are theoretical from a fit of eq 2 to the data.

and FORTRAN programs developed by Cleland (1979). The individual saturation curves used to obtain pH profiles were fitted by using eq 1. Data for pH profiles that showed a decrease with a slope of +1 as the pH is decreased or −1 as the pH is increased were fitted by using eq 2 or 3. Data for pH profiles that decreased with a slope of +1 at low pH and a slope of −1 at high pH were fitted by using eq 4. Data for pH profiles that decreased with slopes of +2 and −1 were fitted by using eq 5, while data for pH profiles that decreased with slopes of +2 and −2 were fitted in halves by using eq 6 and 7. In eq 1–7, A and H are reactant and hydrogen ion concentrations, respectively, K is the K_m for A, and V is the maximum velocity. The constants K_1 and K_2 represent acid dissociation constants for enzyme or reactant functional groups reflected on the acid side of the pH profiles; K_3 and K_4 represent acid dissociation constants for enzyme or reactant functional groups reflected on the basic side of the pH profiles.

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

$$\log Y = \log (C/(1 + H/K_1)) \quad (2)$$

$$\log Y = \log (C/(1 + K_3/H)) \quad (3)$$

$$\log Y = \log (C/(1 + H/K_1 + K_3/H)) \quad (4)$$

$$\log Y = \log (C/(1 + H/K_1 + H^2/K_1K_2 + K_3/H)) \quad (5)$$

$$\log Y = \log (C/(1 + H/K_1 + H^2/K_1K_2)) \quad (6)$$

$$\log Y = \log (C/(1 + K_3/H + K_3K_4/H^2)) \quad (7)$$

Y is the value of the parameter observed as a function of pH, and C is the pH-independent value of Y.

Results

The PP_i-PFK is stable over the pH range 5–8.5 for at least 15 min (Cho & Cook, 1988). The only significant decrease in activity occurs at high pH, yielding a k_{inact} of 0.034 min^{-1} at pH 9.0 and 0.041 min^{-1} at pH 9.5. However, even at these pH values where the enzyme is less stable, initial velocity data can be obtained since the pH-jump technique was utilized for all assays in this study; i.e., a small volume of enzyme maintained in dilute buffered solution at pH 7 was added to a reaction mixture at the desired pH with 100 mM buffer.

The kinetic mechanism for the PP_i-PFK is rapid equilibrium random with E:$MgPP_i$:P_i, E:P_i:F6P, and E:FBP:$MgPP_i$ dead-end complexes allowed (Bertagnolli & Cook, 1984; Cho et al., 1988). Diagnostic for the above mechanism are the competitive inhibition patterns obtained by P_i vs F6P and $MgPP_i$ or by FBP vs $MgPP_i$ at saturating concentrations of the fixed substrate. The diagnostic patterns were repeated along with the initial velocity patterns in the absence of products and dead-end inhibitors at pH 5 and 9 to be sure the kinetic mechanism did not change with pH. All patterns were qualitatively identical with those obtained at pH 8, suggesting that the kinetic mechanism does not change with a change in pH.

pH Dependence of Kinetic Parameters. The pH dependence of kinetic parameters in the direction of F6P phosphorylation is shown in Figure 1. The maximum velocity is pH inde-

Table I: Summary of pK Values Obtained from the pH Dependence of Kinetic Parameters

parameter	pK_a ± SE[a,b]	pK_b ± SE[a,b]
V/K_{F6P}	5.7 ± 0.1 (2) [6.0]	8.4 ± 0.1 [8.7]
V/K_{MgPP_i}	5.2 ± 0.1	9.0 ± 0.1 [8.7]
	6.0 ± 0.1 [5.5]	
V/K_{FBP}	5.8 ± 0.1 (2) [5.5, 6.0]	8.6 ± 0.1 (2) [8.7 (2)]
V/K_{P_i}	6.4 ± 0.2	8.5 ± 0.2
$pK_{i,T6P}$	5.4 ± 0.2	8.4 ± 0.1
	6.1 ± 0.1	
$pK_{i,MgPCP}$	5.4 ± 0.3	8.8 ± 0.1
	6.4 ± 0.1	
$pK_{i,HBP}$	5.6 ± 0.1 (2)	8.4 ± 0.1 (2)
$pK_{i,sulfate}$		8.4 ± 0.2

[a] Values in parentheses represent the minimum number of groups detected. [b] Values in brackets are the pK values that were subtracted from the profiles indicated to generate the profiles given in Figure 1 (inset).

pendent. The V/K values for F6P and $MgPP_i$ decrease at low and high pH with slopes >1 and −1, respectively. A fit of eq 5 to the data gives two pKs with an average value of 5.7 on the acid side and a pK of 8.4 on the basic side for V/K_{F6P} and two pKs of 5.2 and 6 on the acid side and a pK of 9 on the basic side for V/K_{MgPP_i}. The pH dependence of kinetic parameters in the direction of P_i phosphorylation is also shown in Figure 1. The maximum velocity is again pH independent. The V/K for FBP decreases at low and high pH with slopes of >1 and <−1. A fit of eq 6 and 7 to the data gives two pKs with an average value of 5.8 on the acid side and two pKs with an average value of 8.6 on the basic side. The V/K for P_i decreases at low and high pH with slopes of 1 and −1. A fit of eq 4 to the data gives pK values of 6.4 and 8.5, respectively. All pK values are summarized in Table I. The pH-independent values obtained in the present case are as follows: V_f/E_t, 955 ± 11 s^{-1}; $V/K_{F6P}E_t$, (2 ± 0.2) × 10^7 M^{-1} s^{-1}; $V/K_{MgPP_i}E_t$, (2.2 ± 0.2) × 10^8 M^{-1} s^{-1}; V_r/E_t, 1000 ± 12 s^{-1}; $V/K_{FBP}E_t$, (7 ± 0.5) × 10^7 M^{-1} s^{-1}; $V/K_{P_i}E_t$, (4 ± 0.5) × 10^6 M^{-1} s^{-1}. These values are in excellent agreement with values reported previously at pH 8 once the latter are corrected to the pH optimum (Bertagnolli & Cook, 1984).

As can be seen, the activity of the enzyme-catalyzed reaction decreases sharply at low and high pH as a result of the multiple groups titrated to decrease activity. This complicates the interpretation of the results. However, since pK values for enzyme and reactant functional groups important for binding are known on the basis of pH dependence of dissociation constants (Cho & Cook, 1988), the profiles can be corrected for these pK values. The V/K_{F6P} profile is obtained with saturating $MgPP_i$ so that the predominant enzyme form is E:$MgPP_i$, and thus the pK for $MgPP_i$ and the lysine in the vicinity of the transferred phosphate should not titrate. The pK values expected in the V/K_{F6P} profile for enzyme and reactant functional groups important for binding are 6.0 for the 6-phosphate of F6P and 8.7 for a lysine at the 6-phosphate site. These pK values were subtracted (to better allow observation of the remaining pH dependence) from the observed pH dependence of V/K_{F6P} by multiplying the observed value at each pH by the factor $\{1 + [H]/(10^{-6}\ M) + (2 \times 10^{-9}\ M)/[H]\}$ to give the corrected pH profile shown in Figure 1(A inset). A fit of eq 2 to the data of Figure 1(A inset) gives a pK value of 5.4 ± 0.3. The V/K_{MgPP_i} profile is obtained at saturating F6P, giving E:F6P as the predominant enzyme form so that the pK for F6P and the lysine near the 6-phosphate should not be observed. Expected pK values in the V/K_{MgPP_i} profile for enzyme and reactant functional groups important for binding include one for $MgPP_i$ (5.5) and a lysine (8.7) in the vicinity of the phosphate to be transferred to C-1 of F6P.

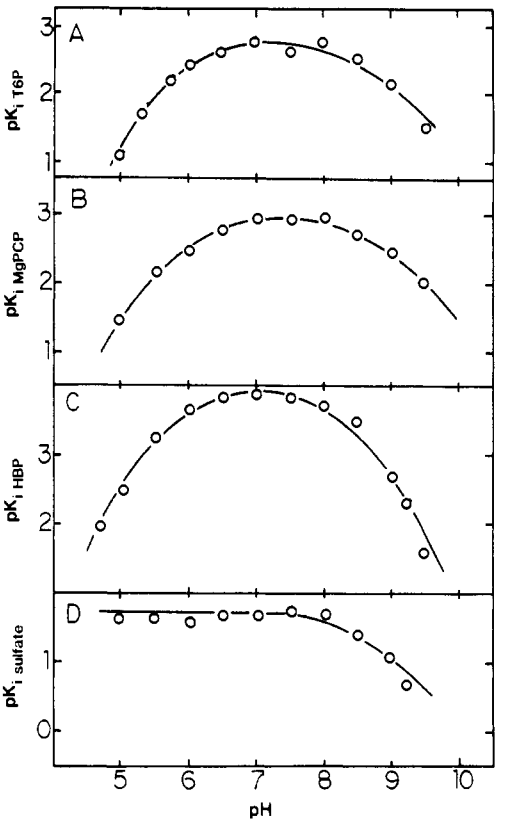


FIGURE 2: pH dependence of the reciprocal of inhibitor dissociation constants. (A) $1/K_i$ for T6P, an inhibitor competitive against F6P; (B) $1/K_i$ for MgPCP, an inhibitor competitive against $MgPP_i$; (C) $1/K_i$ for HBP, an inhibitor competitive against FBP; (D) $1/K_i$ for sulfate, an inhibitor competitive against P_i. All data were obtained from inhibition patterns with the fixed reactant maintained at a saturating concentration and the other reactant varied around its K_m. All reactions were carried out at 25 °C with conditions and buffers as discussed under Materials and Methods. The points represent experimental values, while the curves are theoretical from a fit of the data as discussed under Results.

Correction of the profile using the factor $\{1 + [H]/(3 \times 10^{-6}\ M) + (2 \times 10^{-9}\ M)/[H]\}$ gives the profile shown in Figure 1(B inset). A fit of eq 2 to the data gives a pK of 6.0 ± 0.1, likely the same group observed in the V/K_{F6P} profile with a pK of 5.5. The V/K_{FBP} profile is obtained at saturating P_i so that the predominant enzyme form is E:P_i. Only the pK for inorganic phosphate should not be observed under these conditions. Expected pK values for enzyme and reactant functional groups important for binding include those for the phosphates at C-1 (5.5) and C-6 (6.0) of FBP and the two lysines in the vicinity of these phosphates (8.7). The profile shown in Figure 1B was corrected by using the factor $\{1 + [H]/(10^{-6}\ M) + [H]^2/(3.2 \times 10^{-12}\ M) + (2 \times 10^{-9}\ M)/[H] + \{(2 \times 10^{-9}\ M)/[H]\}^2\}$, giving the profile shown in Figure 1(C inset). A fit of eq 2 to the data in Figure 1(C inset) gives a pK value of 5.2 ± 0.2. The V/K_{P_i} profile is obtained at saturating FBP concentration with the predominant enzyme form being E:FBP so that the C-1 and C-6 phosphates and the two lysine pKs may not be observed. The expected pK value of a group important for binding is that of phosphate (~6.5). An additional enzyme group with a pK of 8.5 is observed in this profile.

pH Dependence of pK_i Profiles. To corroborate the V/K profiles and aid in further distinguishing between binding and catalytic groups, the pK_i (log $1/K_i$) profiles for inhibitors competitive against each of the reactants were obtained. The pK_i profiles for tagatose 6-phosphate (competitive vs F6P) and MgPCP (competitive vs $MgPP_i$) are shown in Figure 2A,B.

The pH dependencies in the case of these two inhibitors are less complex than those obtained for the reactants, F6P and $MgPP_i$. The pK_i for T6P decreases on the acid side with a limiting slope of 2 and on the basic side with a slope of −1. A fit of eq 5 to the data in Figure 2A gives pK values of 5.4, 6.1, and 8.5. The pK_i for MgPCP decreases on the acid side with a limiting slope of 2 and on the basic side with a limiting slope of −1. A fit of eq 5 to the data in Figure 2B gives pK values of 5.4, 6.4, and 8.8. The pK_i profile for hexanediol 1,6-bisphosphate (competitive vs FBP) is somewhat more complex, decreasing at low and high pH with slopes of at least 2 and −2. A fit of the data shown in Figure 2C using eq 6 and 7 gives two pKs on the acid side with an average value of 5.6 and two pKs on the basic side with an average value of 8.4. The pK_i for sulfate (competitive vs P_i) decreases only at high pH with a slope of −1, giving a pK value of 8.4. All pK values are summarized in Table I. The pH-independent values of K_i are as follows: 1.4 ± 0.3 mM for T6P; 0.9 ± 0.04 mM for MgPCP; 120 ± 10 μM for HBP; 18 ± 3 mM for sulfate.

Discussion

Protection against inactivation by pyridoxal 5′-phosphate (PLP) has recently been used to obtain the dissociation constants for all reactants for the pyrophosphate-dependent phosphofructokinase-catalyzed reaction (Cho & Cook, 1988). The stoichiometry of incorporation of PLP was measured by reduction of the Schiff base with NaB^3H_4 in the absence and presence of FBP, giving two lysine residues protected from modification. The pH dependence of the reactant dissociation constants indicated the requirement for $MgPP_i$ and the phosphate esters at C-1 and C-6 of F6P and FBP to be fully ionized with inorganic phosphate at the HPO_4^{2-} protonation state. In addition, both lysine residues must also be protonated.

The present studies indicate that the maximum velocity in either the direction of F6P phosphorylation or P_i phosphorylation is pH independent. The V/K profiles for reactants exhibit pK values for enzyme and reactant functional groups important for binding as well as enzyme catalytic groups. Thus, reactants bind only when correctly protonated and only to the correctly protonated form of the enzyme (Cleland, 1977). In addition to the pK values for potential general acid–base catalysts, the pK values for binding groups are also expected to be observed in the pH–rate profiles as mentioned above. All pK values should be intrinsic and not perturbed as a result of slow steps other than the chemical interconversion steps since Cho et al. (1988) have shown that phosphoryl transfer is likely the only rate-determining step. The pK values for binding groups can then be subtracted from the log V/K vs pH profiles to determine whether general base and general acid residues are observed.

Interpretation of the pH Dependence of Kinetic Parameters. Once correction of the V/K profiles was carried out as discussed under Results, the remainder of the V/K profiles were analyzed for the presence of additional pH dependencies. As seen in Figure 1(inset), the V/K_{F6P}, V/K_{MgPP_i}, and V/K_{FBP} profiles contain a single pK on the acidic side once corrected for known binding groups. If an additional pK is observed on the basic side of the V/K_{F6P} and V/K_{MgPP_i} pH profiles, it must be ≥9.5. The value of the pK on the acid side is essentially identical for all three profiles and in both reaction directions must be unprotonated for activity. If there is an additional pH dependence reflecting a general acid, it would be expected in the V/K_{P_i} and V/K_{MgPP_i} profiles but not in the V/K_{F6P} and V/K_{FBP} profiles since P_i and $MgPP_i$ should lock the protonation state of this group once bound. The V/K_{P_i} profile does reflect a single pK on the basic side once corrected for the pK of inorganic phosphate but no pKs are observed on the basic side of the V/K_{MgPP_i}, V/K_{F6P}, or V/K_{FBP} profiles. The pK on the basic side of the V/K_{P_i} profile is thus most likely the lysine in the vicinity of the C-1 phosphate of FBP. Unlike the lysine near the C-6 phosphate whose protonation state is apparently locked upon binding F6P, the protonation state of the one near C-1 is apparently not locked upon binding of FBP.

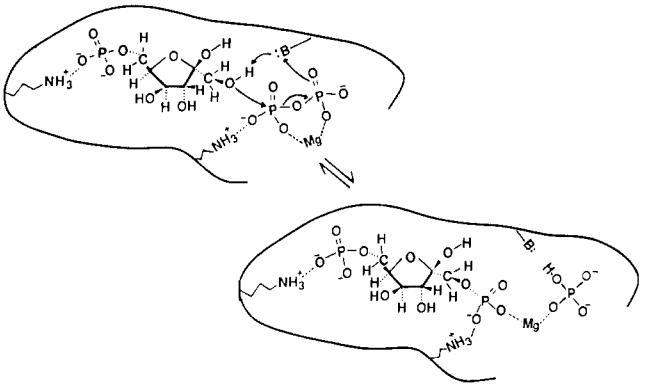
Thus, it appears that the PP_i-PFK catalyzes the phosphoryl transfer via general acid–base proton shuttle mechanism (Scheme IB,C). In the direction of F6P phosphorylation, the general base has a pK around 5.4, and this is perturbed to a slightly higher value of 6 when F6P is bound. This group functions as an acceptor of the proton on the C-1 hydroxyl concomitant with nucleophilic attack on $MgPP_i$. The leaving group phosphate is then protonated via the same group, leaving the general base unprotonated at the end of the catalytic cycle prepared for another round of catalysis. In the opposite reaction direction the catalytic group will have the same protonation state. As seen in the V/K_{FBP} profile, the general base is observed with a pK of 5.2. With FBP saturating (V/K_{P_i}), the protonation state of the general base is locked since it does not titrate. The groups observed represent phosphate (6.4) and, as suggested above, a lysine with a pK of 8.4, presumably the one identified in the vicinity of the C-1 phosphate.

It is interesting to note that although the protonation state of all titrable groups is locked when all reactants are bound, the general base titrates in all cases except the case where FBP is bound. In the latter case, the pK of the general base must be perturbed to a low pH. The presence of the phosphate at C-1 may allow a conformational change that partially closes the site.

Interpretation of the pH Dependence of Inhibitor K_i Values. The pK_i for T6P exhibits pK values of 5.4 and 6.1 on the acid and 8.4 on the basic sides. The pK of 6.1 is likely that of the 6-phosphate, essentially identical with F6P while the pK of 8.4 is likely a lysine that ion pairs the 6-phosphate. The remaining pK of 5.4 is that of the general base, so that T6P binds optimally when it is unprotonated. A similar situation exists with MgPCP. The pK of MgPCP should be approximately 1.3 pH units higher than that of $MgPP_i$ (Yoon & Cook, 1987). The pK for $MgPP_i$ in the present system is approximately 5.5 (Cho & Cook, 1988), so that the pK for MgPCP is about 6.8. This value is in reasonable agreement with the value of 6.4 observed on the acid side of the $pK_{i,MgPCP}$ profile. The pK of 8.8 observed on the basic side of the profile likely represents that of a lysine suggested to be in the vicinity of the phosphate to be transferred (Cho & Cook, 1988), while the remaining pK of 5.4 is probably the general base. Thus, in addition to the requirement for the phosphate of F6P and $MgPP_i$ to be ionized and for lysine ε-amine groups near C-6 of F6P and $MgPP_i$ to be ionized, F6P and $MgPP_i$ bind optimally when the general base is unprotonated.

In the case of HBP, an analogue of FBP, both phosphates and the lysines near them are required in the protonated form. It is difficult to determine whether additional groups are required in a given protonation state from the profile. However, on the basis of results obtained for MgPCP and T6P, it is likely that the protonation state of the group that will act as the general base is required unprotonated. The pK_i profile for sulfate is very easily interpreted. Sulfate is fully ionized over the entire pH range. As a result, the $pK_{i,sulfate}$ profile would be expected to show only the pK of the group that interacts with inorganic phosphate. The lysine with a pK of 8.5 also interacts with the negatively charged sulfate.

Scheme II: Possible Chemical Mechanism for Pyrophosphate-Dependent Phosphofructokinase from *P. freudenreichii*



Another piece of evidence consistent with the proton-shuttle or single-base mechanism is the absolute values of the kinetic parameters. One can fit the pH profiles shown in Figure 1 (inset) to the equation for a bell-shaped pH profile. If this is done, a basic pK of $\geq$9.5 is obtained (at least in the case of V/K_{F6P}). If this were a general acid while the group with a pK around 5.5 was a general base consistent with Scheme IA, these two groups would presumably be in their optimum protonation state in the direction of F6P phosphorylation, that is, the general base unprotonated and the general acid protonated. In the reverse reaction direction, these two groups would have to have reverse protonation states; that is, the group with a pK of 5.5 would have to be protonated, while the one with a pK of 9.3 would have to be unprotonated. If the general acid and general base were not in contact, this would require that between the two pK values only approximately 0.01% of the total enzyme would be correctly protonated. Thus, if one corrects to 100% correctly protonated enzyme, rates must be multiplied by a factor of 10000. The absolute values of kinetic parameters in the direction of P_i phosphorylation calculated above are as follows: V_r/E_t, 1000 s^{-1}; $V/K_{FBP}E_t$, 7×10^7 M^{-1} s^{-1}; $V/K_{P_i}E_t$, 4×10^6 M^{-1} s^{-1}. Correcting to 100% optimally protonated enzyme gives the following values: V_r/E_t, 10^7 s^{-1}; $V/K_{FBP}E_t$, 7×10^{11} M^{-1} s^{-1}; $V/K_{P_i}E_t$, 4×10^{10} M^{-1} s^{-1}. Note that both V/K values are well above the predicted diffusion limit of 10^8–10^9 M^{-1} s^{-1} and the calculated turnover number would make the PP_i-PFK one of the fastest enzymes known, somewhat surprising for an enzyme known to have a rapid equilibrium random kinetic mechanism (Bertagnolli & Cook, 1984; Cho et al., 1988) working in the nonglycolytic reaction direction.

There is a possibility that the two groups are in contact with one another, e.g., as a carboxyl/lysine hydrogen-bonded pair. In this case the proton could move to either the lysine or the carboxyl dependent on the reactant direction. The percentage of optimally protonated enzyme in this case would be 100% in either reaction direction, and no violation of the diffusion limit would occur. If this scenario were correct, the pK observed on the basic side of the V/K_{MgPP_i} and V/K_{P_i} profiles could be a lysine general acid, and the data would be consistent with Scheme IA. This would require that a total of three lysines be present in the active site, one in the vicinity of C-1, another near C-6, and a third acting as a general acid. Only two lysines were protected against inactivation by reactants (Cho & Cook, 1988). However, protection was not carried out with a ternary complex such as E:FBP:SO_4, in which case protection of a third residue may have been observed. In any case, if an active-site carboxyl is present, this alternative possibility cannot be excluded. Experiments designed to test for the presence of an active-site carboxyl are presently under way. We presently favor the single general acid–base proton-shuttle mechanism since there is no evidence for a pK on the basic sides of the corrected V/K_{MgPP_i}, V/K_{F6P}, and V/K_{FBP} profiles.

Chemical Mechanism. In the direction of F6P phosphorylation enzyme exists initially with the two enzyme ϵ-amine groups of lysine protonated and a general base (Scheme II). The binding of F6P requires the 6-phosphate to be ionized, while binding of $MgPP_i$ requires that it should also be ionized. Once reactants are bound, the protonation state of the general base is locked. The general base then accepts a proton from the hydroxyl at C-1 of F6P concomitant with nucleophilic attack of the hydroxyl oxygen on $MgPP_i$. The proton is then transferred to the leaving inorganic phosphate. Whether this occurs in the same transition state or in two separate steps is not known. The enzyme with product bound then has the general base unprotonated; that is, it is in the same state as it started in the direction of F6P phosphorylation.

In conclusion, it appears that the PP_i-PFK catalyzes its reaction via general acid–base catalysis using a single base as a proton shuttle. It will be interesting to contrast these results with those obtained for an ATP-dependent phosphofructokinase when available.

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