Importance in Catalysis of the 6-Phosphate-binding Site of 6-Phosphogluconate in Sheep Liver 6-Phosphogluconate Dehydrogenase*

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The 6-phosphate of 6-phosphogluconate (6PG) is proposed to anchor the sugar phosphate in the active site and aid in orienting the substrate for catalysis. In order to test this hypothesis, alanine mutagenesis was used to probe the contribution of residues in the vicinity of the 6-phosphate to binding of 6PG and catalysis. The crystal structure of sheep liver 6-phosphogluconate dehydrogenase shows that Tyr-191, Lys-260, Thr-262, Arg-287, and Arg-446 contribute a mixture of ionic and hydrogen bonding interactions to the 6-phosphate, and these interactions are likely to provide the majority of the binding energy for 6PG. All mutant enzymes, with the exception of T262A, exhibit an increase in $K_{\text{H}}$ that ranges from 5- to 800-fold. There is also a less pronounced increase in $K_{\text{NADP}}$, ranging from 3- to 15-fold, with the exception of T262A. The R287A and R446A mutant enzymes exhibit a dramatic decrease in $V_{\text{max}}$, respectively, as well as in $V/n_{\text{H}}$, and therefore no further characterization was carried out with these two mutant enzymes. No change in $V/n_{E}$ was observed for the Y191A mutant enzyme, whereas 2- and 3-fold decreases were obtained for the K260A and T262A mutant enzymes, respectively, resulting in a decrease in $V/K_{\text{H}}$, ranging from 3- to 120-fold. All mutant enzymes also exhibit at least an order of magnitude increase in $K_{\text{NADP}}$, indicating that the decarboxylation step has become more rate-limiting. Data are consistent with significant roles for Tyr-191, Lys-260, Thr-262, and Arg-446 in providing the binding energy for 6PG. In addition, these residues also likely ensure proper orientation of 6PG for catalysis and aid in inducing the conformation change that precedes, and sets up the active site for, catalysis.

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6-Phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.44) catalyzes the reversible oxidative decarboxylation of 6-phosphogluconate (6PG), producing ribulose 5-phosphate (Ru5P) and CO$_2$ with the concomitant reduction of NADP to NADPH. The kinetic mechanism is rapid equilibrium random in the basis of a complete kinetic characterization of the sheep liver enzyme and Candida utilis (1, 2). The pH dependence of kinetic parameters indicates a general acid-general base chemical mechanism (1, 2), and site-directed mutagenesis (3, 4) studies suggest that Lys-183 and Glu-190 are likely the general base and the general acid, respectively. In this mechanism, the general base (Lys-183) is required to accept a proton from the 3-hydroxyl group of 6PG concomitant with hydride transfer from C-3 of 6PG to the coenzyme. Reduction of the nicotinamide ring is accompanied by rotation around the $N$-glycosidic bond such that the ring occupies the position formerly occupied by the 1-carboxylate of the substrate (5). The resulting 3-keto-6-phosphogluconate intermediate is decarboxylated to produce the enediol of Ru5P with the general base used to protonate the carboxyl oxygen. A general acid (Glu-190) is needed to facilitate the tautomerization of the enediol of Ru5P to the keto product (Fig. 1).

In the E6PG binary complex, the 6-phosphate of 6PG is surrounded by the following five active-site residues that are within hydrogen-bond distance: Tyr-191, Lys-260, Thr-262, Arg-287, and Arg-446 (6). Multiple sequence alignment of 6PGDH shows that these five residues are completely conserved (Fig. 2), and Fig. 3 shows these residues in the protein environment. Notably, Lys-260 donates a hydrogen bond to the 6-phosphate from its backbone NH and also provides significant hydrophobic interaction to substrate via its side chain. Arginine 446 is from the C-terminal tail of the second subunit and together with Arg-287 balances the charge of the phosphate and may contribute most to anchoring the substrate. Mutations were made at Arg-447 in 6PGDH from Lactococcus lactis (7) (the homolog is Arg-446 in sheep liver 6PGDH), and all the mutants were devoid of enzyme activity, indicating that this residue is critical for activity. Interestingly, Arg-287 participates in a hydrogen-bond network through water 528 to Glu-190 and the 1-carboxyl of 6PG. It is suggested that this network is important in defining the bound conformation of 6PG (6).

In this study we investigate the role of each of the residues that interact with the 6-phosphate of 6PG. Site-directed mutagenesis was used to change Tyr-191, Lys-260, Thr-262, Arg-287, and Arg-446 to Ala, one at a time. Initial velocity and isotope effects studies were carried out to characterize the mutant enzymes. Data are consistent with the hypothesis that these residues are important in providing the binding energy for the 6-phosphate of 6PG. They also play a role in catalysis in the 6PGDH reaction as a result of properly orientating the substrate.
MATERIALS AND METHODS

Chemicals and Reagents—Oligonucleotide primers for mutagenesis and sequencing were from Biosynthesis Inc. and Invitrogen. The QuikChange® site-directed mutagenesis kit and Pfu polymerase were from Stratagene. The Geneclean® II kit was from Bio 101, Inc. The PerfectPrep® plasmid mini kit was from Eppendorf. The DNA molecular ladder was from New England Biolabs. Restriction endonucleases, deoxynucleoside triphosphates, and protein molecular mass markers were purchased from Invitrogen.

Acetaldehyde, acetate kinase, ADP-agarose resin, ATP, ampicillin, glucose-6-phosphate dehydrogenase, hexokinase, kanamycin, lithium potassium acetyl phosphate, the trisodium salt of 6PG, NADP, and pyrophosphate were from Sigma. β-d-(3-deuterio)-glucose (98 atom % D) was from Omicron Biochemicals, Inc. NiNTA-agarose resin was purchased from Qiagen. Glycerol and sulfuric acid were from Fisher. Isopropyl β-D-thiogalactopyranoside was from Gold Biotechnology, Inc. Hepes, BisTris, and Ches buffers were from Research Organics, Inc. Deuterium oxide (99 atom % D) was from Cambridge Isotope Laboratories, Inc. All other chemicals were the highest quality available.

Bacterial Strain and Plasmids—The Escherichia coli strain XL1-Blue was used to transform the mutated plasmid, and M15[pREP4] was the host strain for expression of the mutant proteins. The plasmid pQE-30 was used as both mutagenesis and expression vector.

Site-directed Mutagenesis—The QuikChange® site-directed mutagenesis kit from Stratagene was used to perform site-directed mutagenesis. Double-stranded DNA prepared from recombinant plasmid pPG-DHLC4 (8) was used as a template, and the synthetic oligonucleotide primers are listed in Table 1. Whole gene sequencing was performed for every mutation at the Laboratory for Genomics and Bioinformatics of the University of Oklahoma Health Science Center in Oklahoma City. The resulting sequence was compared with that of the wild type 6PGDH using BLAST. Successfully mutated plasmids were transformed into M15[pREP14] competent cells, the expression host. Frozen stocks of strains harboring plasmid were stored in LB/ampicillin/kanamycin medium containing 15% glycerol at −80 °C.
Active-site Mutations in 6-Phosphogluconate Dehydrogenase

Growth and Purification Conditions—The bacterial strain containing the mutated plasmid was first grown in 50–200 ml of LB medium, containing 100 μg/ml ampicillin and 25 μg/ml kanamycin at 37 °C, overnight in a water bath shaker. The next day, this culture was transferred into 2–10 liters of LB/Amp/Kan medium and was incubated at 25 °C for 8 h or until the $A_{600}$ reached 0.6. Isopropyl β-D-thiogalactopyranoside was then added to a final concentration of 1 mM to initiate expression. After 16 h of growth at 25 °C with shaking, the cells were harvested by centrifugation at 5,000 $\times$ g for 30 min. The cell pellet was resuspended in 4 volumes of Buffer A (50 mM triethanolamine, 2 mM β-mercaptoethanol, pH 7.5), sonicated on ice for 3 min (pulsing 30 s with 30 s of rest) using a MISONIX Sonicator® XL, and centrifuged at 25,000 $\times$ g for 30 min. The supernatant was combined with 2–10 ml of Ni-NTA resin pre-equilibrated with Buffer A and stirred at 4 °C for 2 h. The mixture was then loaded onto a 25-ml column, and the column was washed with 5 volumes of Buffer A containing 20 mM imidazole. Dependent on the number of uses of the Ni-NTA resin prior to regeneration, the imidazole concentration will be higher for fresh resin, and lower once the resin has been used. As a result, a short gradient of 0.2–0.4 M was used for elution, rather than an isocratic elution at a fixed concentration of imidazole. Protein concentrations were measured for all fractions using the method of Bradford (9). Fractions containing protein were pooled and applied to an ADP-agarose affinity column, which takes advantage of the NADP-binding site in the protein. Pooled fractions from the Ni-NTA column were applied to the ADP-agarose column directly, with a flow rate of 2 ml/min. The column was then washed with 10 volumes of Buffer A, and protein was eluted with 150 mM pyrophosphate buffer, pH 7.5, containing 1 mM EDTA and 2 mM β-mercaptoethanol. Fractions were collected, and protein concentration was determined by reading the absorbance at 280 nm (ε = 62,160 M$^{-1}$ cm$^{-1}$ for His$_6$-tagged 6PGDH). Fractions containing protein were again pooled and concentrated using an Amicon ultrafiltration apparatus with a PM-30 semipermeable membrane. SDS-PAGE was performed for wild type and all mutant enzymes to analyze the purity of the protein.

The wild type and mutant proteins were purified in an identical manner, and all enzymes were stored at 4 °C in the same buffer used for elution from the ADP-agarose column. All mutant enzymes are stable and do not lose activity for more than 1 year.

Synthesis of 3-d-6PG—3-deutério-Glucose was converted to 3-deutério-glucose 6-phosphate enzymatically by using hexokinase as described previously (10). The extent of conversion to the product of the reaction was determined by end point assay using glucose-6-phosphate dehydrogenase. Upon completion of the reaction, the pH of the solution was adjusted to 4.5 to denature the enzymes in the mixture, and Amicon ultrafiltration was used to remove the enzymes. The resulting solution was treated with acid-washed and heat-activated charcoal, stirred for 1 h, and filtered to remove nucleotides, and then Dowex 50W-H$^+$ resin was used to remove Mg$^{2+}$. The solution, containing 3-d-glucose 6-phosphate, was oxidized with bromine to yield the final product 3-d-6PG, which was purified as described previously (5).
Active-site Mutations in 6-Phosphogluconate Dehydrogenase

Initial Velocity Studies—Initial velocity studies were performed using a Beckman DU640 UV/visible spectrophotometer. The appearance of NADPH ($\epsilon_{340} = 6.22 \text{ mm}^{-1} \text{ cm}^{-1}$) was monitored over time using 1-cm path length semi-micro quartz cuvettes. The temperature was maintained at 25°C using a Beckman temperature controller. Initial velocity patterns were measured for all mutant and wild type enzymes in 100 mM Hepes, pH 7.5, using variable concentrations of 6PG (0.5–5 $K_m$) and NADP (0.5–5 $K_m$) dependent on the mutant enzyme.

$pH$ Studies—Initial velocity data were obtained as a function of pH under conditions in which 6PG was varied, and NADP was fixed at a saturating level (40 $K_m$). The pH was maintained using the following buffers at 100 mM concentrations: BisTris, 5–6.5; Hepes, 6.5–8.5; Chaps, 8.5–9.5. Sufficient overlap was obtained upon changing buffers to eliminate buffer effects. The pH was determined before and after the initial velocity measurements. All enzymes are stable for at least 5 min at all pH values studied.

Primary Deuterium Kinetic Isotope Effects—The concentrations of 3-protio-6PG (3-$h$-6PG) and 3-$d$-6PG were determined enzymatically in triplicate by end point assay using wild type 6PGDH. The concentrations from three determinations were in agreement within 2%. Primary deuterium isotope effects with 3-$d$-6PG were obtained for all the mutant enzymes as well as for the wild type enzyme by direct comparison of initial velocities (11). $D_1V$ and $D_1(V/K_{NADP})$ were obtained in triplicate by varying NADP at saturating levels of either 3-$h$-6PG or 3-$d$-6PG (20 $K_m$), whereas $D_2V$ and $D_2(V/K_{NADP})$ were measured in triplicate varying 3-$h$-6PG or 3-$d$-6PG at saturating concentrations of NADP (40 $K_m$).

Solvent Deuterium Kinetic Isotope Effects—Initial velocities for the wild type and mutant enzymes were measured in both H$_2$O and D$_2$O solutions. Substrates (NADP, 3-$h$-6PG, and 3-$d$-6PG) and buffers used in D$_2$O solutions were first dissolved in a small volume of D$_2$O and lyophilized overnight to remove exchangeable protons. The lyophilized powders were then dissolved in D$_2$O to give the desired concentrations, and pH was adjusted using either DCl or NaOD. Primary solvent deuterium isotope effects were obtained by direct comparison of initial velocities in the pH/pD independent region.

$^{13}$C-Kinetic Isotope Effects—These effects were measured using the natural abundance of $^{13}$C at C-1 of 6PG (12). Both high conversion (100% reaction, which represents $^{12}$C/$^{13}$C in substrate) and low conversion samples were used to measure the $^{12}$C/$^{13}$C isotope ratios in the CO$_2$ produced from the reaction of 3-$h$-6PG or 3-$d$-6PG. From these ratios, the $^{13}$C-ketonic isotope effect was calculated (13).

For high conversion samples, in 40 ml of H$_2$O or D$_2$O, the reaction mixtures contained 2 mM 3-$h$-6PG or 3-$d$-6PG, 0.5 mM NADP, and 10 mM oxidized glutathione. The low conversion reactions contain 6.5 mM 3-$h$-6PG or 3-$d$-6PG for the enzymes that have a $K_{NADP}$ close to that of the wild type 6PGDH (28 $M$), or 15 mM, if $K_{NADP}$ was higher. The NADP concentration was 0.25 mM for enzymes with a $K_{NADP}$ about 2 $M$ or 1 mM if $K_{NADP}$ was higher (greater than 20 $M$). All low conversion samples contained 5 mM oxidized glutathione in a final volume of 40 ml. The pH of the reaction solutions was titrated to 7.6, followed by sparging with CO$_2$–free nitrogen overnight. Before starting the reactions, aliquots were withdrawn to determine the initial concentrations of 6PG by end point assay in triplicate. Then the reactions were initiated by the addition of 2 units of 6PGDH and 100 units of glutathione reductase to recycle NADP for both high and low conversion samples.

The high conversion samples were allowed to proceed for more than 16 h, and end point assays were used to confirm the completion of the reaction. For the low conversion samples, extents of reaction were monitored by end point, assaying the remaining 6PG. Reactions were quenched when 50 $\mu$mol of CO$_2$ were produced. For both high and low conversion samples, 0.2 ml of concentrated sulfuric acid was added to quench the reactions, and the generated CO$_2$ was isolated by vacuum distillation (14). Aliquots of the samples were withdrawn after the CO$_2$ isolation and incubated at pH 8 overnight. The next day, end point assays were performed in triplicate to determine the exact extent of the low conversion reactions. Isotopic composition of the CO$_2$ was measured on a Finnigan Delta $E$ isotope-ratio mass spectrometer in the laboratory of Dr. Michael Engel, Department of Geophysics, University of Oklahoma. All ratios were corrected for $^{17}$O according to Craig (15).

Nomenclature—Isotope effects are expressed using the nomenclature developed by Northrop (19) and Cook and Cleland (20). Deuterium, $^{13}$C, and solvent deuterium isotope effects are written with a leading superscript D, 13, or D$_2$O, e.g. a primary deuterium isotope effect on V/K is written $D_iV/K$. Multiple isotope effects are written with a leading subscript to depict the isotope varied, and a following subscript to depict the fixed isotope, e.g. a solvent deuterium isotope effect measured with deuterated 6PG would be written $D_{iO}(V/K)_D$.

Data Processing—Double-reciprocal plots were used to analyze the data, and all plots and replots were linear. Data were fitted using the appropriate rate equations and programs developed by Cleland (16). Data for substrate saturation curves obtained at a fixed concentration of the second substrate were fitted using Equation 1.

$$v = \frac{VA}{K_a + A}$$

(Eq. 1)

Initial velocity patterns were fitted using Equation 2.

$$V = \frac{K_{A,V}K_B + K_A + AB}{K_{A,V} + A(1 + F_{E,V})}$$

(Eq. 2)

Deuterium kinetic isotope effect data in H$_2$O and D$_2$O were fitted using Equation 3.

$$v = \frac{VA}{K_a(1 + F_{E,UV}) + A(1 + F_{E,UV})}$$

(Eq. 3)

In Equations 1 and 2, $v$ is the initial velocity; $V$ is the maximum velocity; A and B are reactant concentrations; $K_a$ and $K_b$ are the Michaelis constants for NADP and 6PG, respectively; and $K_{AV}$ is the dissociation constant for NADP. In Equation 3, $F_i$ is the fraction of deuterium label in the substrate or solvent, and $E_V$ and $E_{UV}$ are the isotope effects $-1$ on $V$ and $V/K$. The pH dependence of $V$ and $V/K$ were fitted using Equation 4,

$$\log y = \log \left( \frac{C}{1 + H/K_a + K_H/H} \right)$$

(Eq. 4)
in which \( y \) is \( V/V/K \); \( C \) is the pH-independent value of \( y \); \( H \) is the hydrogen ion concentration; and \( K_y \) and \( K_H \) are the acid dissociation constants for enzyme or substrate groups important in a given protonation state for optimal binding and/or catalysis.

Calculation of \(^{13}\text{C}\)-kinetic isotope effects was performed according to Equation 5,
\[
^{13}(V/K) = \frac{\log(1 - f)}{\log(1 - f(R_p/R_s))}
\]
where \( f \) is the fraction of completion of the reaction, and \( R_p \) and \( R_s \) are the \(^{13}\text{C}:/^{12}\text{C}\) isotope ratios for \( \text{CO}_2 \) at partial and complete conversion, respectively. Isotope ratios, given as \( \delta^{13}\text{C} \), were calculated from Equation 6,
\[
\delta^{13}\text{C} = \left( \frac{R_{\text{emp}}}{R_{\text{std}}} - 1 \right) \times 10^3
\]
where \( R_{\text{emp}} \) and \( R_{\text{std}} \) are \(^{12}\text{C}:/^{12}\text{C}\) isotope ratios for sample and standard, respectively. The standard for \( \text{CO}_2 \) was calibrated from Pee Dee Belemnite with a \(^{13}\text{C}:/^{12}\text{C}\) of 0.0112372 (15).

RESULTS

Spectral Properties of Mutant Enzymes—There are 16 tryptophan residues in 6PGDH spread throughout the protein structure. Identical tryptophan fluorescence emission spectra were obtained for all mutant and wild type enzymes, upon excitation at 298 nm (data not shown), indicating that the microenvironment of tryptophan residues of the proteins is the same for all enzymes. As a result, there are no major changes in the overall structure of the enzyme resulting from the mutation. Changes are restricted to the local area within the active site.

Kinetic Parameters of the Mutant Enzymes—Initial velocity patterns were obtained by measuring the initial rate at pH 7.5 using variable concentrations of 6PG (0.5–5 \( K_m \)) and NADP (0.5–5 \( K_m \)) dependent on the mutant enzymes. Data are summarized in Table 2.

\( K_{\text{NADP}} \) increased for all but Thr-262 mutation, which showed no change compared with that of wild type enzyme. The increase in \( K_{\text{NADP}} \) for Y191A and K260A is about 3-fold, whereas more than an order of magnitude increase is observed for R287A and R446A mutant enzymes. Data suggest that changes in the 6PG-binding site somehow affect the binding of NADP, i.e. there is a linkage between the two sites. In comparison, the value of \( V/K_{\text{NADP}}E_s \) for the R287A and R446A mutant enzymes is decreased 10\(^3\)–10\(^4\)-fold, and \( V/E_s \) is decreased by at least 2 orders of magnitude with respect to the wild type enzyme.

Data indicate that elimination of either of the arginine residues dramatically impairs enzyme activity. On the other hand, \( V/K_{\text{NADP}}E_s \) is decreased 30- and 120-fold, respectively, compared with the wild type enzyme, for Y191A and K260A mutant enzymes. Changes in \( V/E_s \) are smaller with no change in the case of Y191A and a 20-fold change in the case of K260A. The T262A mutant enzyme exhibits a 3-fold change in \( V/E_s \) and \( V/K_{\text{NADP}}E_s \), reflecting the 3-fold change in the former.

Kinetic Primary Deuterium Isotope Effects—The kinetic deuterium isotope effects on \( V \) and \( V/K_{\text{NADP}} \) were measured at saturating NADP (40 \( K_m \)) (Table 3). The isotope effects on both kinetic parameters are small in all cases, and within error equal to one another, consistent with the proposed rapid equilibrium random kinetic mechanism (1). Isotope effects on R287A and R446A were not measured because of the very low activity.

Solute Deuterium Kinetic Isotope Effects—The solute deuterium kinetic isotope effects on \( V \) and \( V/K_{\text{NADP}} \) were measured at saturating NADP (40 \( K_m \)), comparing the rates in \( \text{H}_2\text{O} \) and \( \text{D}_2\text{O} \) (Table 3). Equal isotope effects, within error, on \( V \) and \( V/K_{\text{NADP}} \) were obtained for the wild type and all mutant enzymes, with the exception of the T262A mutant enzyme. For all three mutant enzymes, the isotope effects are smaller than those

### Table 2

Summary of the kinetic parameters for 6PGDH wild type and mutant enzymes

|         | WT       | Y191A    | K260A    | T262A    | R287A    | R446A    |
|---------|----------|----------|----------|----------|----------|----------|
| \( V/E \) (s\(^{-1}\)) | 9.4 ± 0.4 | 11.0 ± 0.4 | 0.43 ± 0.05 | 3.1 ± 0.3 | 0.015 ± 0.001 | 0.03 ± 0.01 |
| Fold decrease | 22       | 3        | 3        | 3        | 3        | 3        |
| \( K_{\text{NADP}} \) (μM) | 5 ± 1    | 14 ± 2   | 16 ± 3   | 4.2 ± 0.5 | 73 ± 10  | 64 ± 6   |
| Fold increase | 2.8      | 2.8      | 2.8      | 2.8      | 2.8      | 2.8      |
| \( K_{\text{NADP}} \) (μM) | 28 ± 4   | 1,040 ± 60 | 150 ± 8 | 24 ± 1 | 23000 ± 2000 | 3,400 ± 400 |
| Fold increase | 37       | 37       | 37       | 37       | 37       | 37       |
| \( V/K_{\text{NADP}}E_s \) (s\(^{-1}\)) | 3.4 × 10\(^5\) | 1.1 × 10\(^3\) | 2.8 × 10\(^3\) | 1.3 × 10\(^5\) | 0.6 | 9.2 |
| Fold decrease | 31       | 31       | 31       | 31       | 31       | 31       |
| \( \Delta G^0 \) (kcal mol\(^{-1}\)) | -6.2     | -4.1     | -5.2     | -6.3     | -2.2     | -3.4     |
| \( \Delta G^0 \) (kcal mol\(^{-1}\)) | 2.1      | 2.1      | 2.1      | 2.1      | 2.1      | 2.1      |

### Table 3

Summary of isotope effects for 6PGDH wild type and mutant enzymes

|         | WT       | Y191A    | K260A    | T262A    | R287A    | R446A    |
|---------|----------|----------|----------|----------|----------|----------|
| \( ^{13}(V/K) \) | 1.0 ± 0.1 | 1.06 ± 0.02 | 1.24 ± 0.09 | 1.25 ± 0.07 |        |
| \( ^{13}(V/K_{\text{NADP}}) \) | 1.0 ± 0.1 | 1.06 ± 0.02 | 1.24 ± 0.09 | 1.25 ± 0.07 |        |
| \( ^{13}(V/K_{\text{NADP}}) \) | 1.0002 | 1.0269 | 1.0397 | 1.0325 |        |
| \( ^{13}(V/K_{\text{NADP}}) \) | 1.0002 | 1.0269 | 1.0397 | 1.0325 |        |
| \( ^{13}(V/K_{\text{NADP}}) \) | 1.0001 | 1.0116 | ND | 1.0206 |        |
| \( ^{13}(V/K_{\text{NADP}}) \) | 1.0001 | 1.0116 | ND | 1.0206 |        |
| \( ^{13}(V/K_{\text{NADP}}) \) | 2.10 ± 0.05 | 1.58 ± 0.06 | 1.22 ± 0.04 | 1.15 ± 0.04 |        |
| \( ^{13}(V/K_{\text{NADP}}) \) | 2.10 ± 0.05 | 1.58 ± 0.06 | 1.22 ± 0.04 | 1.15 ± 0.04 |        |
| \( ^{13}(V/K_{\text{NADP}}) \) | 2.28 ± 0.05 | ND | 0.94 ± 0.01 | 0.77 ± 0.04 |        |
| \( ^{13}(V/K_{\text{NADP}}) \) | 6.40 ± 0.04 | 6.2 ± 0.1 | 9.1 ± 0.1 | 8.5 ± 0.1 | 8.0 ± 0.1 |
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observed for the wild type protein. Multiple primary deuterium/solvent deuterium isotope effects were measured for K260A and T262A mutant enzymes at saturating level NADP (40 $K_m$) (Table 3). For the K260A mutant enzyme $D_2O$ within error, is equal to $D_2O/V_{fit}$, whereas $D_2O/(V/K)_D$ is slightly smaller than $V_{fit}/(V/K)_H$. In the case of T262A mutation, $D_2O/V_{fit}$ is within error equal to $V_{fit}$, and inverse solvent isotope effects are obtained for $D_2O/(V/K)_D$ and $D_2O/(V/K)_H$.

$^{13}$C-Kinetic Isotope Effects—Data for $^{13}$C-kinetic isotope effects obtained with 3-$d$-6PG and 3-$d$-6PG are shown in Table 3. For all of the mutant enzymes, the value of $^{13}$C-kinetic isotope effect $1/(V/K_{6PG})$ is at least an order of magnitude higher than that of the wild type enzyme. Deuteration of 6PG decreases the observed $^{13}$C-kinetic isotope effects for the wild type enzyme as well as that for K260A and T262A, and in all cases the equality for a stepwise mechanism with oxidation preceding decarboxylation is satisfied within error (13). $^{13}$C-Kinetic isotope effects were also measured in H$_2$O and D$_2$O to determine the multiple solvent deuterium $^{13}$C isotope effects, as summarized in Table 3. Note that there is a decrease in the $^{13}$C-kinetic isotope effects when measured in D$_2$O.

pH Dependence of Kinetic Parameters—The pH dependence of the kinetic parameters of the mutant enzyme Y191A was measured by varying 6PG at saturating level of NADP (40 $K_m$). Enzyme is stable at pH 5.5 and 9.5. Bell-shaped pH-rate profiles with limiting slopes of 1 and −1 was obtained for $V/K_{6PG}$ and $V$. The $pK_a$ values are summarized in Table 3. The pH-rate profiles for the Y191A mutant enzyme is shown in Fig. 4.

DISCUSSION

The main aim of this research was to determine the importance of residues Tyr-191, Lys-260, Thr-262, Arg-287, and Arg-446, which interact with the 6-phosphate of 6PG, and their role in providing binding energy and proper orientation of the substrate. On the basis of the crystal structure of the 6PG binary complex, interactions between each of the five residues listed and the 6-phosphate of 6PG is suggested. A multiple sequence alignment of 6PGDHs from a variety of species from bacteria to man indicates a complete conservation of all of the residues considered (Fig. 2). Site-directed mutagenesis was used to change Tyr-191, Lys-260, Thr-262, Arg-287, and Arg-446 to alanine one at a time to eliminate the interaction between each of the residues and the 6-phosphate of 6PG. Steady-state kinetic parameters and isotope effects were measured to determine the effect of the substitutions on the ability of 6PGDH to use 6PG as a substrate.

Theory for interpretation of kinetic parameters and isotope effects in the 6PGDH reaction has been developed previously, and equations are reproduced here for aid in data interpretation (5, 10). The oxidative decarboxylation reaction catalyzed by 6PGDH is stepwise with oxidation preceding decarboxylation as suggested by multiple deuterium/$^{13}$C kinetic isotope effect studies (10). Multiple solvent deuterium kinetic isotope effect and proton inventory studies indicate the presence of an isomerization of the enzyme complex prior to hydride transfer and decarboxylation (17). The kinetic mechanism of 6PGDH at saturating NADP can be written as indicated in Reaction 1, where $A, B, X, Q,$ and $R$ represent NADP, 6PG, 3-keto-6PG, ribulose 5-phosphate, and NADPH, respectively. The rate constants $k_5$ and $k_4$ are for binding and dissociation of 6PG, and $k_3$ and $k_2$ are for binding and dissociation of NADP; $k_5$ and $k_6$ are for an isomerization of $ENADP$-6PG complex; $k_7$ and $k_8$ are for forward and reverse hydride transfer, and $k_9$ is the rate constant for decarboxylation of the 3-keto intermediate and release of CO$_2$. The deuterium-sensitive step, hydride transfer, may exhibit an isotope effect upon deuteration of 6PG at the 3-position, depicted by $D_3k_6$ and $D_3k_5$, which can be related by the equilibrium isotope effect $D_3k_{eq} = D_3k_7/D_3k_6$. The $^{13}$C-kinetic sensitive step, $k_{19}$, may exhibit a primary isotope effect, $^{13}k_{19}$.

Given the rapid equilibrium nature of the mechanism, central complex interconversion is rate-limiting (steps included from EAB to $E'QR$, and thus $k_3$, $k_4 > k_2$). Finally, because the kinetic isotope effects on $V$ and $V/K$ are equal to one another for the wild type and each of the mutant enzymes, $k_6 = (k_7/k_8)/(1/k_5 + 1/k_6)$. It is possible that the kinetic mechanism of the enzyme changed upon making the mutations discussed above. However, the kinetic mechanism is equilibrium random for the WT enzyme (1, 2). In addition, $V$ is decreased for all of the mutant enzymes, with the exception of Y191A. The only real possibility for a change in kinetic mechanism for the remaining mutant enzymes is to an equilibrium ordered addition of reactants, and this would have been observed when initial velocity patterns were performed (the $K_m$ for one of the reactants would be 0). In the case of the Y191A mutant enzyme, the $V/K$ values for both

![Figure 4. pH dependence of kinetic parameters for the Y191A mutant of 6-PGDH. Data were obtained for $V(A)$ and $V/K_{6PG}(B)$. The points shown are the experimentally determined values, and the curves are from a fit of the data using Equation 4.](image-url)
reactants decrease, and this would be difficult to rationalize for other than an equilibrium mechanism.

On the basis of the above, the following Equations 7–11 have been developed.

\[ V = \frac{k_f}{1 + c_{cf} + c_r} \]  
\[ \frac{V}{K_{6PG}} = \frac{k_f k_6}{k_5} \]  
\[ c_{cf} = \frac{k_1}{k_5 + k_6} \]  
\[ c_f = \frac{k_6}{k_5} \]  
\[ c_r = \frac{k_5}{k_5} \]

A kinetic deuterium isotope effect is observed on the hydride transfer step, depicted by \( \text{D}^7 \) and \( \text{D}^9 \), which can be related by the equilibrium isotope effect \( \text{D}^7 \text{K}_{eq} = \text{D}^7 \text{k}_{cf} \). Expressions for the primary kinetic deuterium isotope effects are given in Equations 12 and 13.

\[ \text{D}^7 \text{V} = \frac{\text{D}^7 k_f + c_{cf} + (\text{D}^7 \text{k}_{eq})c_r}{1 + c_{cf} + c_r} \]  
\[ \frac{\text{D}^7 \text{V}}{K_{6PG}} = \frac{\text{D}^7 k_f + c_f + (\text{D}^7 \text{k}_{eq})c_r}{1 + c_f + c_r} \]

Because \( \text{D}^7 \text{V} = \text{D}^7 (V/K) \) (Table 3), \( c_f = c_{cf} \) and the equation for the isotope effect on \( V \) (Equation 14) is equal to that on \( V/K \), i.e. Equation 15. The rate constant \( k_f \) will reflect a \( \text{^{13}C} \)-kinetic isotope effect given by \( \text{^{13}K}_{eq} \). Expressions for the primary \( \text{^{13}C} \)-kinetic isotope and the multiple \( \text{^{13}C}/\text{D} \) multiple isotope effect, i.e. the \( \text{^{13}C} \)-kinetic isotope effect with 3-d-6PG, are given in Equations 14 and 15.

\[ \frac{13}{K_{6PG/H}} = \frac{13 k_f + 1 + c_f}{1 + c_f} \]  
\[ \frac{13}{K_{6PG/D}} = \frac{13 k_f + \left( \frac{\text{D}^7 k_f}{c_f} \text{D}^7 \text{k}_{eq} \right) \left( 1 + \frac{c_f}{\text{D}^7 \text{k}_{eq}} \right)}{1 + \left( \frac{13 k_f}{c_f} \text{D}^7 \text{k}_{eq} \right) \left( 1 + \frac{c_f}{\text{D}^7 \text{k}_{eq}} \right)} \]

where \( \text{D}^7 k_f \) is the intrinsic deuterium isotope effect on the hydride transfer step, and \( \text{D}^7 \text{K}_{eq} \) is the equilibrium isotope effect on hydride transfer (1.18 for oxidation of a secondary alcohol (17)).

Because \( K_{6PG} \) is the ratio of \( V \) and \( V/K_{6PG} \), and taking into account \( k_f = (1 + k_f/k_6)/(1/k_5 + 1/k_6) \) (from \( \text{D}^7 \text{V} = \text{D}^7 (V/K) \)), we get Equation 16.

\[ K_{6PG} = \left( \frac{k_f}{k_5} \right) \frac{k_6}{(k_5 + k_6)} \]

where \( k_f/k_6 \) is the \( K_0 \) of 6PG for the \( E^*\text{NADP-6PG} \) complex, and \( k_f/(k_5 + k_6) \) corrects for the distribution of the central complexes between \( E^*\text{NADP-6PG} \) and \( E^*\text{NADP-6PG} \). Therefore, \( K_{6PG} \) is the equilibrium constant for dissociation of 6PG from \( E^*\text{NADP-6PG} \) and \( E^*\text{NADP-6PG} \). By using the above rate equations, we discuss the results for each of the mutant enzymes.

The key to interpretation of the data obtained for the mutant enzymes lies in the changes in \( K_{6PG} \) \( \text{D}(V/K_{6PG}) \), and \( ^{13}(V/K_{6PG}) \). The increase in \( K_{6PG} \) and \( K_{\text{NADP}} \) likely results from an increase in the net off-rates for 6PG and NADP from \( E^*\text{NADP-6PG} \); \( k_f k_5/(k_5 + k_6) \) in the case of 6PG and \( k_f k_6/(k_5 + k_6) \) in the case of NADP, that is an increase in \( k_5 \), \( k_6 \), or both. Because the \( K_{eq} \) values of both substrates are affected, it is likely to be both. The conformational change that precedes the catalytic steps represented by \( k_5 \) and \( k_6 \) inReaction 1, requires both reactants bound. This is suggested by a superposition of the \( E^*\text{Nbr}^a\text{ADP} \) and \( E^*\text{6PG} \) complexes (6), which gives a hydride transfer distance of 5.3 Å, which is much too long. Therefore, a conformational change that brings the two substrates closer together is required.

The R287A and R446A Mutant Enzymes—As shown in Table 1, \( K_{6PG} \) increases by about 800- and 120-fold for R287A and R446A, respectively, compared with the value of the wild type enzyme. In addition, \( V/E_2 \) decreases about 600- and 300-fold respectively, resulting in a \( V/K_{6PG}E_2 \) more than 10,000 times lower than that of the wild type protein. Because of the very low activity, no further characterization was carried out on the two mutant enzymes. On the basis of the measured kinetic parameters, the affinity of the mutant enzymes for their substrate 6PG decreases substantially (\( \Delta G^0 \) 3.95 and 2.81 kcal/mol for R287A and R446A, respectively), and the ability of the mutant enzymes to catalyze reaction is severely impaired, as a result of eliminating hydrogen bonding and ionic interactions contributed by the arginine residues. Given the expression for \( K_{6PG} \) (see Equation 16), the net rate constant for release of 6PG is increased. Because there is also an observed greater than 10-fold increase in \( K_{\text{NADP}} \) it is likely, as suggested above, that part of the increase results from a change in the equilibrium position of the conformational change that precedes the hydride transfer step to favor \( \text{EAB} \) (see Reaction 1).

The Y191A Mutant Enzyme—Data for replacement of Tyr-191 with Ala are interesting in that \( V/E_s \) is increased by about 20% (Table 2). One would thus not expect much change in the isotope effects. This is not the case, and significant changes in the magnitude of the isotope effects are observed. The \( V \) and \( V/K_{6PG} \) pH-rate profiles for the Y191A mutant enzyme exhibits \( pK \) values that are closer together than those observed for the WT enzyme (Table 3). (The phenomenon is easier to see and explain using the \( V/K \) profiles. The same phenomenon is appar-
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The large increase in $K_{\text{6PG}}$ for the mutant enzyme exhibits a 3-fold increase in $K_{\text{NADP}}$, suggesting a change in the rate of the conformational change that precedes hydride transfer. Data further suggest that occupancy of the 6-phosphate-binding site is needed to generate the optimum catalytic conformation for the enzyme.

The T262A Mutant Enzyme—Only in the case of the T262A mutant enzyme is there no observed change in $K_{\text{6PG}}$, suggesting no significant binding energy is provided by this side chain. This is not surprising, given the 3.7 Å distance from the T side chain to the 6-phosphate (6) (Fig. 3). However, a 3-fold decrease in $V/E_e$ is observed, and the isotope effects differ significantly from those obtained for the wild type enzyme. The large value of $13(V/K_{\text{6PG}})$, 1.0325, indicates the decarboxylation step has become predominantly rate-limiting overall, and the primary kinetic deuterium isotope effect is close to the value of the equilibrium deuterium isotope effect of 1.18 on the hydride transfer step (17). The multiple deuterium/$^{13}$C isotope effect is decreased slightly consistent with the small deuterium isotope effect. The increase in the $^{13}$C isotope effect to 1.0325 (the effect for WT is 1.0028) can be used to estimate the partition ratio for the 3-keto-6PG intermediate in the case of the WT enzyme. Substituting 1.0325 for $^{13}k_o$ in Equation 13 and 1.0028 for $^{13}(V/K_{\text{6PG}})$ gives a value of 10.6 for $k_o/k_p$. Thus, in order to see the increased value of $^{13}(V/K_{\text{6PG}})$, the partition ratio for the 3-keto-6PG intermediate must decrease by a factor of 10. The change in partition ratio is much greater than the observed decrease in $V/E_e$ (3-fold), and thus it is likely that the fraction of enzyme that is catalytically competent has increased in the case of the mutant enzyme as suggested above for the Y191A mutant enzyme or that a step within the catalytic pathway has increased in rate (this would most likely be $k_e$, because it is included in both $V$ and $V/K$).

An inverse solvent deuterium kinetic isotope effect is observed on $V/K$, and this likely reflects a medium effect on the conformation change that precedes hydride transfer (18). Deuteration of the substrate has no significant effect on the inverse solvent effect on $V/K$; this is not surprising given the small value of $^{13}(V/K)$. Of interest, the solvent deuterium isotope effect on $V$ is normal, and this may be indicative of some rate limitation by tautomerization of the enediol of ribulose 5-phosphate, but this will require additional study.

The K260A Mutant Enzyme—Lysine 260 donates a hydrogen bond from its backbone NH to the 6-phosphate and also provides van der Waals interactions via its side chain and the backbone of 6PG. Changing Lys 260 to alanine gives a greater than 5-fold decrease in the affinity of 6PGDH for 6PG. This change in binding energy likely reflects the van der Waals interactions because the backbone NH will still be present (although it may...
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not be in position to donate a hydrogen bond). As stated above, the increase in both $K_{\text{NADP}}$ and $K_{\text{6PG}}$ suggests a decrease in the rate of the conformational change to set the active site up for catalysis. Data further suggest either a requirement for occupancy of the 6-phosphate site or proper binding of 6PG to generate an optimum catalytic conformation, i.e. utilization of the binding energy of 6PG to catalyze the reaction.

The 20-fold decrease in $V$ likely results from a decrease in the rate of the decarboxylation step, $k_d$, consistent with the estimated 13-fold decrease in $k_d/k_r$ calculated as above for the T262A mutant enzyme. The largest value of $^{13}V$/$K_{\text{6PG}}$ is observed for this mutant enzyme. The decreased value of the deuterium isotope effects is also consistent with the increased rate limitation of the decarboxylation step. However, the values of $D^2V$ and $D^1V$/$K_{\text{6PG}}$ are greater than $D^0V$ and $D^0V$/$K_{\text{6PG}}$, suggesting a small contribution to rate limitation by the hydride transfer step. In agreement, the values of $D^2O$/$K_{\text{6PG}}$ and $D^1O$/$K_{\text{6PG}}$, are finite and normal, suggesting a contribution from either hydride transfer, decarboxylation, or both.

The observed decrease in the solvent isotope effect to a value of 1 is consistent with a decrease in the observed solvent isotope effects on decarboxylation, as a result of the inverse effect on the conformational change preceding hydride transfer. The finite value of the effect on $V$ may again suggest a contribution from the tautomerization step. Data are also consistent with the structural data of Adams et al. (6), which show that the alkyl side chain of Lys-260 rests against the backbone of 6PG. The effect of removing the lysine side chain could introduce a significant positional change of the bound substrate, resulting in a poorly oriented 6PG, a subsequent decrease in the rate of the conformational change to establish the catalytic conformation and decrease the rate of decarboxylation.

Evidence for Interaction between the Two Substrate Binding Sites—A summation of the $\Delta\Delta G^0$ values estimated from all of the mutations greatly exceeds the total binding energy of 6PG. It is apparent that the 6-phosphate of 6PG is a very important binding determinant for the substrate. Occupancy of the 6-phosphate-binding site appears to be critical for proper positioning of the substrate for the subsequent catalytic steps, most importantly decarboxylation (see above). Another way of putting this is that occupancy of the 6-phosphate-binding site is important for the reaction, likely to induce the conformational change in enzyme preceding catalysis. In agreement with this suggestion, there is an increase in $K_{\text{NADP}}$ for all the mutant enzymes, with the exception of T262A, ranging from 3- to 15-fold. According to the crystal structures of wild type protein with ligands bound, the coenzyme-binding pocket is separated from the 6PG-binding site (6). Because all of the mutations reside in the 6PG-binding site and no significant change in the overall structure of the mutant enzymes was detected, it is suggested that the two sites are linked, likely via the induced conformational change. To this point, there is no direct evidence to suggest where and how the two sites are linked. In addition, a ternary complex of 6PGDH with both substrates bound is not available. An overlay of the two binary complexes (E-Nbr$^8$ADP and E6PG) (5) can be used as a starting point for identification of a proposed pathway that connects the sites, as shown in Fig. 5. The green colored structure in Fig. 5 is from the E6PG binary complex and the yellow colored structure is the E-Nbr$^8$ADP complex. The highlighted region (Fig. 5, red), including loops (residue 8–12, 31–34, 73–78, 100–103, 128–131, and 258–261) and a helix (reside 79–83) on both sides of the dinucleotide, provides a possible linkage. Further experiments, including site-directed mutagenesis in the proposed region and structure analysis, will help to test the putative linkage between sites.

However, one of the things we anticipated is that the bound substrate would change in its position within its binding site in the mutant enzymes compared with the wild type enzyme. This is likely what has occurred in most cases given the change in the relative rates of hydride transfer and decarboxylation. Care must be taken in interpreting data for mutant enzymes when alanine is substituted for a residue with a larger volume side chain, because the position of the bound reactant can change when large changes are made by site-directed mutagenesis. Isotope effects and multiple isotope effects, when they can be measured, greatly facilitate the interpretation of the data.

Addendum—Consistent with the suggestion that decarboxylation is rate-limiting for the mutant enzymes, we have recently measured pre-steady-state time courses following the appearance of NADPH, mixing enzyme with both substrates. A rapid burst is observed followed by a slow steady-state rate that is equivalent to $V/E_r$. The burst amplitude is equal to $E_i$ in the case of the K260A and T262A mutant enzymes.

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