# Oxidative Decarboxylation of 6-Phosphogluconate by 6-Phosphogluconate Dehydrogenase Proceeds by a Stepwise Mechanism with NADP and APADP as Oxidants†

Chi-Ching Hwang,‡ Anthony J. Berdis,‡,§ William E. Karsten,‡ W. W. Cleland,|| and Paul F. Cook*,‡

*Department of Chemistry and Biochemistry, University of Oklahoma, 620 Parrington Oval, Norman, Oklahoma 73019, and the Institute for Enzyme Research, University of Wisconsin—Madison, Madison, Wisconsin 53705*

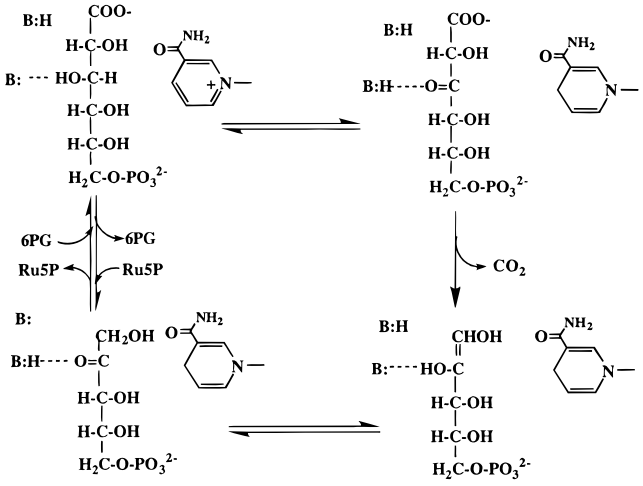
*Received March 17, 1998; Revised Manuscript Received July 8, 1998*

ABSTRACT: Primary kinetic deuterium, $^{13}C$, and multiple deuterium/$^{13}C$-isotope effects on $V/K_{6PG}$ have been measured for the *Candida utilis* (*cu*) and sheep liver (*sl*) 6-phosphogluconate dehydrogenases (6PGDH). With NADP as the dinucleotide substrate, the following values of $^{D}(V/K_{6PG})$, $^{13}(V/K_{6PG})_H$, and $^{13}(V/K_{6PG})_D$ were measured at pH 8 for *cu*6PGDH (*sl*6PGDH): 1.57 ± 0.08 (1.87 ± 0.10), 1.0209 ± 0.0005 (1.0059 ± 0.000 10), 1.0158 ± 0.0001 (1.0036 ± 0.0008). With APADP as the dinucleotide substrate, values for the above isotope effects at pH 8 are as follows: 2.98 ± 0.08 (2.47 ± 0.06), 1.0106 ± 0.0002 (1.0086 ± 0.000 09), and 0.9934 ± 0.0003 (0.9950 ± 0.0003). Results indicate the oxidative decarboxylation of 6PG to the 1,2-enediol of ribulose 5-phosphate proceeds via a stepwise mechanism with hydride transfer preceding decarboxylation in all cases. The inverse $^{13}C$-isotope effect observed with APADP and 6PG-3d may reflect a preequlibrium isotope effect on the binding of 6PG preceding hydride transfer. Deuterium-isotope effects on $V$, $V/K_{NADP}$, and $V/K_{6PG}$ are identical at all pHs and for both enzymes. The primary deuterium-isotope effect on $V/K_{6PG}$ for both enzymes is constant at pH values below the p$K$ in the pH profile for $V/K_{6PG}$, and decreases as the pH increases. Data suggest the development of rate limitation by a step or steps other than the hydride-transfer step as the pH is increased.

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*). Steady-state kinetic studies of 6PGDH from the yeast *Candida utilis* and from sheep liver suggest a rapid equilibrium random kinetic mechanism with dead-end E:NADP:ribulose 5-phosphate and E:NADPH:6-phosphogluconate complexes (*3*−*5*).

An acid−base chemical mechanism for 6PGDH (Scheme 1) has been proposed based on the pH dependence of kinetic parameters and dissociation constants of competitive inhibitors (*5*, *6*). The mechanism requires an active-site general base to accept a proton from the 3-hydroxyl concomitant with transfer of a hydride from $C_3$ of 6PG to $C_4$ of the nicotinamide ring of NADP. 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.

Scheme 1




The reaction catalyzed by 6PDGH is similar in nature to those catalyzed by isocitrate dehydrogenase (*7*, *8*), malic enzyme (*9*, *10*), tartrate dehydrogenase (*11*), and isopropylmalate dehydrogenase (*12*, *13*) in that all yield a ketone, $CO_2$, and NAD(P)H as products. However, unlike other enzymes in the class, 6PDGH does not require a divalent metal ion

† This work was supported by grants from the National Institutes of Health to P.F.C. (GM 50872) and W.W.C. (GM 18938).

* Author to whom correspondence should be addressed.
‡ University of Oklahoma.
§ Present address: Department of Chemistry, Pennsylvania State University, 152 Davey Laboratory, University Park, PA 16802-6300.
|| University of Wisconsin—Madison.

[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; thio-NADP, 3-thionicotinamide adenine 2′-phosphate; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; Mes, 2-(*N*-morpholino)propanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

S0006-2960(98)00611-4 CCC: $15.00 
Published on Web 08/20/1998

for activity (*1*, *14*, *15*). The oxidative decarboxylation reaction catalyzed by all of the above is stepwise with hydride transfer preceding decarboxylation with NAD(P) as the dinucleotide substrate (*3*, *16*−*18*). The mechanism for the oxidative decarboxylation of malate by malic enzyme changes to a concerted reaction with APAD(P) as the dinucleotide substrate (*19*−*21*). Data in this case suggest an asynchronous transition state with decarboxylation ($C_3$−$C_4$ bond cleavage) less advanced than oxidation ($C_2$−H cleavage). The change in mechanism presumably results from the combination of a higher energy barrier for hydride transfer and a lower overall free energy level for products giving a collapse of the energy well for the oxalacetate intermediate (*20*, *21*).

As stated above, multiple isotope effects on the reaction catalyzed by 6PGDH from *C. utilis* (*3*) suggest a stepwise mechanism for the oxidative decarboxylation of 6PG, but data were not well conditioned, and did not adhere to the equalities derived by Hermes et al. (*16*). In the present study, the technique of multiple isotope effects has been applied to the reaction catalyzed by 6PGDH from *C. utilis* and sheep liver to better understand its mechanism and test whether a change in mechanism is observed when APADP is used as the dinucleotide substrate. Data suggest a stepwise oxidative decarboxylation of 6PG irrespective of the dinucleotide substrate.

## METHODS AND MATERIALS

*Enzyme.* Cytoplasmic 6-phosphogluconate dehydrogenase from *Candida utilis* (*cu*6PGDH) was purchased from Sigma. Recombinant sheep liver 6PGDH was expressed and purified by the method of Chooback et al. (*22*). The *sl*6PGDH was stored at −20 °C in a storage buffer containing 20 mM Hepes, pH 7, 1 mM $\beta$-mercaptoethanol, and 20% glycerol.

*Chemicals*. 6-Phosphogluconate, NADP, APADP, $\beta$-D-glucose, ATP, acetyl phosphate, hexokinase, and acetate kinase were from Sigma. $\beta$-D-Glucose-3d with 95 at. % D was from Isotec, Inc. The buffers, Mes, Hepes, and Ches, were from Research Organics. All other chemicals and reagents were obtained from commercial sources and were of the highest purity available. Glucose 6-phosphate-3d was prepared from $\beta$-D-glucose-3d using hexokinase and ATP with acetyl phosphate and acetate kinase present in order to recycle the nucleotide (*5*). 6-Phosphogluconate-3d was prepared from glucose 6-phosphate-3d according to Horecker (*23*) with the following modifications. Glucose 6-phosphate-3d (G6P-3d; 6.4 mmol) was dissolved in 20 mL of water and 0.4 mL of concentrated HCl, followed by the addition of 8 mL of 0.57 M $Na_2SO_4$. The pH was adjusted to 5.4 by the addition of 4 N NaOH and an excess of $Br_2$ (0.8 mL, 15.5 mmol) was added to initiate the reaction. The pH was maintained between 4.8 and 6.2 for at least 20 min by addition of 4 N NaOH. The remaining $Br_2$ was then removed by sparging with $N_2$ for at least 1 h. The mixture was then applied to a Bio-Rad AG1x8 (200−400 mesh) anion-exchange column in the formate form, and 6PG-3d was eluted using an 800 mL linear gradient (0 to 6 N) of formic acid, followed by washing with 400 mL of 6 N formic acid. The presence of 6PG-3d was determined using the 6PGDH assay (see below) at pH 8. Fractions containing 6PG-3d were pooled and concentrated to give 4.6 mmol, an overall yield of 72%.

*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. Values of $^{D}(V/K_{Nuc})$ were measured by varying NADP (or APADP) at saturating levels of 6PG or 6PG-3d, while $^{D}(V/K_{6PG})$ were obtained varying 6PG or 6PG-3d at saturating levels of the dinucleotide. 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. Assays contained 100 mM Hepes, pH 8, 0.5 mM NADP, 20 units 6PGDH, and variable amounts of deuterated and unlabeled substrate. The concentrations from several determinations were in agreement within 1%.

*$^{13}$C-Isotope Effects.* The technique employed for the determination of $^{13}$C-isotope effects is that of O'Leary (*24*) 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 (*16*). 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 either 40 or 20 mL: 6.5 mM 6PG or 6PG-3d, 0.25 mM NADP (or APADP), and 5 mM oxidized glutathione. Reaction mixtures for high-conversion reactions contained the following in 20 mL: 2 mM 6PG or 6PG-3d, 0.5 mM NADP (or APADP), 10 mM oxidized glutathione. The reaction mixtures were titrated with a saturated NaOH solution to pH 8, following by sparging with $CO_2$-free nitrogen overnight. Aliquots were withdrawn prior to reaction to determine the initial concentration of 6PG or 6PG-3d by end-point assay. Reaction was then initiated by the addition of 8.3 units 6PGDH 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 0.1 mL and sulfuric acid and isolation of the $CO_2$. Isolation and analysis of all samples was carried out on the same day the $CO_2$ was generated. Isotopic composition of the $CO_2$ was determined on a isotope-ratio mass spectrometer (Finnigan Delta E). All ratios were corrected for $^{17}$O according to Craig (*25*).

*Data Processing.* Reciprocal initial velocities were plotted against reciprocal substrate concentrations, and all plots were linear unless otherwise stated. Data were fitted using the appropriate rate equations and computer programs developed by Cleland (*26*). Data for substrate saturation curves at a fixed concentration of the second substrate were fitted using

eq 1. Deuterium kinetic isotope effect data, obtained by direct comparison of initial velocities, and with equal or independent effects on $V$ and $V/K$ were fitted using eq 2.

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

$$v = VA/[(K_a + A)(1 + F_i v_i)] \quad (2)$$

In eqs 1 and 2, $v$ and $V$ are the initial and maximal velocity, $K_a$ is the Michaelis constant for $A$, $A$ is the concentration of the variable substrate. In eqs 2 and 3, $F_i$ is the fraction of deuterium label in the substrate and $v_i$ is the isotope effect minus 1 for equal effects on $V$ and $V/K$, and $E_{V/K}$ and $E_V$ are the isotope effects minus 1 for the respective parameters.

$$v = VA/[(K_a(1 + F_i E_{V/K}) + A(1 + F_i E_V)] \quad (3)$$

Calculation of $^{13}$C-isotope effects were according to eq 4, where $f$ is the fraction of the reaction, $R_p$ and $R_\infty$ are the isotopic ratios of the product $CO_2$ at partial and complete reaction, respectively.

$$^{13}(V/K) = \log(1 - f)/\log[1 - f(R_p/R_\infty)] \quad (4)$$

Isotope ratios are given as $\delta^{13}$C, calculated from eq 5, where $R_{smp}$ and $R_{std}$ are $^{13}$C/$^{12}$C isotopic ratios for sample and standard, respectively.

$$\delta^{13}C = (R_{smp}/R_{std} - 1) \times 10^3 \quad (5)$$

The standard for $CO_2$ was calibrated from Pee Dee Belemnite (*25*) with $^{13}$C/$^{12}$C of 0.011 237 2.

*Theory*. The kinetic mechanism of 6PGDH is rapid equilibrium random (*3*−*5*). However, $^D(V/K_{6PG})$, $^{13}(V/K_{6PG})_H$, and $^{13}(V/K_{6PG})_D$ are obtained at a saturating concentration of NADP (or APADP), and under these conditions, the following mechanistic scheme applies.

$$EA \underset{k_4}{\overset{k_3B}{\rightleftharpoons}} EAB \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E'AB \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E'XR \xrightarrow{k_9} E'QR \xrightarrow{k_{11}} E \quad (6)$$

where A, B, R, Q, and X represent NADP, 6PG, NADPH, ribulose 5′-phosphate, and 3-keto-6PG, respectively. The rate constants $k_3$ and $k_4$ are for binding of 6PG and dissociation of E:6PG, $k_5$ and $k_6$ are for an isomerization of the E:6PG complex, $k_7$ and $k_8$ are for the oxidation of 6PG and reduction of the 3-keto intermediate, respectively, $k_9$ is the rate constant for the decarboxylation of the 3-keto intermediate, and $k_{11}$ is a net rate constant for dissociation of products, which is rapid. The rate processes depicted by $k_7$ and $k_8$ will reflect a kinetic deuterium-isotope effect given by $^Dk_7$ and $^Dk_8$, respectively, and related by the equilibrium isotope effect, $^DK_{eq} = {}^Dk_7/{}^Dk_8$, while $k_9$ will reflect a $^{13}$C-isotope effect given by $^{13}k_9$. In addition, $^{13}K_{i,6PG}$ represents an equilibrium isotope effect on $K_{i\ 6PG}$.

For a rapid equilibrium mechanism, the rate equation for the deuterium-isotope effect, tritium-isotope effect[2] on $V/K_{6PG}$ and those for the $^{13}$C-isotope effect on $V/K_{6PG}$ with protium- and deuterium-labeled 6PG are given in eqs 7−10

$$^D(V/K_b) = [^Dk_7 + k_7/k_6 + (k_8/k_9)(^DK_{eq})](1 + k_7/k_6 + k_8/k_9) \quad (7)$$

$$^T(V/K_b) = [^Dk_7{}^{1.44} + k_7/k_6 + (k_8/k_9)(^DK_{eq}{}^{1.44})]/(1 + k_7/k_6 + k_8/k_9) \quad (8)$$

$$^{13}(V/K_b)_H = \{^{13}K_{i,6PG}{}^{13}k_9 + (k_9/k_8)[^{13}K_{i,6PG} + (k_7/k_6)(^{13}k_5)]\}/[1 + (k_9/k_8)[1 + (k_7/k_6)] \quad (9)$$

$$^{13}(V/K_b)_D = \{^{13}K_{i,6PG}{}^{13}k_9 + (k_9{}^Dk_7/k_8{}^DK_{eq})[^{13}K_{i\ 6PG} + (k_7/{}^Dk_7k_6)(^{13}k_5)]\}/[1 + (k_9{}^Dk_7/k_8{}^DK_{eq})[1+ (k_7/{}^Dk_7k_6)] \quad (10)$$

$c_f$ and $c_r$, the forward and reverse commitments to catalysis (*27*) are defined for our purposes in terms of the primary deuterium-isotope effect as $k_7/k_6$ and $k_8/k_9$, respectively. The inverse $^{13}$C-isotope effect obtained when 6PG-3d is the substrate suggests a rate-limiting hydride transfer (see below). Under these conditions, $k_9 \gg k_8$; $k_6 \gg k_7$, and the expression for $^{13}(V/K)_D$ is reduced to eq 11.

$$^{13}(V/K_b)_D = {}^{13}K_{i,6PG} \quad (11)$$

*Estimation of Intrinsic Isotope Effects and Commitment Factors*. Assuming a stepwise oxidative decarboxylation of 6PG eqs 7−10 apply where the forward commitment to catalysis, $c_f$, is $k_7/k_6$, the reverse commitment to catalysis, $c_r$, is $k_8/k_9$, $^Dk_7$ is the intrinsic primary deuterium-isotope effect, and $^{13}k_9$ is the primary $^{13}$C-isotope effect. There are four equations and four unknowns, but eqs 9 and 10 are not independent, and consequently, an absolute solution is not possible. Estimates for the commitments to catalysis and the intrinsic isotope effects are obtained by fixing the value of $^Dk_7$ and calculating values for the remaining unknowns. A computer program has been written to make these calculations.

## RESULTS

*Deuterium Isotope Effects*. Kinetic parameters for both *cu*6PGDH and *sl*6PGDH using several dinucleotide substrates are shown in Table 1. The kinetic deuterium-isotope effect on $V$ and $V/K_{6PG}$ with saturating NADP or APADP for *cu*6PGDH and *sl*6PGDH are equal at pH 8. With NADP, $^DV$ and $^D(V/K_{6PG})$ are 1.57 ± 0.08 and 1.87 ± 0.10 for *cu*6PGDH and *sl*6PGDH, respectively, while the effects are larger with saturating concentrations of APADP, 2.98 ± 0.08 and 2.47 ± 0.06, respectively. Kinetic parameters and deuterium-isotope effects were also measured with thio-NADP for *cu*6PGDH. The increase in the magnitude of deuterium-isotope effects correlates with the decrease in $V$ and $V/K_{6PG}$ as the dinucleotide substrate is changed.

The pH dependence of kinetic isotope effects for *cu*6PGDH and *sl*6PGDH is also provided in Table 2. With NADP as the oxidant, the isotope effect is pH-independent below pH 8 for *cu*6PGDH and decreases above pH 7. The kinetic deuterium-isotope effect for *sl*6PGDH shows a trend similar to that shown by *cu*6PGDH, Table 2. Due to a decreased enzyme stability and the higher $K_{6PG}$ at the pH extremes, a

[2] On the basis of the Swain−Schaad relationship (*28*), $^Dk^{1.44} = {}^Tk$.

Table 1: Kinetic Parameters for *Cu*6PGDH and *sl*6PGDH

| parameter | enzyme | nucleotide |
|---|---|---|
| | *cu*6PGDH[a] | |
| $V/K_{Nuc}E_t$ ($M^{-1}$ $s^{-1}$) | $(3.0 \pm 0.7) \times 10^5$ | NADP |
| $V/K_{6PG}E_t$ ($M^{-1}$ $s^{-1}$) | $(1.4 \pm 0.1) \times 10^4$ | |
| $V/E_t$ ($s^{-1}$) | $4.0 \pm 0.2$ | |
| $V/K_{Nuc}E_t$ ($M^{-1}$ $s^{-1}$) | $(2.5 \pm 1.2) \times 10^5$ | APADP |
| $V/K_{6PG}E_t$ ($M^{-1}$ $s^{-1}$) | $(3.0 \pm 0.6) \times 10^3$ | |
| $V/E_t$ ($s^{-1}$) | 0.71 | |
| $V/K_{Nuc}E_t$ ($M^{-1}$ $s^{-1}$) | $(2.7 \pm 0.2) \times 10^4$ | thioNADP |
| $V/K_{6PG}E_t$ ($M^{-1}$ $s^{-1}$) | $(1.8 \pm 0.2) \times 10^3$ | |
| $V/E_t$ ($s^{-1}$) | $0.30 \pm 0.01$ | |
| | *sl*6PGDH | |
| $V/K_{Nuc}E_t$ ($M^{-1}$ $s^{-1}$) | $(5 \pm 1) \times 10^5$ | NADP[b] |
| $V/K_{6PG}E_t$ ($M^{-1}$ $s^{-1}$) | $(1.4 \pm 0.8) \times 10^5$ | |
| $V/E_t$ ($s^{-1}$) | 4.5 | |
| $V/K_{Nuc}E_t$ ($M^{-1}$ $s^{-1}$) | $(1.8 \pm 0.1) \times 10^4$ | APADP[c] |
| $V/K_{6PG}E_t$ ($M^{-1}$ $s^{-1}$) | $(1.5 \pm 0.1) \times 10^3$ | |
| $V/E_t$ ($s^{-1}$) | $0.45 \pm 0.02$ | |

[a] Kinetic parameters were determined at pH 7, 25 °C (*4*). [b] pH independent values from ref *5*. [c] Kinetic parameters determined in this study at at pH 7, 25 °C.

Table 2: Primary Deuterium Kinetic Isotope Effects for 6-PGDH

| enzyme | pH | nucleotide | $^D(V/K_{NADP})$ | $^D(V/K_{6PG})$ |
|---|---|---|---|---|
| *cu*6PGDH | 5.5 | NADP[a] | $2.04 \pm 0.18$ | |
| | 6.0 | | $1.75 \pm 0.08$ | $1.79 \pm 0.08$ |
| | 6.5 | | $1.74 \pm 0.10$ | |
| | 7.0 | | $1.70 \pm 0.09$ | $1.75 \pm 0.09$ |
| | 8.0 | | $1.57 \pm 0.08$ | $1.63 \pm 0.07$ |
| | 9.0 | | $1.59 \pm 0.11$ | $1.58 \pm 0.07$ |
| | 9.5 | | $1.42 \pm 0.09$ | $1.43 \pm 0.06$ |
| | 10.0 | | $1.36 \pm 0.08$ | |
| | 6.0 | APADP[a] | | $3.90 \pm 0.30$ |
| | 7.8 | | | $2.98 \pm 0.08$ |
| | 8.0 | | $3.75 \pm 0.12$ | $3.60 \pm 0.08$ |
| | 9.5 | | $3.17 \pm 0.11$ | |
| | 6.0 | thioNADP | $3.69 \pm 0.09$ | $3.96 \pm 0.10$ |
| | 8.0 | | $2.64 \pm 0.11$ | $2.53 \pm 0.08$ |
| | 9.5 | | $2.38 \pm 0.10$ | $2.22 \pm 0.08$ |
| *sl*6PGDH | 5.9 | NADP | | $3.2 \pm 0.2$ |
| | 6.9 | | | $2.1 \pm 0.1$ |
| | 7.8 | | | $1.87 \pm 0.10$ |
| | 5.9 | APADP | | $4.10 \pm 0.20$ |
| | 6.9 | | | $3.9 \pm 0.20$ |
| | 7.8 | | | $2.50 \pm 0.10$ |
| | 7.8 | | | $2.47 \pm 0.06$ |

[a] Assays were carried at 25 °C and saturating concentrations ($20K_m$) of the fixed substrate. In all cases $^D(V/K) = {}^DV$.

quantitative analysis of the deuterium-isotope effects could not be performed accurately below pH 5.5. At pH 9, a sigmoid double reciprocal curve for both unlabeled and deuterated substrates was observed which make the analysis of kinetic isotope effects difficult.[3] Data obtained with other dinucleotides gives a similar, albeit less pronounced, trend with the exception of data obtained with APADP and the *cu*6PGDH.

*$^{13}$C-Isotope Effects*. Data for $^{13}$C-isotope effects on the oxidative decarboxylation of 6PG or 6PG-3d are shown in Table 3. It is apparent from the results that the isotope effect is independent of the fraction of the reaction, indicating that there was little or no $CO_2$ contamination. The value of $^{13}(V/K_{6PG})$ is generally higher when measured for *cu*6PGDH than for *sl*6PGDH, e.g., the isotope effect minus 1 obtained with 6PG/NADP is 3.4-fold greater. In all cases, deuteriation of 6PG decreases the observed $^{13}$C-isotope effect. The inverse $^{13}$C-isotope effect obtained with APADP and 6PG-3d will be discussed below.

A notable aspect of the $^{13}$C-isotope effect data is the more negative $\delta^{13}$C obtained for 6PG-3d than for 6PG. Thus, the glucose-3-$\delta$ obtained from the commercial source is depleted in $^{13}$C at C-1. However, the $\delta^{13}$C values measured for replicates are consistent under a variety of conditions.

Table 3: Primary $^{13}$C Kinetic Isotope Effects for 6-PGDH at pH 8, 25 °C[a]

| enzyme | nucleotide | substrate | $\delta^{13}$C (low conversion) | *f* | $^{13}(V/K)$ |
|---|---|---|---|---|---|
| *cu*6PGDH | NADP | 6PG-3h | −42.28 | 0.135 | 1.0212 |
| | | | −42.291 | 0.12 | 1.0212 |
| | | | −42.086 | 0.067 | 1.0203 |
| | | | | | $1.0209 \pm 0.0005$[b] |
| | NADP | 6PG-3d | −64.909 | 0.524 | 1.0156 |
| | | | −64.96 | 0.535 | 1.0159 |
| | | | −65.105 | 0.517 | 1.0158 |
| | | | | | $1.0158 \pm 0.0001$[b] |
| | APADP | 6PG-3h | −32.45 | 0.18 | 1.0105 |
| | | | −32.66 | 0.138 | 1.0105 |
| | | | −32.84 | 0.167 | 1.0109 |
| | | | | | $1.0106 \pm 0.0002$[b] |
| | APADP | 6PG-3d | −48.676 | 0.022 | 0.9932 |
| | | | −48.68 | 0.007 | 0.9933 |
| | | | −49.34 | 0.07 | 0.9938 |
| | | | | | $0.9934 \pm 0.0003$[b] |
| *sl*6PGDH | NADP | 6PG-3h | −28.57 | 0.127 | 1.0058 |
| | | | −28.39 | 0.169 | 1.0058 |
| | | | −28.15 | 0.29 | 1.0060 |
| | | | | | $1.0059 \pm 0.0001$[b] |
| | NADP | 6PG-3d | −58.498 | 0.21 | 1.0042 |
| | | | −57.266 | 0.18 | 1.0026 |
| | | | −58.18 | 0.28 | 1.0040 |
| | | | | | $1.0036 \pm 0.0008$[b] |
| | APADP | 6PG-3h | −31.22 | 0.116 | 1.0085 |
| | | | −31.18 | 0.105 | 1.0086 |
| | | | | | $1.0086 \pm 0.000\ 09$[b] |
| | APADP | 6PG-3d | −50.148 | 0.06 | 0.9947 |
| | | | −50.427 | 0.05 | 0.9950 |
| | | | −50.744 | 0.08 | 0.9953 |
| | | | | | $0.9950 \pm 0.0003$[b] |

[a] The 100% conversion samples yielded the following $\delta$ $^{13}$C values for 6-PG: −23.42, −23.39, and −23.00 with an avg of $-23.3 \pm 0.2$. The 100% conversion samples yielded the following $\delta$ $^{13}$C values for 6-PG-3d: −55.07, −55.06, and −54.96 with an avg of $-55.03 \pm 0.06$. [b] Average values.

## DISCUSSION

*Stepwise Oxidative Decarboxylation of 6PG.* The theory and methodology of multiple isotope effects on enzyme-catalyzed reactions developed by Hermes et al. (*16*) allows one to distinguish between a concerted and stepwise mechanism and provides estimates of the intrinsic isotope effects on the bond-breaking steps. Briefly, three isotope effects

[3] 6-Phosphogluconate dehydrogenase is a homodimer with subunits of approximately 50 kDa (*1*, *29*). The sigmoid double reciprocal plot at high pH may result from dissociation of the dimer to monomers with the dimer stabilized by 6PG binding, or site−site cooperativity. These aspects will require further study.

are measured, $^{D}(V/K_{6PG})$, $^{13}(V/K_{6PG})_H$, a $^{13}$C-isotope effect using 6PG, and $^{13}(V/K_{6PG})_D$, a $^{13}$C-isotope effect with 6PG-3d. If $^{13}(V/K_{6PG})_D$ is equal to or greater than $^{13}(V/K_{6PG})_H$, the same step reflects the deuterium and $^{13}$C effects; that is, the reaction is concerted. If $^{13}(V/K_{6PG})_H$ is equal to $^{13}(V/K_{6PG})_D$, the step is rate limiting, while if $^{13}(V/K_{6PG})_D$ is greater than $^{13}(V/K_{6PG})_H$, the chemical step was not completely rate limiting with protium-labeled reactant, and deuteriation slows it down and increases the $^{13}$C-isotope effect. A decrease in $^{13}(V/K_{6PG})_D$ compared to $^{13}(V/K_{6PG})_H$ signals a stepwise mechanism. In the stepwise case deuteriation of substrate increases rate-limitation of the hydride-transfer step, partially masking the $^{13}$C-sensitive step, and decreasing the observed $^{13}$C-isotope effect.

Qualitatively, the oxidative decarboxylation reaction catalyzed by 6PGDH is stepwise whether NADP or APADP is the dinucleotide substrate for both *cu*6PGDH and *sl*6PGDH. The most likely sequence of steps for the oxidative decarboxylation of a $\beta$-hydroxy acid is oxidation preceding decarboxylation. Equalities have been developed by Hermes et al. (*16*) for cases in which the deuterium sensitive step precedes the $^{13}$C-sensitive step, and the opposite case. For the case where the deuterium sensitive step precedes the $^{13}$C-sensitive step, $[^{13}(V/K_{6PG})_H - 1]/[^{13}(V/K_{6PG})_D - 1] = [^{D}(V/K_{6PG})/^{D}K_{eq}]$. Substitution of the values for the isotope effects and their associated errors into the equality gives $1.34 \pm 0.001 = 1.33 \pm 0.07$ for *cu*6PGDH and $1.64 \pm 0.001 = 1.58 \pm 0.08$ for *sl*6PGDH, so the equation holds within experimental error for both enzymes.[4] With APADP as the dinucleotide substrate, data again adhere to a stepwise mechanism since $^{13}(V/K_{6PG})_D < ^{13}(V/K_{6PG})_H$. However, unexpectedly, a very well-defined inverse $^{13}$C-isotope effect is observed in the presence of APADP/6PG-3d, and thus the above equality cannot be used. The inverse effect will be discussed further below. Support for a stepwise mechanism also comes from the work of Rippa et al. (*30*) who showed that 2-deoxy-6-phosphogluconate is oxidized to 3-keto-2-deoxy-6-phosphogluconate, which is released from the enzyme active site and only slowly decarboxylated by 6PGDH.

For *cu*6PGDH, $V/E_t$ is 6-fold lower with APADP than with NADP, and the deuterium-isotope effect observed with APADP/6PG-3d is ~3 at pH 8, compared with ~1.6 with NADP/6PG-3d. Thus, it is likely that the hydride-transfer step becomes slower as conditions change along the series NADP/6PG, NADP/6PG-3d, APADP/6PG, and APADP/6PG-3d. Over this same series, the $^{13}$C-isotope effect decreases from 2 to 1.6 to 1% and finally becomes inverse with a value of 0.993 with APADP-6PG-3d. The hydride-transfer step has likely become rate-limiting overall with APADP/6PG-3d, and the inverse $^{13}$C-isotope effect likely reflects a change in the fractionation factor at C-1 of 6PG upon binding. A slightly different trend is observed with *sl*6PGDH. The $V/E_t$ value decreases by a factor of 10 as the deuterium-isotope effect increases from 1.9 with NADP to 2.5 with APADP and the $^{13}$C effect increases from 0.6 to 0.9%. Since both isotope effects increase, the most likely explanation is that the chemical portion of the reaction has become slower than a physical step. The kinetic mechanism for *sl*6PGDH is rapid equilibrium (*5*), and the physical step must then be a conformational change that closes the active site in preparation for catalysis. The decrease in the $^{13}$C-isotope effect obtained in the presence of 6PG-3d to become inverse with a value of 0.995 with APADP/6PG-3d again suggests that hydride transfer has become completely rate determining under the latter conditions and that the inverse value reflects an isotope effect on 6PG binding.

Table 4: Calculated Values of Intrinsic Isotope Effects and Commitment Factors for *cu*6PGDH

| $^{D}k$ | $^{13}k$ | $c_f$ | $c_r$ |
|---|---|---|---|
| 2.8 | 1.110 | 1.76 | 1.52 |
| 2.9 | 1.062 | 1.52 | 0.79 |
| 3.0 | 1.045 | 1.23 | 0.51 |
| 3.1 | 1.036 | 0.94 | 0.38 |
| 3.2 | 1.031 | 0.62 | 0.29 |
| 3.3 | 1.027 | 0.15 | 0.23 |
| 3.4 | 1.025 | −0.12 | 0.20 |

Using the equations derived in the Theory, estimates for the intrinsic isotope effects and commitments to catalysis are obtained for *cu*6PGDH and given in Table 4. Estimates were generated using the data given in Tables 2 and 3 and the value of $2.05 \pm 0.12$ for $^{T}(V/K_{6PG})$ reported by Rendina et al. (*3*). Four isotope effects are measured and, as discussed in the Theory, four equations with a total of four unknowns are generated. However, the equations for $^{13}(V/K)_H$ and $^{13}(V/K)_D$ are related, and thus an absolute solution cannot be obtained (*16*). Estimates in Table 4 are obtained by fixing the value of $^{D}k$ as given and calculating the remaining parameters. Values of $>3.4$ for $^{D}k$ are rejected since a negative $c_f$ value is calculated, and values of $\leq 2.8$ for $^{D}k$ are rejected since the value of about 1.11 calculated for $^{13}k$ is higher than the theoretical maximum of about 1.07 for transfer reactions (*31*). (Actually for breaking a single C−C bond as in a decarboxylation reaction, values up to 1.05 are predicted.) Taking into account errors in the isotope effect measurements, estimates were recalculated at the limits obtained by subtracting and adding the standard error to the average values reported. The following range of values is obtained: $^{D}k$, 2.9−3.3; $c_f$, 0.15−1.52; $c_r$, 0.23−0.79; and $^{13}k$, 1.027−1.062. (Similar estimates for the sheep liver enzyme cannot be obtained until the primary tritium isotope effect has been measured.)

On the basis of the ranges calculated for the intrinsic isotope effects and commitment factors, some statements can be made concerning the *cu*6PGDH reaction. The best defined parameter is the intrinsic deuterium-isotope effect (2.9−3.3) on transfer of a hydride from $C_3$ of 6PG to $C_4$ of the nicotinamide ring of NADP. The value, though narrowly defined, does not allow a distinction between an early or late transition state for hydride transfer. Westheimer (*32*) has suggested that the deuterium-isotope effect in hydrogen-transfer reactions will become equal to the semiclassical maximum of 7−10 (*33* and references therein) only when the forces in the transition state on both sides of the transferring atom balance. The lower than maximum value

[4] The data of Rendina et al. (*3*), although qualitatively suggestive of a stepwise oxidative decarboxylation, did not fit the above equality. A comparison of the $^{13}$C-isotope effects obtained in these studies and those obtained by Rendina et al. (*3*) indicates it was in error in the previously determined isotope effects that caused the data to be ill-conditioned. Values of $^{13}(V/K_{6PG})_H$ and $^{13}(V/K_{6PG})_D$ are 1.0209 and 1.0158 in these studies compared to values of 1.0096 and 1.0081 in the previous studies. The reason for the discrepancy is unknown.

for *cu*6PGDH could thus reflect an early or late transition state for hydride transfer, and additional data will have to be obtained to further define the transition-state structure. As discussed above, the $^{13}$C-isotope effect is expected to increase from a value very close to 1 for a very early transition state to a value of 1.05−1.07 for a late transition state. The range of calculated $^{13}$C effects (1.024−1.07) spans the range from early to late transition states for $C_1-C_2$ bond cleavage, with an average value more consistent with a later transition state.

The partition ratio for the 3-keto-6PG intermediate obtained upon oxidation of 6PG is given by $c_r$, which is the ratio of the rates of reduction to 6PG to the rate of decarboxylation, (see Theory). The partition ratio varies between a value of 0.2 and 1.5 ($c_r$ in Table 4) in the case of *cu*6PGDH, compared to a value of about 16 obtained for the partition ratio of the oxalacetate intermediate in the malic enzyme reaction. Either an increase in the energy barrier for the reduction of 3-keto-6PG intermediate, a decrease in the energy barrier for its decarboxylation, or both can result in the smaller value of $c_r$. The differences likely reflect an increased stability of the 3-keto intermediate as a result of the flanking α- and γ-hydroxyls (see below), but this aspect requires additional experimentation. The cu6PGDH has a rapid equilibrium random kinetic mechanism (*4*) so that off-rates for reactants are much faster than the net rate constant for conversion of the E:NADP:6PG complex to products. Thus, the finite $c_f$ value, if present, must reflect an internal commitment to catalysis, likely resulting from some rate limitation by the conformational change proposed to close the active site prior to catalysis. Better estimates of all of the above values will be obtained when additional isotope effects are measured, e.g., the α-tritium secondary isotope effect predicted upon labeling the 4-position of the nicotinamide ring of NADP.

*Metal- vs Nonmetal-Requiring Oxidative Decarboxylases.* The results obtained in the present study differ from those obtained for malic enzyme. With the latter, a change in chemical mechanism from a stepwise oxidative decarboxylation with NAD(P) to a concerted reaction with APAD(P) is observed (*20*, *21*). The proposed reason for the change in the malic enzyme mechanism from stepwise with NADP to concerted with APADP is the combination of a higher energy barrier for hydride transfer and a lower overall free energy level for products, generating a collapse of the energy well for the oxalacetate intermediate (*20*, *21*). In the case of the 6PGDH reaction, the hydride-transfer step becomes more rate limiting as the dinucleotide substrate is changed from NADP to APADP, and the free energy level of products likewise changes due to the more positive redox potential of APADP compared to NADP. There is, however, no change in mechanism as the dinucleotide substrate is changed from NADP to APADP in the 6PGDH reaction, even though an increase in the measured deuterium kinetic isotope effect is observed. Thus, the 3-keto-6PG intermediate in the 6PGDH reaction must be more stable than the oxaloacetate intermediate in the malic enzyme reaction. There are two main differences between the malic enzyme and 6PGDH reactions: (1) the presence of electron-withdrawing α- and γ-hydroxyl groups in 6PG flanking the β-hydroxyl (as stated above) compared to a methylene and the 1-carboxylate in malate; and (2) the presence of a divalent metal ion activator in the malic enzyme reaction that is thought to act as a Lewis acid in the decarboxylation step. The differences must contribute to the differences in stability of the keto intermediate in the two enzyme-catalyzed reactions. The hydroxyls α and γ to the 1-carboxyl of 6PG are thought to contribute to the decarboxylation of 6PG, based on their electron-withdrawing ability. The divalent metal ion activator, on the other hand, is thought to coordinate directly to the keto group of the intermediate and act as a Lewis acid to facilitate decarboxylation. We propose that the metal-coordinated β-keto acid is activated to a greater extent than the α, γ-dihydroxy β-keto acid.

*Inverse $^{13}$C-Isotope Effect.* With APADP as the dinucleotide, an inverse $^{13}$C-isotope effect is observed when 6PG-3d is the substrate. Under these conditions, the hydride-transfer step is thought to completely limit the overall reaction. Whatever the explanation for the inverse isotope effect, it must result from an increase in the fractionation factor for the carboxylate of 6PG reflect a preequilibrium isotope effect on binding of 6PG, or must occur in the transition state for hydride transfer. It is difficult to envision an increase in the fractionation factor for the carboxylate in the transition state for hydride transfer, which precedes decarboxylation, and thus a preequilibrium isotope effect on binding of 6PG is the more likely explanation. The isotope effect on 6PG binding could reflect desolvation of the carboxylate as it is translated to the relatively hydrophobic enzyme active site or may reflect an increase in the fractionation factor at $C_1$ of 6PG as it is bound to 6PGDH compared to the unbound molecule. It is possible, for example, that the causative factor for the isotope effect is an increase in the force constants for some of the torsional modes of 6PG, specifically those involving rotation of the carboxyl group or rotation of the $C_2-C_3$ bond, which would be frozen upon binding. If the effect reflects torsional mode changes, they may result from the formation of the initial interaction complex or from the isomerization of the E:NADP:6PG complex that is proposed to close the site in preparation for catalysis. To provide an estimate of the magnitude of the $^{13}$C-isotope effect that could be expected based on torsional mode changes, ab initio calculations are being carried out.

*pH Dependence of Deuterium Isotope Effects*. Given the constant value of $^{D}(V/K_{6PG})$ at low pH (over a pH range in which $V/K_{6PG}$ decreases), and the decrease in $^{D}(V/K_{6PG})$ as the pH increases above 7−8, data adhere to a model in which the pH sensitive step(s) is(are) not the same as the isotope sensitive step(s) (*34*). The most likely pH dependent step, by analogy to the alcohol dehydrogenase reaction is deprotonation of the $C_3$-hydroxyl to produce an alkoxide prior to its oxidation to the 3-keto, as originally proposed by Rendina et al. (*3*). However, in the absence of a metal ion or some other group that could serve to decrease the p*K* of the 3-hydroxyl, it is unlikely that preequilibrium formation of an alkoxide occurs. (The general base is thought to be Lys183 based on the crystal structure of the sheep liver E:6PG binary complex (*35*). A more likely explanation for the decrease in the value of the deuterium-isotope effect at high pH is that some other nonisotope-dependent step begins to become rate-limiting as the pH increases. An explanation for the pH dependence of the deuterium-isotope effect will have to await further study.

## 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. Berdis, A. J., and Cook, P. F. (1993) *Biochemistry 32*, 2041−2046.
7. Villafranca, J. J., and Colman, R. F. (1972)*J. Biol. Chem. 247*, 209−214.
8. Villafranca, J. J., and Colman, R. F. (1974) *Biochemistry 13*, 1152−1160.
9. Viega Salles, J. B., and Ochoa, S. (1950) *J. Biol. Chem. 187*, 849−861.
10. Hsu, R. Y., and Lardy, H. A. (1967) *J. Biol. Chem. 242*, 520−526.
11. Tipton, P. A., and Peisach, J. (1990) *Biochemistry 29*, 1749−1756.
12. Rabin, R., Salamon, I. I., Bleiweis, A. S., Carlin, J., and Ajl, S. J. (1968) *Biochemistry 7*, 377−388.
13. Pirrung, M. C., Han, H., and Nunn, D. S. (1994) *J. Org. Chem. 59*, 2423−2429.
14. Siebert, G., Carsiotis, M., and Plaut, G. W. E. (1957) *J. Biol. Chem. 226*, 977−991.
15. Rutter, W. J., and Lardy, H. A. (1958) *J. Biol. Chem. 233*, 374−382.
16. Hermes, J. D.,Roeske, C. A., O'Leary, M. H., and Cleland, W. W. (1982) *Biochemistry 21*, 5106−5114.
17. Grissom, C. E., and Cleland, W. W. (1988) *Biochemistry 27*, 2927−2934.
18. Tipton, P. A. (1996) *Biochemistry 35*, 3108−3114.
19. Weiss, P. M., Gavva, S. R., Harris, B. G., Urbauer, J. L., Cleland, W. W., and Cook, P. F. (1991) *Biochemistry 30*, 5755−5762.
20. Karsten, W. E., and Cook, P. F. (1994) *Biochemistry 33*, 2096−2103.
21. Edens, W. A., Urbauer, J. C., and Cleland, W. W. (1997) *Biochemistry 36*, 1141−1147.
22. Chooback, L., Price, N. E., Karsten, W. E., Nelson, J., Sundstrom, P. R., and Cook, P. F. (1998) *Protein Expression Purif. 13*, 251−258.
23. Horecker, B. L., and Wood, W. A. (1957) *Methods Enzymol. 3*, 172−174.
24. O'Leary, M. H. (1980) *Methods Enzymol. 64*, 83−104.
25. Craig, N. (1957) *Geochim. Cosmochim. Acta 12*, 133−140.
26. Cleland, W. W. (1979) *Methods Enzymol. 63*, 103−108.
27. Northrop, D. B. (1975) *Biochemistry 14*, 2644−2651.
28. Swain, C. G., Stivers, E. C., Reuwer, J. F., Jr., and Schaad, L. J. (1958) *J. Am. Chem. Soc. 80*, 5885−5893.
29. Somers, D. O'N., Hajdu, J., and Adams, M. J. (1991) *Protein Expression Purif. 2*, 385−389.
30. Rippa, M., Signorini, M., and Dallocchio, F. (1973) *J. Biol. Chem. 248*, 4920−4925.
31. Huskey, W. P. (1991) in *Enzyme Mechanism from Isotope Effects* (Cook, P. F., Ed.) pp 37−72, CRC Press, Boca Raton, FL.
32. Westheimer, F. H. (1961) *Chem. Rev. 61*, 265−273.
33. Sühnel, J., and Schowen, R. L. (1991) in *Enzyme Mechanism from Isotope Effects* (Cook, P. F., Ed.) pp 3−35, CRC Press, Boca Raton, FL.
34. Cook, P. F., and Cleland W. W. (1981) *Biochemistry 21*, 1805−1816.
35. Adams, M. J., Ellis, G. H., Gover, S., Naylor, C. E., and Phillips, C. (1994) *Structure 2*, 651−668.


BI980611S