Chemical Mechanism of 6-Phosphogluconate Dehydrogenase from *Candida utilis* from pH Studies†

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ABSTRACT: The pH dependence of kinetic parameters and dissociation constants for competitive inhibitors was determined in order to obtain information on the chemical mechanism for the 6-phosphogluconate dehydrogenase reaction from *Candida utilis*. A mechanism is proposed in which an active site general base accepts the proton from the 3-hydroxyl concomitant with hydride transfer at C-3; the resulting 3-keto intermediate is decarboxylated to give the enol of ribulose 5-phosphate, followed by tautomerization of the enol to the keto product with the assist of a second enzyme residue acting as a general acid. There is also a requirement for an ionized phosphate of 6-phosphogluconate and ribulose 5-phosphate for optimum binding. The maximum velocity is pH dependent, decreasing at high and low pH giving pK values of 6 and 10, while the V/K for 6-phosphogluconate decreases at low pH with a slope of 2 yielding pK values of 6.4 and 7.6, respectively, and at high pH with a slope of −1 yielding a pK of 8.2. The 6-sulfogluconate pK_i profile decreases at low and high pH giving pK values of 7.1 and 8.5, respectively. The 5-phosphoribonate and 5-phosphoarabonate pK_i profiles show similar behavior giving pK values of 6.5 and 8.8, respectively, for the former and 6.8 and 8.8, respectively, for the latter. The V/K for NADP also decreases at low and high pH giving pKs of 7.5 and 8.1, while the ATP-Ribose pK_i profile decreases at low and high pH giving pKs of 7.2 and 8.0. Studies of 6-phosphogluconate and ribulose 5-phosphate analogs provide information on the regiochemistry of the sugar–phosphate substrate for 6-phosphogluconate dehydrogenase. Basically, *S* stereochemistry at C-4 is required for optimum binding while *R* stereochemistry at C-2 is greatly preferred for substrate activity.

6-Phosphogluconate dehydrogenase from *Candida utilis* has a rapid equilibrium random mechanism with dead-end E:NADP:Ru 5-P[1] and E:NADPH:6-PG complexes (Berdis & Cook, 1993). Topham and Dalziel (1986) have shown that [2-^{18}O]ribulose 5-phosphate is enzymatically converted to 6-phosphogluconate by sheep liver 6-PGDH with the complete retention of the heavy atom. These results indicate that a Schiff-base intermediate is not involved in the reductive carboxylation reaction. Use of the alternate substrate 2-deoxy-6-phosphogluconate for the enzyme from *Candida utilis* (Rippa et al., 1973) results in oxidation of the substrate in the presence of NADP to yield 3-keto-2-deoxy-6-phosphogluconate which is released into solution. As the 3-keto product builds up, it is then decarboxylated by the enzyme to 1-deoxyribulose 5-phosphate. Incubation of the intermediate 3-keto compound with 6-PGDH and NADPH results in either reduction to 2-deoxy-6-phosphogluconate or decarboxylation to 1-deoxyribulose 5-phosphate. The overall chemical mechanism thus appears to be a two-step oxidative decarboxylation reaction in which oxidation precedes decarboxylation. Consistent with this hypothesis is the result of Rendina et al. (1984), who made use of multiple isotope effects in the 6-PGDH reaction. A decrease in the primary ^{13}C isotope effect for decarboxylation when 3-deuterio-6-PG is used in place of the 3-protio compound clearly indicates a stepwise mechanism.

The 6-PGDH catalyzes a reaction very similar to those catalyzed by metal-dependent malic enzyme and isocitrate dehydrogenase, but the former has no apparent metal ion requirement. It is of particular interest to determine the nature of the acid–base catalysis in view of the fact that there is no metal ion or cofactor requirement for this reaction. In this study, the pH dependence of kinetic parameters is used to determine the acid–base chemistry of 6-PGDH from *Candida utilis*.

MATERIALS AND METHODS

Enzyme and Chemicals. 6-PGDH is as previously described (Berdis & Cook, 1993). All substrates and reagents are as previously described (Berdis & Cook, 1993) with the following additions; mannose 6-phosphate, allose, galactose 6-phosphate, xylulose 5-phosphate, arabinose 5-phosphate, adenosine 5′-monophosphate, fructose 6-phosphate, acetyl phosphate, hexokinase, and acetate kinase were from Sigma while Ches and Mes were obtained from Ultrol. All other chemicals and reagents were obtained from commercial sources and were of the highest purity available. Allose 6-phosphate was prepared from allose using hexokinase and ATP with acetyl phosphate and acetate kinase to recycle the nucleotide. The nucleotide and metal were removed by the addition of heat activated acid-washed charcoal and Dowex 50 W (H^+) followed by lypholization. 6-Sulfogluconate, 5-phosphoribonate, 6-phosphogalactonate, 6-phosphoallonate, 5-phosphoarabonate, and

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[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Ches, 2-(cyclohexylamino)ethanesulfonic acid; SDS sodium dodecyl sulfate; BME, 2-hydroxyethylmercaptan; 6-PG, 6-phosphogluconate; 6-PGDH, 6-phosphogluconate dehydrogenase; 6-SG, 6-sulfogluconate; 5-PRib, 5-phosphoribonate; ATP-ribose, 2′-monophosphoadenosine 5′-diphosphoribose; 6-PGal, 6-phosphogalactonate; 6-PMan, 6-phosphomannonate; Xu 5-P, xylulose 5-phosphate; R 5-P, ribose 5-phosphate; F 6-P, fructose 6-phosphate; 6-PAll, 6-phosphoallonate; 5-PAra, 5-phosphoarabonate; NADP, nicotinamide adenine dinucleotide 2′-phosphate (the plus sign is omitted for convenience).

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6-phosphomannonate were synthesized by bromine oxidation as previously described (Berdis & Cook, 1993).

pH Studies. All assays were carried out using a Gilford 250 spectrophotometer equipped with a strip-chart recorder to measure the appearance of NADPH at 340 nm. The temperature was maintained at 25 °C using a circulating water bath to maintain constant temperature of the thermospacers of the cell compartment. Reaction cuvettes were 1 cm in path length and 1 mL in volume. All cuvettes were incubated for at least 10 min in a water bath prior to initiation of reaction.

Initial velocity data were obtained as a function of pH under conditions in which one substrate was varied while the other was maintained at a saturating concentration. In order to be certain that the kinetic mechanism is pH independent and to obtain estimates of the K_m values for 6-PG and NADP, initial velocity patterns in the absence of products were obtained at pH 5.5 and 10. Patterns were obtained by measuring the initial velocity at different levels of NADP and several fixed levels of 6-PG. The pH was maintained using the following buffers at 100 mM concentrations: Mes, 5.5–6.5; Hepes, 6.5–8.5; Ches, 8.5–10. The pH was recorded before and after initial velocity data were recorded.

Inhibition data were obtained for inhibitors competitive with the variable substrate at a saturating concentration of the fixed substrate. Full inhibition patterns for ATP-ribose and 5-PRib were obtained at pH 5.5, 7, and 10 with the substrate varied at several different levels of the inhibitor, including zero. Full inhibition patterns for 6-SG and 5-PAra vs 6-PG were obtained at pH 7 and 9. Once the competitive nature of the inhibition was determined to be pH independent, Dixon experiments were performed in which the variable substrate was fixed at a concentration equal to its K_m and the inhibitor concentration was varied over a range (including zero) that gave inhibition. The measured K_i was then divided by 2 to obtain the true K_i.

Data Processing. Reciprocal initial velocities were plotted against reciprocal substrate concentrations, and all plots and replots were linear. Data were fitted using the appropriate rate equations and computer programs developed by Cleland (1979). Data conforming to a general sequential mechanism were fitted using eq 1. Data obtained for substrate saturation curves for the pH dependence of the kinetic parameters were fitted using eq 2, while data for competitive inhibition were fitted using eq 3. Data for bell-shaped pH profiles in which the limiting slopes were +1 and −1 were fitted using eq 4, while data for $V/K_{6\text{-PG}}$ which decreases with a limiting slope of +2 at low pH and −1 at high pH were fitted using eq 5. Data from Dixon plot analysis were fitted using eq 6.

$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB) \quad (1)$$

$$v = VA/(K_a + A) \quad (2)$$

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

$$\log y = \log [C/(1 + H/K_1 + K_2/H)] \quad (4)$$

$$\log y = \log [C/(1 + H/K_1 + K_2/H + H^2/K_0)] \quad (5)$$

$$y = Ax + B \quad (6)$$

In eqs 1–3, v is the initial velocity, V is the maximum velocity, A, B, and I are reactant and inhibitor concentrations, K_a and K_b are the Michaelis constants for A and B, K_{ia} is the dissociation constant for A, and K_{is} and K_{ii} are slope and intercept inhibition constants respectively. In eqs 4 and 5, y is the observed value of the parameter of interest, e.g., V/K_{NADP} at a given pH, C is the pH-independent value of y, H is the hydrogen ion concentration, K_1 and K_2 are acid dissociation constants for enzyme, substrate, or inhibitor functional groups, and K_0 is the product of K_1 and a second dissociation constant (K_3) which results in a further decrease in $V/K_{6\text{-PG}}$ at low pH. In eq 6, y is $1/v$, x is inhibitor concentration, A is the slope, and B is the ordinate intercept. Values for K_i are obtained by determining the abscissa intercept (B/A) divided by 2. Standard errors were determined by the following:

$$SE = [\text{int/slope}[(SE \times \text{int/int})^2 + (SE \times \text{slope/slope})^2]^{1/2}]/2 \quad (7)$$

where all values are determined by the computer fit.

RESULTS

In order to obtain information concerning the acid–base chemistry catalyzed by 6-PGDH, the pH dependence of kinetic parameters was determined. First, however, it is necessary to ensure that the kinetic mechanism is pH independent. Initial velocity patterns obtained at pH 5.5 and 10 intersect to the left of the ordinate as observed at pH 7 (Berdis & Cook, 1993). Product inhibition is competitive by Ru 5-P vs 6-PG at saturating NADP and by NADPH vs NADP at saturating 6-PG, consistent with a pH-independent rapid equilibrium random kinetic mechanism.

pH Dependence of Kinetic Parameters. The pH dependence of the kinetic parameters for the 6-PGDH reaction in the direction of oxidative decarboxylation are shown in Figure 1. The maximum velocity decreases at high and low pH giving pK values of 6 and 10. The V/K for NADP also decreases at both low and high pH with slopes of 1 and −1, respectively, giving pK values of 7.5 and 8.1. The V/K for 6-PG decreases at high pH with a slope of −1 giving a pK of 8.2, but with a slope of 2 at low pH giving pK values of 6.4 and 7.6. pH-independent values of the kinetic parameters are as follows: V/E_t, 140 s^{-1}; $V/K_{NADP}E_t$, 1.7×10^5 M^{-1} s^{-1}; and $V/K_{6\text{-PG}}E_t$, 1.3×10^4 M^{-1} s^{-1}.

pH Dependence of the K_i for Inhibitory Analogs. Dead-end inhibitors competitive with the variable substrate bind to the same enzyme form as the variable substrate. Since addition of the variable substrate is limiting under conditions in which inhibition is observed, addition of the inhibitor and protonation–deprotonation of enzyme come to equilibrium, and thus dissociation constants are obtained and the pH dependence of the dissociation constants will yield true pK values (Cleland, 1977). The pK_i profile[2] for ATP-ribose is shown in Figure 2A. The value of $1/K_i$ decreases at low and high pH with slopes of 1 and −1, respectively, giving pK values of 7.2 and 8. The pH-independent value for $K_{i\ ATP\text{-ribose}}$ is 8 μM.

The pK_i profile for 6-SG is shown in Figure 2B. The value of $1/K_i$ decreases at low and high pH with a slope of 1 and −1, respectively, giving pK values of 7.1 and 8.5. The pK_i profile for 5-PRib is depicted in Figure 3A. The value of $1/K_i$ decreases at low and high pH with slopes of 1 and −1, respectively, giving pK values of 6.5 and 8.8. The pK_i profile for 5-PAra is depicted in Figure 3B. The value of $1/K_i$ decreases at low and high pH with slopes of 1 and −1, respectively, giving pK values of 6.8 and 8.8. The pH-independent values of $K_{i\ 6\text{-SG}}$, $K_{i\ 5\text{-PRib}}$, and $K_{i\ 5\text{-PAra}}$ are 3.5, 3.5, and 3.2 mM, respectively. Data are summarized in Table I.

Substrate Analogs. Various analogs of 6-PG and Ru 5-P were tested as either substrates or inhibitors for the 6-PGDH

[2] Plotted as $\log 1/K_i$ so that a decrease represents a decrease in affinity.

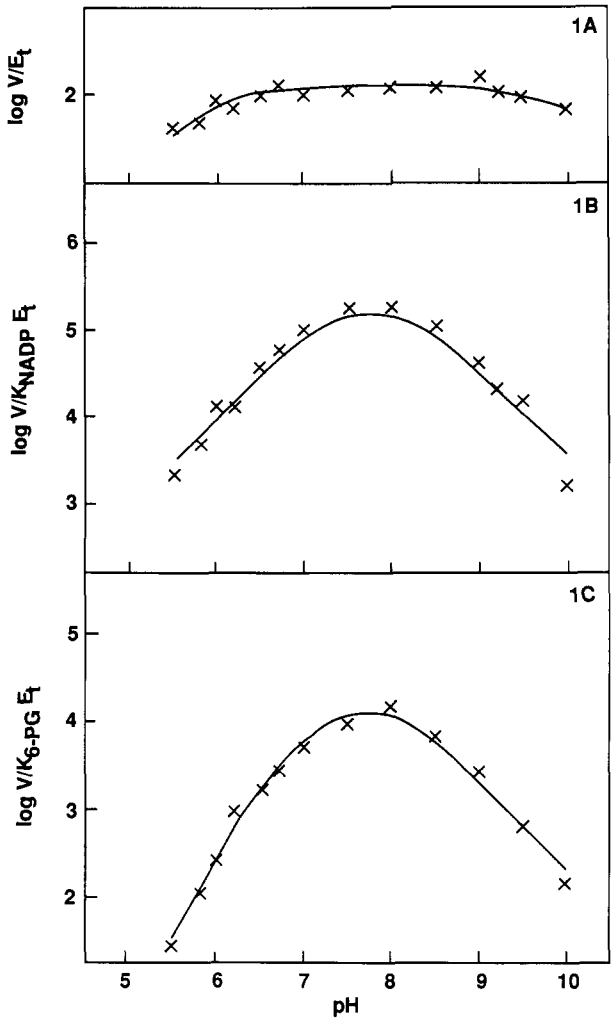


FIGURE 1: pH dependence of kinetic parameters for the 6-PGDH reaction from *Candida utilis*. Data were obtained at 25 °C for V (A), $V/K_{6\text{-PG}}$ (B), and V/K_{NADP} (C). The points shown are the experimentally determined values, while the curves are from a fit of the data using eq 4 for V and V/K_{NADP} and eq 5 for $V/K_{6\text{-PG}}$.

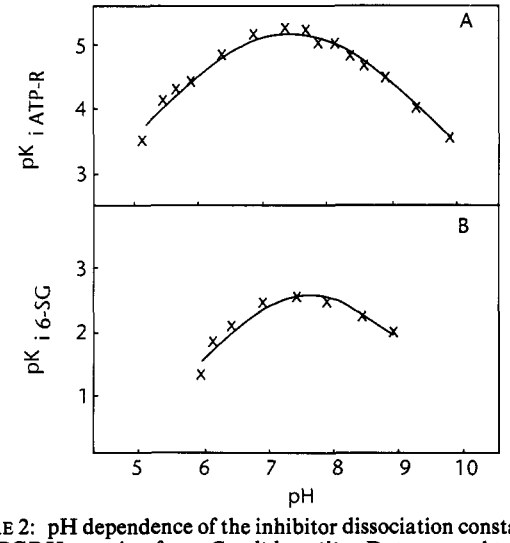


FIGURE 2: pH dependence of the inhibitor dissociation constants for the 6-PGDH reaction from *Candida utilis*. Data were obtained at 25 °C for ATP-ribose (A) and 6-sulfogluconate (B). The points shown are the experimentally determined values, while the curves are from a fit of the data using eq 4 in all cases.

reaction at pH 7 (Figure 4). As indicated above and in the preceding paper in this issue, 6-SG, 5-PAra, and 6-PAll are competitive inhibitors vs 6-PG and 5-PRib is competitive vs

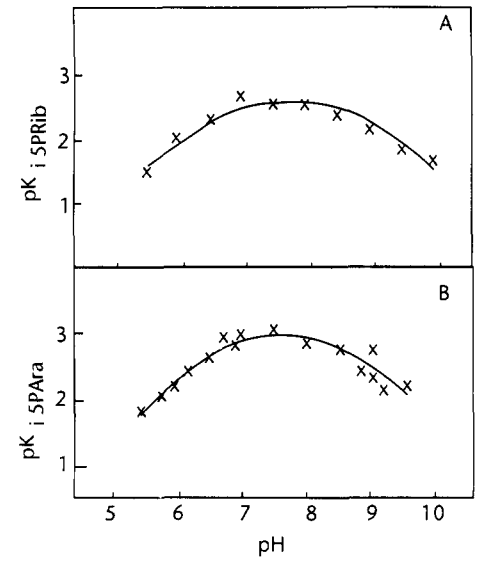


FIGURE 3: pH dependence of the inhibitor dissociation constants for the 6-PGDH reaction from *Candida utilis*. Data were obtained at 25 °C for 5-phosphoribonate (A) and 5-phosphoarabonate (B). The points shown are the experimentally determined values, while the curves are from a fit of the data using eq 4 in all cases.

Table I: pH Dependence of Kinetic Parameters for 6-Phosphogluconate Dehydrogenase from *Candida utilis*

parameter	acid side (pK ± SE)	basic side (pK ± SE)	parameter	acid side (pK ± SE)	basic side (pK ± SE)
V	6.0 ± 0.2	10.0 ± 0.4	$pK_{i\ 6\text{-SG}}$	7.1 ± 0.1	8.5 ± 0.1
$V/K_{6\text{-PG}}$	6.4 ± 0.3	8.2 ± 0.2	$pK_{i\ 5PRib}$	6.5 ± 0.4	8.8 ± 0.4
	7.6 ± 0.2		$pK_{i\ 5PAra}$	6.8 ± 0.3	8.8 ± 0.3
V/K_{NADP}	7.5 ± 0.1	8.1 ± 0.1	$pK_{i\ ATP\text{-}R}$	7.2 ± 0.3	8.0 ± 0.3

6-PG and Ru 5-P. 6-PMan acts as a substrate and has a K_m value of 23 mM while 6-PGal, Xu 5-P, R 5-P, and F 6-P do not act as substrates and do not inhibit the 6-PGDH reaction up to concentrations of 30 mM. There may be a slight activation of the reaction by F 6-P (data not shown). Data are summarized in Table II.

DISCUSSION

Interpretation of pH Dependence of Kinetic Paramters. V/K profiles are obtained at saturating levels of one substrate while varying the other. In the case of the V/K_{NADP} profile (Figure 1B), the predominant enzyme form is E:6-PG while E:NADP is the predominant enzyme form in the case of the $V/K_{6\text{-PG}}$ profile (Figure 1C). The V/K for NADP yields pK values of 7.5 and 8.1. Since only the 2′-phosphate of NADP (6.1; Dawson et al., 1979) is included in the range covered, it is likely that these pKs must reflect enzyme residues. The V/K for 6-PG yields pK values of 6.4, 7.6, and 8.2, respectively. The pK of 6.4 most likely reflects the 6-phosphate group of 6-PG. The 6-phosphate of glucose-6-phosphate has a pK value of approximately 6.1 (Dawson et al., 1979), and there should be little difference between it and 6-PG. The pK values of 7.6 and 8.2 are nearly identical to those obtained in the V/K_{NADP} profile, and these pK values most likely reflect enzyme residues. The pH dependence of the V profile will be discussed below.

Interpretation of the pK_i Profiles. As stated above, pK_i profiles give intrinsic pK values (Cleland, 1977). Since the kinetic mechanism appears to be rapid equilibrium random, true pK values are also expected from the V/K profiles. To test this hypothesis, pK_i profiles were obtained for inhibitors competitive against NADP and 6-PG. The pK_i profile for

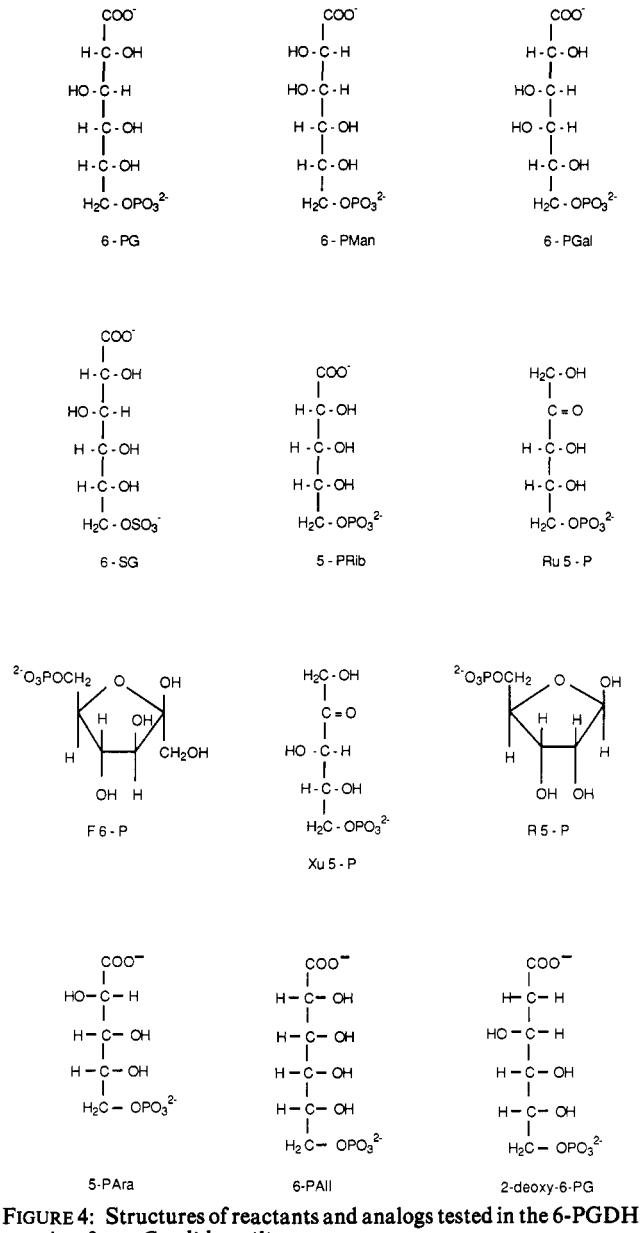


FIGURE 4: Structures of reactants and analogs tested in the 6-PGDH reaction from *Candida utilis*.

ATP-ribose, a competitive inhibitor of NADP, gives p*K*s of 7.2 and 8, respectively. These values are identical within error to those obtained in the V/K profile for NADP, and these are consistent with true p*K* values being observed for the latter.[3] The pK_i profile for 6-SG gives p*K* values of 7.1 and 8.5, lower and higher, respectively, than the p*K*s observed in the $V/K_{6\text{-PG}}$ profile, but again, identical within error. The agreement is not as good in this case, but if substrates were sticky, the p*K*s observed in the V/K profile would be lower and higher than the low and high p*K*s observed in the pK_i profile (Cleland, 1977). This is not the case, and the lack of agreement must be a result of experimental error. The pK_i profile for 5-PRib gives p*K* values of 6.5 and 8.8, respectively. The basic p*K* again agrees with that seen in the $V/K_{6\text{-PG}}$ profile (see above). Thus, the p*K* of the group seen on the acid side of the profile is 7.1–7.6, and that of the group seen on the basic side is 8–8.5. The p*K* values of 6.5 and 6.8 determined using 5-PRib and 5-PAra most likely reflect the p*K* value of the 5-phosphate

[3] Small commitment factors could still be present for this system with values equal to the antilog of the largest error on the p*K* values compared, i.e., from V/K and pK_i profiles.

Table II: Comparison of Substrates and Analogs for 6-Phosphogluconate Dehydrogenase from *Candida utilis*

molecule	effect	K_m or $K_i \pm$ SE
(A) Oxidative Decarboxylation[a]		
6-phosphogluconate	substrate	220 ± 20 μM[c]
6-sulfogluconate	inhibitor	1.6 ± 0.2 mM[c]
5-phosphoribonate	inhibitor	2.5 ± 0.6 mM[c]
6-phosphomannonate	substrate	23 ± 5 mM[d]
6-phosphoallonate	inhibitor	4.1 ± 0.5 mM[c]
5-phosphoarabonate	inhibitor	3.2 ± 0.4 mM[c]
fructose 6-phosphate	slight activation	
ribose 5-phosphate	none	
6-phosphogalactonate	none	
(B) Reductive Carboxylation[b]		
ribulose 5-phosphate	substrate	1 mM[e]
5-phosphoribonate	inhibitor	4.9 ± 1.2 mM[c]
xylulose 5-phosphate	none	
ribose 5-phosphate	none	

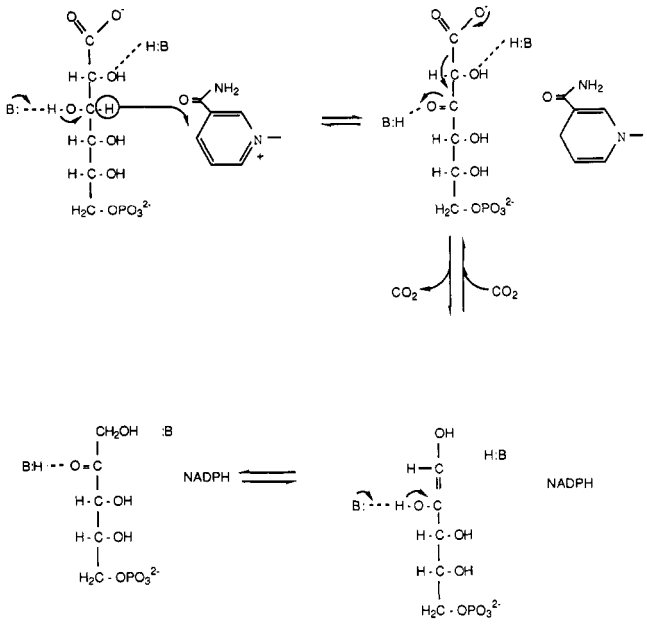
[a] Assays were performed at pH 7 with 1 mM NADP and 1.4 μg of 6-PGDH for substrate determination and 1 mM NADP, 0.3 mM 6-PG, and 1.4 μg of 6-PGDH for Dixon plot analysis unless otherwise stated. Refer to Figure 3 for structures of molecules. [b] Assays were performed at pH 7 with 100 μM NADPH, 20 mM CO_2, and 110 μg of 6-PGDH for substrate determination and 100 μM NADPH, 1 mM Ru 5-P, 20 mM CO_2, and 110 μg of 6-PGDH for Dixon plot analysis unless otherwise stated. [c] Values were determined as described previously (Berdis & Cook, 1993). [d] Value obtained by Lineweaver–Burk analysis. [e] Value obtained from graphical analysis as described previously (Berdis & Cook, 1993).

group and agree within error with the p*K* value of 6.4 observed in the $V/K_{6\text{-PG}}$ profile. The p*K* of 7.1–7.6 is thus not observed in the $pK_{i\ 5\text{-PRib}}$ profile. As seen below, the group with a p*K* of 7.1–7.6 is proposed to be a general base and will be located in the vicinity of the 3-hydroxyl and hydrogen-bonded to it. There is also an acidic group in the vicinity of the 2-hydroxyl that will donate its proton to the enol intermediate after decarboxylation. 5-PRib is one carbon smaller than 6-PG so that if the 5-phosphate anchors 5-PRib in a manner similar to 6-PG, the C-1 and C-2 positions of the former correspond to C-2 and C-3 of the latter. Thus, the general acid likely hydrogen bonds the C-1 carboxyl of 5-PRib while the general base should hydrogen bond the C-2 hydroxyl. The stereochemistry at C-3 of 6-PG is S and that of 5-PRib and 5-PAra at C-2 are *R* and *S*, respectively. Thus, the site can apparently accommodate both stereochemistries at C-2 of the 5-carbon analogs. In addition, since there is no longer any effect of protonating the general base on the affinity of these two analogs, the general base likely can also donate a hydrogen bond to the 1-carbonyl.

Chemical Mechanism. A chemical mechanism for the 6-PGDH reaction consistent with the pH dependence of the V/K profiles is shown in Scheme I. In this mechanism, an enzyme general base (p*K* ~ 7.5) accepts the proton from the 3-hydroxyl group of 6-PG concomitant with hydride transfer to NADP to form a β-keto-acid intermediate (3-keto-6-phosphogluconate) and NADPH. The 3-keto-6-phosphogluconate is then decarboxylated to form the 1,2-enediol intermediate with the general base donating the proton abstracted in the first step to the keto oxygen. The enediol tautomerizes to give Ru 5-P with a proton donated by an enzymatic general acid (p*K* ~ 8) to C-1, while the general base (p*K* ~ 7.5) again abstracts the proton from C-2.

6-PGDH, NAD-malic enzyme, and isocitrate dehydrogenase all catalyze similar reactions, oxidative decarboxylation of a β-hydroxyl acid using NAD(P) as a hydride acceptor yielding a ketone, CO_2, and NAD(P)H as products. 6-PGDH differs from the aforementioned enzymes in that it requires no metal or cofactors for activity. pH studies (Kiick et al.,

Scheme I: Proposed General Acid/General Base Chemical Mechanism of 6-PGDH from *Candida utilis*



1986) and multiple isotope effects (Wiess et al., 1991) for the NAD-malic enzyme from the *Ascaris suum* suggest the requirement for a general base (pK ~ 4.9) and a general acid (pK ~ 9) with the same general function as those proposed for the 6-PGDH reaction. The divalent metal ion Mg^{2+} has been proposed to act as a Lewis acid polarizing the carbonyl of the keto intermediate oxalacetate in the decarboxylation step. This function may be carried out by some other Lewis acid in 6-PGDH, e.g., either the positively charged side chain of an amino acid residue or the positive dipole of an α-helix or the acid–base catalysis is sufficient. In agreement with the proposed mechanism for malic enzyme, keto acids bind with higher affinity with the general base protonated (Park et al., 1986). The position of the general acid in malic enzyme was shown to be near C-3 based on the pH dependence of the inhibition constant for tartronate and oxalate (Kiick et al., 1986). The V profile for malic enzyme gives a pK of 4.8 as a result of slowing down a step thought to be the release of NADH. Recent results of R. Rajapaska in this lab, however, suggest a slow pH-dependent conformational change in the E:NAD complex.

The NADP-isocitrate dehydrogenase also apparently has a similar divalent metal dependent mechanism based on pH (Cook & Cleland, 1981) and intermediate partitioning (Grissom & Cleland, 1988) studies. The V profile shows behavior similar to that of malic enzyme with a decrease only at low pH apparently for the same reason as shown by recent pre-steady-state studies (Kumosinski et al., 1990). The V/K profile (Cook & Cleland, 1981) reflects the third pK of isocitrate (pK ~ 6.7), a general acid (pK ~ 9.5), and an additional pK at low pH (<5). The latter pK is that of the general base perturbed to low pH as a result of the slow dissociation of isocitriate relative to catalysis (Grissom & Cleland, 1988). The true pK for this general base is 5.7. The metal ion again acts as a Lewis acid to polarize the carbonyl of the oxalosuccinate intermediate in the decarboxylation step.

It thus appears that all three of the pyridine nucleotide linked dehydrogenases have a stepwise chemical mechanism with general acid/general base catalysis. The decarboxylation step is catalyzed by a Lewis acid in the malic enzyme and isocitrate dehydrogenase reaction, while there is no evidence for a Lewis acid in the 6-PGDH reaction. However, the malic enzyme and isocitrate dehydrogenase reactions involve an enol intermediate while the 6-PGDH reaction involves an enediol intermediate. Thus, metal ion coordination is required for the former enzymes since it is difficult to form an enolate, while acid–base catalysis is likely sufficient for the more facile formation of an enediolate as with 6-PGDH. Consistent with this suggestion, Rippa et al. (1973) have shown that the decarboxylation of 2-deoxy-6-phosphogluconate is slow enough to allow the majority of the 3-keto intermediate to dissociate from enzyme with very little of the 1-deoxyribulose 5-phosphate being formed.

Interpretation of Substrate Analog Data. Various analogs of 6-PG and Ru 5-P were used in the 6-PGDH reaction to determine their roles as possible substrates or inhibitors based upon structural similarities to the natural substrate. The chemical structures of these molecules are shown in Figure 3. In all cases, it is assumed that the phosphate (or sulfate) anchors the ω-carbon in the same position on enzyme. The analog 6-PMan is an alternative substrate for 6-PGDH in the direction of oxidative decarboxylation, yielding the same product, Ru 5-P, as does the natural substrate 6-PG. The V/K for 6-PMan is 10^5-fold lower than 6-PG, and the V is 10^3-fold lower, giving a K_m that is 100-fold higher than 6-PG. The 100-fold decrease in the affinity likely reflects a loss of interaction at the 2-hydroxyl group. (There may also be a change in the interactions at C-1 and C-3 resulting from steric considerations.) These results are consistent with the data obtained with 2-deoxy-6-phosphogluconate using the enzyme from *C. utilis* (Rippa et al., 1973). The K_m for 2-deoxy-6-phosphogluconate is 10-fold higher than that of 6-PG while the V_{max} is approximately 75 times slower, giving a V/K 750 times slower than that with 6-PG. The absence of the C-2 hydroxyl in the case of 2-deoxy-6-PG as opposed to a change in stereochemistry at C-2 as in the case of 6-PMan provides a better substrate, suggesting that there may be some steric interference with the 6-PMan analog. A change in the steriochemistry at C-4, as seen in the case of 6-PGal, results in a complete loss of binding indicating an absolute requirement for the S configuration at this position. In agreement with this, the equivalent 3 epimer of Ru 5-P, xyulose 5-phosphate (Xu 5-P), also does not bind. A change in the stereochemistry at C-3, as seen in 6-PAll, gives a competitive inhibition with a K_i about 20-fold higher than $K_{6\text{-PG}}$. The site will thus accommodate changes in the stereochemistry at C-2 and C-3 to S and R, but the correct stereochemistry is greatly preferred. The structurally similar 6-sulfogluconate is a competitive inhibitor of 6-PG. The substitution of sulfate for phosphate results in a 7-fold decrease in the affinity compared to 6-PG,[4] likely due to the loss of a negative charge. In addition, however, the binding of 6-SG must be nonproductive since it does not act as a substrate. The 5-carbon analogs, 5-PRib and 5-PAra, are competitive inhibitors of 6-PG. In both cases, there is a 10- and 20-fold decrease in the affinity, likely as a result of the C-1 carboxyl taking the place of C-2 in 6-PG. The lack of inhibition by F 6-P is likely a result of the loss of a combination of effects. The predominant structure of F 6-P is the furanose, and it additionally has no negative charge at C-1. This also likely explains the the lack of inhibition by R 5-P. (The slight activation observed by F 6-P is unexplainable at this time but is currently being investigated.) Although these studies are limited with respect to the number of analogs tested, they do provide information on the requirements for

[4] The K_m for 6-PG is equal to its dissociation constant since the kinetic mechanism is rapid equilibrium random.

productive binding of 6-PG. It can be seen that the phosphate group at the ω-carbon and the *S* stereochemistry at C-4 are required for binding, and *R* stereochemistry at C-2 is greatly preferred for activity.

Interpretation of the pH Dependence of V. Examination of the *V* profile indicates two p*K*s with values of 6 and 10. These p*K*s likely reflect the same groups as those in the V/K_{NADP} profile and the enzyme groups in the $V/K_{6\text{-PG}}$ profile but perturbed to lower (6 compared to 7.5 and 7.6) and higher (10 compared to 8.1 and 8.2) pH, respectively. There are several possibilities for the perturbation in the p*K* to lower and higher pH. (1) A slow step could be present that is not included in the rate expression for V/K such as an isomerization of E, EA, or EB or slow release of the last product. (2) A change to a more hydrophobic environment around the ionizable residues could occur (it could also be the opposite, but it is unlikely that as the site closes into its catalytic conformation, the environment would become more hydrophilic). (3) Hydrogen bonding of the enzyme residues to the reactant 6-PG could occur. The first explanation is unlikely since the kinetic mechanism for 6-PGDH is rapid equilibrium random (Berdis & Cook, 1992a). A change to a more hydrophobic environment is also unlikely given the proposed function of the groups with p*K* values of 7.5 and 8 as general base and general acid, respectively. In order that the p*K* for the general base be perturbed to low pH as a result of the hydrophobic environment, it would have to be a cationic acid such as histidine or lysine, while the general acid would have to be a neutral acid such as aspartate, glutamate, or cysteine. This would require that the group with a p*K* of 8 be the neutral acid, while the group with a p*K* of 7.5 be the cationic acid, an unlikely possibility. The most likely cause of the perturbation is that the catalytic groups hydrogen-bond to the reactant 6-PG. However, the p*K* values are for all intents and purposes identical in the V/K_{NADP} (E:6-PG) and $V/K_{6\text{-PG}}$ (E:NADP) profiles, suggesting that hydrogen bonding, if it occurs, takes place only in the ternary complex once the active site has closed to its catalytic conformation.

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