Multiple Isotope Effects as a Probe of Proton and Hydride Transfer in the 6-Phosphogluconate Dehydrogenase Reaction†

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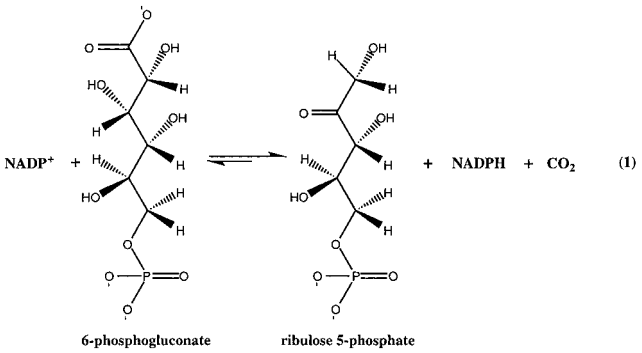
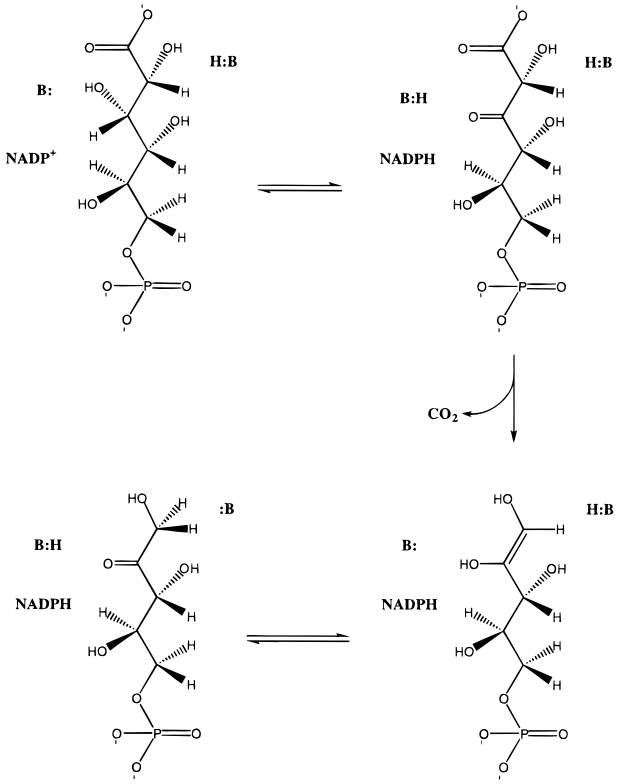
ABSTRACT: Primary solvent deuterium, primary substrate deuterium, multiple solvent deuterium/substrate deuterium, and multiple solvent deuterium/^{13}C isotope effects on V/K_{6PG} have been measured for the *Candida utilis* and sheep liver 6-phosphogluconate dehydrogenases (6PGDH). Proton inventory data suggest the presence of a significant medium effect in a step preceding hydride transfer and the presence of a kinetic solvent deuterium isotope effect on hydride transfer. Multiple isotope effect data confirm the presence of multiple solvent deuterium sensitive steps, likely including a conformational change preceding hydride transfer, hydride transfer, and decarboxylation.

6-Phosphogluconate dehydrogenase (EC 1.1.1.44) catalyzes the reversible oxidative decarboxylation of 6-phosphogluconate to ribulose 5-phosphate and CO_2 with the concomitant generation of NADPH[1] (*1*, *2*).



A rapid equilibrium random kinetic mechanism has been proposed based on steady-state kinetic studies of 6PGDH from the yeast *Candida utilis* (*cu*) and from sheep liver (*sl*) (*3*−*5*). The mechanism of oxidative decarboxylation of 6PG is stepwise with oxidation preceding decarboxylation based on recently measured multiple isotope effect data (*6*). Catalytically, a general base/general acid mechanism has been suggested (Scheme 1) based on the pH dependence of kinetic parameters (*5*, *7*). The general base acts to shuttle the proton between itself and the 3-hydroxyl of 6PG throughout the reaction, ultimately accepting it as ribulose is formed. The general acid presumably plays a role in only the last of three steps, viz. the tautomerization of the enediol of ribulose 5-phosphate to the keto product.

Scheme 1: 6-Phosphogluconate Dehydrogenase Reaction



Recent isotope effect data have shown that the oxidative decarboxylation of 6PG to the 1,2-enediol of ribulose 5-phosphate proceeds via a stepwise mechanism with hydride transfer preceding decarboxylation for both *cu*6PGDH and *sl*6PGDH (*6*). An inverse ^{13}C isotope effect observed with APADP and 6PG-3d suggested a preequilibrium isotope effect on the binding of 6PG preceding hydride transfer.

In the above mechanism, it is possible that the general base may accept the proton from the 3-hydroxyl prior to

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[1] Abbreviations: 6PGDH, 6-phosphogluconate dehydrogenase; *cu*, *Candida utilis*; *sl*, sheep liver; 6PG, 6-phosphogluconate; 6PG-3d, 6-phosphogluconate deuterated at C3; NADP, nicotinamide adenine dinucleotide 2′-phosphate (the plus sign is omitted for convenience); APADP, 3-acetylpyridine adenine dinucleotide 2′-phosphate; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; Mes, 2-(*N*-morpholino)-propanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-N′-*2*-ethanesulfonic acid.

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hydride transfer, facilitating transfer of the hydride to C-4 of the nicotinamide ring of NADP. A preequilibrium proton transfer cannot be ruled out despite the fact that the p*K* of the general base is about 8.5 in free enzyme, while that of the 3-hydroxyl is closer to 16 in solution (*5*, *7*). In the present study, multiple solvent deuterium/substrate deuterium isotope effects are used to probe the relationship between proton and hydride transfer steps. Data also bear on the structure of the transition state for the hydride-transfer step.

METHODS AND MATERIALS.

Enzymes. 6-Phosphogluconate dehydrogenase from *Candida utilis* (*cu*6PGDH) and sheep liver were purchased from Sigma. Recombinant sheep liver 6PGDH was expressed from either the pKK 223-3 or pQE 30 vectors and purified by the method of Chooback et al. (*8*). The *sl*6PGDH was stored at −20 °C in a storage buffer containing 20 mM Hepes, pH 7, 1 mM BME, and 20% glycerol.

Chemicals. 6-Phosphogluconate, NADP, APADP, β-D-glucose, ATP, acetyl phosphate, hexokinase and acetate kinase were from Sigma. β-D-glucose-3d with 95 at. % D was from Isotec, Inc. The buffers, Mes, Hepes, and Ches, were from Research Organics. The D_2O was atom 99.9 at. % D and purchased from Cambridge Isotope Laboratories. Glucose 6-phosphate-3d was prepared according to Hwang et al. (*6*). All other chemicals and reagents were obtained from commercial sources and were of the highest purity available.

Initial Velocity Studies. The rate of the 6PGDH-catalyzed reaction was monitored at 340 nm ($\epsilon_{340} = 6200\ M^{-1}\ cm^{-1}$) with NADP or at 363 nm ($\epsilon_{363} = 9100\ M^{-1}\ cm^{-1}$) with APADP. Primary deuterium isotope effects with 6PG-3d were obtained by direct comparison of initial velocities in H_2O or in D_2O. Values of $^{D}(V/K_{6PG})$ were obtained varying 6PG or 6PG-3d at saturating levels of the dinucleotide substrate. Values for ^{D}V were obtained in all cases at saturating concentrations of reactants. The precision of *V*/*K* isotope effects measured by the direct comparison of initial velocities requires that one accurately know the concentration of the varied reactant, and these were calibrated enzymatically by end-point assay using 6PGDH and measuring the change in absorbance at 340 nm for complete conversion of 6PG to Ru5P and CO_2. Assays contained 100 mM Hepes, pH 8, 0.5 mM NADP, 20 units for 6PGDH, and variable amounts of deuterated or unlabeled substrate. The concentrations from several determinations were in agreement within 1%.

The pH(D) dependence of kinetic parameters was measured according to Price and Cook (*5*). In the case of reactions measured in D_2O, all reactants were prepared and lyophilized twice in D_2O. Buffers were titrated to pD [where pD is equal to the pH meter reading plus 0.4 (*9*)] using KOD.

Proton Inventories. To obtain information on the number and fractionation factor for proton(s) being transferred at the transition state(s), the proton inventory method was utilized (*9*). The technique requires a measurement of *V* and *V*/*K* as a function of the fractional concentration of D_2O in the reaction mixture. Data were then fitted using the form of the Gross−Butler equation given in eq 2 (*9*)

$$^{n}k = {}^{D_2O}k(1 - n + n\phi^{T})Z^{-n} \qquad (2)$$

where ^{n}k is the ratio of the rate constants (*V* or *V*/*K*) measured in different fractional concentrations of D_2O compared to 100% D_2O, ^{D_2O}k is the solvent deuterium isotope effect, i.e., the ratio of the rate constants in H_2O and D_2O, *n* is the fractional concentration of D_2O, ϕ^T is the fractionation factor for protons of import in the transition state, and *Z* represents a medium effect.

^{13}C Isotope Effects. The technique employed for the determination of ^{13}C isotope effects is that of O'Leary (*10*) in which the natural abundance of ^{13}C in the C-1 position of 6PG is used. Both high-conversion (100% reaction) and low-conversion samples were collected. The $^{12}C/^{13}C$ isotope ratios in the CO_2 produced in the reactions were determined for both samples. From these ratios, the relative rates of reaction for ^{12}C vs ^{13}C, and thus the ^{13}C isotope effect, were calculated (*11*). Use of this natural abundance method minimizes the errors caused by atmospheric CO_2 contamination. Reaction mixtures for the low-conversion reactions contained the following in 40 mL: 6.5 mM 6PG, 0.25 mM NADP, and 5 mM oxidized glutathione. Reaction mixtures for high-conversion reactions contained the following in 40 mL: 2 mM 6PG, 0.5 mM NADP, and 10 mM oxidized glutathione. The reaction mixtures were titrated with a saturated NaOH solution to pH 7.4, followed by sparging with CO_2-free nitrogen overnight. Aliquots were withdrawn prior to reaction to determine the initial concentration of 6PG by end-point assay. Reaction was then initiated by the addition of either 2 units of 6PGDH in H_2O solution or 4 units in D_2O solution and 100 units of glutathione reductase for both high- and low-conversion samples.

Low-conversion samples were quenched with 0.2 mL of concentrated sulfuric acid at the appropriate time. The extent of reaction was determined by measuring the remaining 6PG for aliquots of the reaction after quenching. The high-conversion samples were allowed to proceed overnight to ensure completion of the reaction, confirmed by end-point assay prior to addition of 0.2 mL of sulfuric acid and isolation of the CO_2. Isolation and analysis of all samples was carried out on the next day that the CO_2 was generated. Isotopic composition of the CO_2 was determined (in the laboratory of Dr. Michael Engel, Department of Geophysics, University of Oklahoma) on a isotope-ratio mass spectrometer (Finnigan Delta E). All ratios were corrected for ^{17}O according to Craig (*12*).

Data Processing. Reciprocal initial velocities were plotted against reciprocal substrate concentrations and all plots were linear. Data were fitted using the appropriate rate equations and computer programs developed by Cleland (*13*). Data for substrate saturation curves at a fixed concentration of the second substrate were fitted using eq 3. Deuterium kinetic isotope effect data in H_2O or D_2O, obtained by direct comparison of initial velocities, were fitted using eq 4. In eqs 3 and 4, v and *V* are the initial and maximal velocity, K_a is the Michaelis constant for 6PG, *A* is the concentration 6PG, F_i is the fraction of deuterium label in the substrate, and v_i is the isotope effect minus 1 for equal effects on *V* and *V*/*K*. The pH(D) dependence of kinetic parameters were fitted using eq 5 for profiles where the log of the parameter decreased at high and low pH with a unit slope or eq 6 for profiles where the log of the parameter decreased at high pH with a unit slope and at low pH with a slope of 2. In eqs 5 and 6, v is either *V* or *V*/*K*, *C* is the pH(D) independent

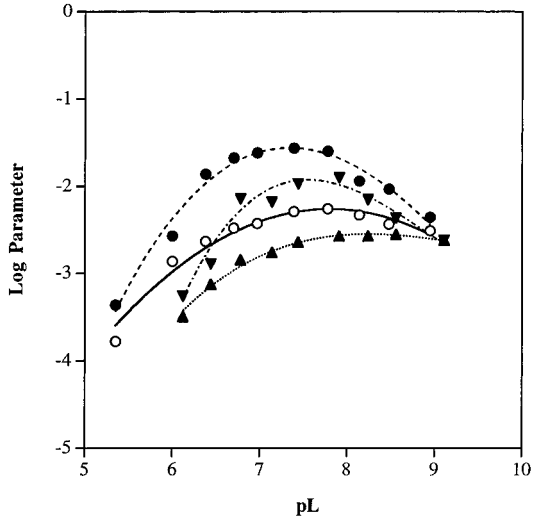

FIGURE 1: pH(D) Dependence of Kinetic Parameters for *cu*6PGDH with NADP as the dinucleotide substrate. Data were obtained for V in H_2O (○) and D_2O (▲) and for V/K_{6PG} in H_2O (●) and D_2O (▼) at 25 °C. Points are experimental, while the curves are theoretical based on a fit using eqs 5 (V) or 6 (V/K).

value of the parameter (it is the ratio of C in H_2O and D_2O that gives the solvent deuterium kinetic isotope effect), L is the hydrogen or deuterium ion concentration, K_1, K_2, and K_o are acid dissociation constants for groups on reactant or enzyme that have a given protonation state for optimum binding and/or catalysis. Calculation of ^{13}C isotope effects were according to eq 7, where f is the fraction of the reaction, R_p and R_∞ are the isotopic ratios of the product CO_2 at partial and complete reaction, respectively. Isotope ratios are given as $\delta^{13}C$, calculated from eq 8, where R_{smp} and R_{std} are $^{12}C/^{13}C$ isotopic ratios for sample and standard, respectively. The standard for CO_2 was calibrated from Pee Dee Belemnite (*12*) with $^{12}C/^{13}C$ of 0.011 237 2.

$$v = VA/(K_a + A) \quad (3)$$

$$v = VA/[(K_a + A(1 + F_i v_i)] \quad (4)$$

$$\log v = \log(C/(1 + L/K_1 + K_2/L) \quad (5)$$

$$\log v = \log(C/(1 + L/K_1 + L^2/K_1K_o + K_2/L) \quad (6)$$

$$^{13}(V/K) = \log(1 - f)/\log[1 - f(R_p/R_\infty)] \quad (7)$$

$$\delta^{13}C = (R_{smp}/R_{std} - 1) \times 10^3 \quad (8)$$

RESULTS

pH(D) Dependence of Kinetic Parameters. The pH(D) dependence of kinetic parameters was measured for both *cu*6PGDH and *sl*6PGDH using NADP and APADP as dinucleotide substrates, over the pH(D) range 5−9 to determine whether a solvent deuterium kinetic isotope effect is observed on kinetic parameters. An example of pH(D) profiles obtained is shown in Figure 1. Data obtained from the pH(D) dependence of kinetic parameters are summarized in Table 1.

There are two types of isotope effects reflected in pH(D) profiles such as those pictured in Figure 1. Each of the acid dissociable functional groups important for binding and/or

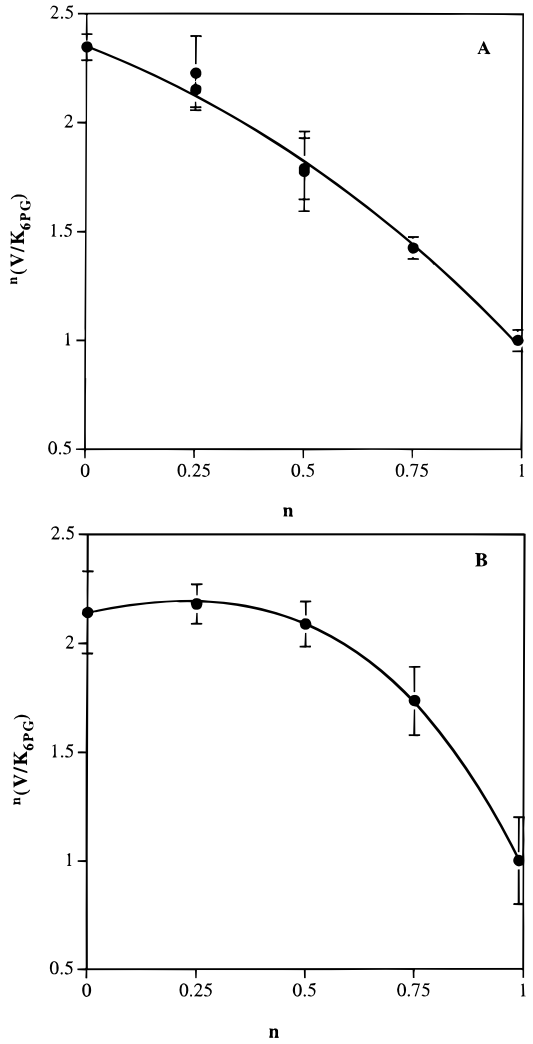

FIGURE 2: Proton Inventories for 6PGDH. Solvent deuterium dependence of $^n(V/K_{6PG})$ (ratio of V/K_{6PG} at n fraction of D_2O to that at 100% D_2O) on the fraction of deuterium (n) in solvent. (A) Data obtained with NADP and 6PG with *cu*6PGDH. A fit of data gives $^{D_2O}k = 2.39 \pm 0.05$, $Z = 0.72 \pm 0.08$ and $\phi^T = 0.29 \pm 0.04$. (B) Data obtained with APADP and 6PG-3D with *sl*6PGDH. A fit of data gives $^{D_2O}k = 2.13 \pm 0.09$, $Z = 0.394 \pm 0.005$ and $\phi^T = 0.158 \pm 0.003$. Points are experimental, while the solid curve is theoretical based on a fit using eq 2.

catalysis will exhibit an equilibrium solvent deuterium isotope effect reflected as an increase in its pK in D_2O compared to H_2O. Assuming the dissociable group is an oxygen or nitrogen, as expected in the case of 6PGDH, an isotope effect of about 3 is predicted, i.e., an increase in the pK by 0.4−0.6 in D_2O (*9*). Although the pH(D) profiles were meant as a screen for the presence of the kinetic isotope effect, one can see, within error, an increase in the pK values of the groups titrated in D_2O compared to H_2O (Table 1). The second type of effect is a kinetic solvent deuterium isotope effect, obtained as the ratio of the pH(D) independent values of the kinetic parameters. The kinetic solvent deuterium isotope effects are summarized in the last column in Table 1, and are significant in every case where an estimate was possible.

Proton Inventory Studies. Information on protons important to the overall reaction can be obtained from the proton inventory method (*9*). In all cases, for both enzymes, the proton inventories can be described as dome-shaped or

Table 1: pH(D) Dependence of Kinetic Parameters for *cu*6PGDH and *sl*6PGDH

enzyme	nucleotide	solvent	parameter	pK_{a1} ± SE	pK_{a2} ± SE	pK_b ± SE	C ± SE	isotope effect
*cu*6PGDH	NADP	H_2O	V		6.7 ± 0.3	8.8 ± 0.5	0.006 ± 0.001	
		D_2O			7.0 ± 0.1	9.7 ± 0.7	0.003 ± 0.0002	2.0 ± 0.4
		H_2O	V/K_{6PG}	6.0 ± 0.2	6.7 ± 0.1	8.0 ± 0.1	0.04 ± 0.006	
		D_2O		ND[a]	7.8 ± 0.3	8.4 ± 0.1	0.013 ± 0.002	3 ± 1
	APADP	H_2O	V		7.0 ± 0.1	9.1 ± 0.2	0.010 ± 0.001	
		D_2O			7.4 ± 0.1	9.6 ± 0.2	0.0068 ± 0.0006	1.67 ± 0.13
		H_2O	V/K_{6PG}	6.1 ± 0.1	6.5 ± 0.1	8.3 ± 0.1	0.076 ± 0.007	
		D_2O			6.8 ± 0.2			ND
*sl*6PGDH	NADP	H_2O	V		6.7 ± 0.1	9.1 ± 0.3	8.8 ± 1.4	
		D_2O			7.3 ± 0.1	ND	3.7 ± 0.2	2.4 ± 0.4
		H_2O	V/K_{6PG}	6.1 ± 0.3	7.1 ± 0.2	7.4 ± 0.2	0.6 ± 0.2	
		D_2O		6.0 ± 1.0	7.5 ± 0.2	8.3 ± 0.2	0.12 ± 0.01	5.0 ± 0.5
	APADP	H_2O	V		6.5 ± 0.1	ND	13 ± 1	
		D_2O			7.1 ± 0.1	ND	8.4 ± 0.8	1.55 ± 0.19
		H_2O	V/K_{6PG}	ND	7.0 ± 0.3	7.6 ± 0.3	540 ± 50	
		D_2O		ND	7.0 ± 0.3	8.1 ± 0.2	150 ± 60	3.6 ± 1.5

[a] ND, not defined.

Table 2: Multiple Solvent Deuterium/Substrate Deuterium Isotope Effects for *cu*6PGDH and *sl*6PGDH[a]

enzyme	nucleotide	$^{D}(V/K_{6PG})_{H_2O}$	$^{D}(V/K_{6PG})_{D_2O}$	$^{D_2O}(V/K_{6PG})_{H}$	$^{D_2O}(V/K_{6PG})_{D}$
*cu*6PGDH	NADP	1.82 ± 0.10	1.62 ± 0.05	2.17 ± 0.08	1.93 ± 0.06
	APADP	3.12 ± 0.12	2.67 ± 0.11	1.42 ± 0.05	1.22 ± 0.04
*sl*6PGDH	NADP	1.61 ± 0.04	1.69 ± 0.03	2.18 ± 0.06	2.28 ± 0.05
	APADP	3.08 ± 0.05	3.00 ± 0.09	1.74 ± 0.02	1.70 ± 0.05
	APADP	3.09 ± 0.09	3.00 ± 0.10	1.66 ± 0.03	1.61 ± 0.06

[a] Assays were carried out at 25 °C and saturating concentrations ($20K_m$) of the nucleotide substrate.

bulging upward. Examples of proton inventories on V/K obtained at the maxima in the pH(D) profiles are shown in Figure 2 with NADP and 6PG with the *cu*6PGDH and with APADP and 6PG-3D with the *sl*6PGDH. In the case of Figure 2A, data are qualitatively consistent with changes in fractionation factor in serial transition states. The dramatic upward bulge in Figure 2B is qualitatively consistent with a medium effect and a change in the fractionation factor in the transition state (*9*). A fit of the Gross−Butler equation (eq 2) to the data in Figure 2A gives $^{D_2O}k = 2.39 \pm 0.05$, $Z = 0.72 \pm 0.08$, and $\phi^T = 0.29 \pm 0.04$ for the reaction catalyzed by *cu*6PGDH with NADP as a nucleotide. Data in Figure 2B gives $^{D_2O}k = 2.13 \pm 0.09$, $Z = 0.394 \pm 0.005$, and $\phi^T = 0.158 \pm 0.003$ for the reaction catalyzed by *sl*6PGDH with APADP as a nucleotide when 6PG-3d as a substrate. The origin of the medium effect will be discussed below.

Multiple Solvent Deuterium/Substrate Deuterium Isotope Effects. Multiple isotope effects allows one to determine whether two isotope effects reflect the same or different steps. A proton must be accepted from the 3-hydroxyl before or concomitant with the transfer of a hydride from C-3 to C-4 of the nicotinamide ring of NADP. Thus, solvent deuterium isotope effects were measured at the maximum in the pH-(D) profiles with 6PG and 6PG-3D to determine whether proton and hydride transfer are concerted in the oxidation of 6PG to the 3-keto intermediate. Multiple isotope effects on V/K_{6PG} are summarized in Table 2 for *cu*6PGDH and *sl*6PGDH.

Multiple Solvent Deuterium/^{13}C Isotope Effects. The decarboxylation of the 3-keto intermediate takes place in a step separate and following the hydride transfer step (*6*). To further define the interrelationship between chemical steps, primary ^{13}C isotope effects, reflecting decarboxylation at C-1 of 6PG, were measured in H_2O and D_2O for *sl*6PGDH and are summarized in Table 3. Note that there is a slight decrease in the ^{13}C isotope effect when measured in D_2O.

Table 3: Multiple ^{13}C/Solvent Deuterium Isotope Effects for *sl*6PGDH[a]

solvent	δ ^{13}C (low conversion)	f	$^{13}(V/K)$
H_2O	−42.847	0.266	1.0228
	−42.828	0.249	1.0225
	−42.914	0.233	1.0223
			1.0225 ± 0.0002[b]
D_2O	−30.139	0.315	1.0190
	−29.960	0.270	1.0182
	−30.311	0.254	1.0184
			1.0185 ± 0.0004[b]

[a] The 100% conversion samples yielded the following δ ^{13}C values for 6-PG in H_2O: −24.299, −24.301, and −24.166 with an avg of −24.255 ± 0.077. The 100% conversion samples yielded the following δ ^{13}C values for 6-PG in D_2O: −14.971, −14.983, and −14.838 with an avg of −14.931 ± 0.081. All experiments were carried out with NADP as the dinucleotide substrate. [b] Average values.

DISCUSSION

Interpretation of Proton Inventory Studies. Since saturation curves yield values for V and V/K_{6PG}, proton inventories are potentially obtained for both parameters. However, because of the difference in pH(D) dependence of V and V/K, only the effects on V/K are without a significant contribution from equilibrium solvent isotope effects on the acid dissociation constants for the general acid and base catalysts. A qualitative interpretation of the proton inventory studies is appropriate because of the significant error on each of the individually determined rate constants.

All of the proton inventories on V/K are dome shaped, most like that pictured in Figure 2A, suggestive of contribu-

tion from proton transfer in two or more serial transition states (*9*). However, when hydride transfer is completely rate limiting (*6*), a pronounced dome-shaped proton inventory such as that shown in Figure 2B is observed. The latter shape is indicative of a medium effect and a transition-state effect. Although the origin of the medium effect is uncertain, the enhancement of the effect when hydride transfer becomes rate limiting suggests that it precedes the hydride-transfer step and may reflect a conformational change known to affect the fractionation factor of the 1-carboxylate of 6PG (*6*). The effect is pronounced as suggested by the shape, and indeed a value of 0.39 is estimated from the data in Figure 2B using the form of the Gross−Butler given in the Materials and Methods. Consistent with the assignment of a conformational change or some steps prior to hydride transfer as the origin of a significant solvent sensitive step are the multiple isotope effect data discussed below. The origin of the transition-state effect is almost certainly the proton transferred from the C3 hydroxyl to a general base as the hydride is being transferred.

Multiple Isotope Effect Data. Multiple isotope effects potentially allow one to define the interrelationship between two isotope sensitive steps (*14*). Two kinds of multiple isotope effects are used in these studies, solvent deuterium/substrate deuterium and solvent deuterium/^{13}C. Data in Table 2 are very informative concerning the 6PGDH reaction. For the *cu*6PGDH, there is a slight decrease in the substrate deuterium isotope effect [$^{D}(V/K_{6PG})$] when obtained in D_2O compared to that in H_2O, or when the solvent deuterium isotope effect [$^{D_2O}(V/K_{6PG})$] is obtained with deuterium-compared to protium-labeled 6PG, in all cases. At face value, these data suggest that a solvent deuterium-sensitive step occurs in a step separate from hydride transfer. One would suspect, then, that proton transfer and hydride transfer do not occur in the same transition state. Similar data obtained for *sl*6PGDH, however, belie the previous statement. There is either no change in the isotope effect or a slight increase upon measuring [$^{D}(V/K_{6PG})$] in D_2O compared to that in H_2O, or measuring [$^{D_2O}(V/K_{6PG})$] with deuterium-compared to protium-labeled 6PG. These data suggest concerted proton and hydride transfer, and indeed in the rate-limiting step (*14*). Different results are thus apparently obtained despite the proposal of very similar mechanisms for the two enzymes (see above).

As a test of the above-mentioned stepwise and concerted proton and hydride transfers catalyzed by the two enzymes, the primary ^{13}C isotope effect was measured in D_2O and compared to the value measured in H_2O for *sl*6PGDH. If hydride transfer was solely rate limiting, as suggested by the multiple isotope effect data in Table 2, the primary ^{13}C isotope effect should be unity, but it is finite with a value of over 2%, a significant effect. In addition, the effect decreases only slightly in D_2O to 1.85%. These data are also in agreement with a solvent deuterium isotope effect on the decarboxylation step partially attenuating the effect of solvent deuterium on other steps.

How can the data for the two enzymes be reconciled? Neither of the two enzymes studied have a single rate-limiting step, and in fact, rate limitation by some step prior to hydride transfer, hydride transfer itself, and decarboxylation of the keto intermediate has been demonstrated (*6*). Thus, the *apparent* rate-limiting concerted proton and hydride transfer for *sl*6PGDH, and the *apparent* stepwise mechanism for the *cu*6PGDH must result from compensatory effects as suggested above. For *sl*6PGDH, a decrease in the primary ^{13}C isotope effect in D_2O compared to that measured in H_2O suggests that transition-state contributions to the medium effect are smaller for the decarboxylation step, while the other steps contribute equally. The reaction catalyzed by *cu*6PGDH, on the other hand, suggests more variability in contributions from the three rate-determining transition states to the medium effect. Thus, a larger transition state contribution from the conformational change, decarboxylation, or both will result in an attenuation of the solvent deuterium isotope effect on the hydride-transfer step.

REFERENCES

1. Pontremoli S., de Flora, A., Grazi, E., Mangiarotti, G., Bonsignore, A., and Horecker, B. L. (1961) *J. Biol. Chem. 236*, 2975−2981.
2. Silverberg, M., and Dalziel, K. E.. (1983) *Eur. J. Biochem. 38*, 229−238.
3. Rendina, A. R., Hermes, J. D., and Cleland, W. W. (1984) *Biochemistry 23*, 6257−6262.
4. Berdis, A. J., and Cook, P. F. (1993) *Biochemistry 32*, 2036−2040.
5. Price, N. E., and Cook, P. F. (1996) *Arch. Biochem. Biophys. 336*, 215−223.
6. Hwang, C.-C., Berdis, A. J., Karsten, W. E., Cleland, W. W., and Cook, P. F. (1998) *Biochemistry 37*, 12596−12602.
7. Berdis, A. J., and Cook, P. F. (1993) *Biochemistry 32*, 2041−2046.
8. Chooback, L., Price, N. E., Karsten, W. E., Nelson, J., Sundstrom, P. R., and Cook, P. F. (1998) *Protein Expression Purif. 13*, 251−258.
9. Quinn, D. M., and Sutton, L. D. (1991) in *Enzyme Mechanism from Isotope Effects* (Cook, P. F., Ed.) pp 73−126, CRC Press, Boca Raton, FL.
10. O'Leary, M. H. (1980) *Methods Enzymol. 64*, 83−104.
11. Hermes, J. D.,Roeske, C. A., O'Leary, M. H., and Cleland, W. W. (1982) *Biochemistry 21*, 5106−5114.
12. Craig, N. (1957) *Geochim. Cosmochim. Acta 12*, 133−140.
13. Cleland, W. W. (1979) *Methods Enzymol. 63*, 103−108.
14. Cleland, W. W. (1991) in *Enzyme Mechanism from Isotope Effects* (Cook, P. F., Ed.) pp 247−265, CRC Press, Boca Raton, FL.

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