Role of the S128, H186, and N187 Triad in Substrate Binding and Decarboxylation in the Sheep Liver 6-Phosphogluconate Dehydrogenase Reaction†

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Received July 6, 2006; Revised Manuscript Received August 23, 2006

ABSTRACT: Crystal structures of 6-phosphogluconate dehydrogenase (6PGDH) from sheep liver indicate that S128 and N187 are within hydrogen-bonding distance of 6PG in the E:6PG binary complex and NADPH in the E:NADPH binary complex. In addition, H186 is also within hydrogen-bonding distance of NADPH in the E:NADPH binary complex, while in the E:6PG binary complex it is within hydrogen-bonding distance of S128 and close to N187. The structures suggest that this triad of residues may play a dual role during the catalytic reaction. Site-directed mutagenesis has been performed to mutate each of the three residues to alanine. All mutant enzymes exhibit a decrease in V/E_t (the turnover number), ranging from 7- to 67-fold. An increase in the K_m for 6PG (K_{6PG}) was observed for S128A and H187A mutant enzymes, while for the H186A mutation, K_{6PG} is decreased by a factor of 2. K_{NADP} remains the same as the wild type enzyme for the S128A and H186A mutant enzyme, while it increases by 6-fold in the N187A mutant enzyme. An increased K_{iNADPH} was measured for all of the mutant enzymes. The primary kinetic ^{13}C-isotope effect is increased, while the primary deuterium kinetic isotope effect is decreased, indicating that the decarboxylation step has become more rate limiting under conditions where substrate is limiting. A quantitative analysis of the data suggests that the S128, H186, and N187 triad is multifunctional in the 6PGDH reaction and contributes as follows. The triad (1) participates in the precatalytic conformational change; (2) provides ground state binding affinity for 6PG and NADPH; and (3) affects the relative rates of reduction or decarboxylation of the 3-keto-6PG intermediate by anchoring the cofactor after hydride transfer, which is accompanied by the rotation of the nicotinamide ring around the N-glycosidic bond and displacement of C1 of 6PG, facilitating decarboxylation.

6-Phosphogluconate dehydrogenase (6PGDH[1]; EC 1.1.1.44) catalyzes the reversible oxidative decarboxylation of 6-phosphogluconate (6PG), to give Ru5P and CO_2 with the concomitant production of NADPH upon reduction of NADP. Previous studies suggest that, in addition to its role in the hydride transfer step, NADPH plays a nonredox role in the 6PGDH catalyzed reaction (*6*−*10*). It has been suggested that, concomitant with hydride transfer, the nicotinamide ring of NADPH rotates around the N-glycosidic bond into a position that is occupied by C1−C3 of 6PG, causing a displacement of this portion of the substrate, Figure 1. In the E:6PG complex, C1 of 6PG is within hydrogen-bonding distance of E190, which is proposed to protonate C1 of the enediol of Ru5P formed upon decarboxylation of the 3-keto-6PG intermediate. The displacement of 6PG after hydride transfer would then facilitate decarboxylation by eliminating the hydrogen-bonding interaction to E190, and better positioning C1 of 6PG out of the C2−C3 plane (*5*).

The structure of the E:6PG binary complex indicates that serine-128 and asparagine-187 are within hydrogen-bonding distance of the 1-carboxylate and 3-hydroxyl of 6PG, respectively, while histidine-186 forms a bridge between them (Figure 2) (*11*). In the E:NADPH binary complex structure, however, S128 and H186 are within hydrogen-bonding distance of the carboxamide oxygen, while N187 is within hydrogen-bonding distance of the carboxamide nitrogen (Figure 3) (*11*). None of these residues is close to the nicotinamide ring of Nbr^8ADP in the E:Nbr^8ADP binary complex (*11*). It would appear that the triad of residues discussed has a multifunctional role in the 6PGDH reaction. Multiple sequence alignment of 6PGDHs from a number of species from bacteria to humans shows that S128, H186, and N187 are completely conserved.

In order to further test the hypothesis that S128, H186, and N187 have a multifunctional role in the 6PGDH reaction, site-directed mutagenesis was used to change each of the residues to A, one at a time. Then initial velocity and isotope effect studies were carried out to compare the native and mutant enzymes in an attempt to deduce the catalytic role of these side chains.

† This work was supported by a grant from the National Science Foundation to P.F.C. (MCB 009127) and by the Grayce B. Kerr endowment to the University of Oklahoma to support the research of P.F.C.

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[1] Abbreviations: 6PGDH, 6-phosphogluconate dehydrogenase; 6PG, 6-phosphogluconate; 3-*d*-6PG, 3-*deutero*-6-phosphogluconate; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide 2-phosphate (the + sign is omitted for convenience); NADPH, reduced nicotinamide adenine dinucleotide 2′-phosphate; Nbr^8-ADP, nicotinamide-8-bromo-adenine dinucleotide 2′-phosphate; Ru5P, ribulose-5-phosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

10.1021/bi0613675 CCC: $33.50
Published on Web 10/03/2006

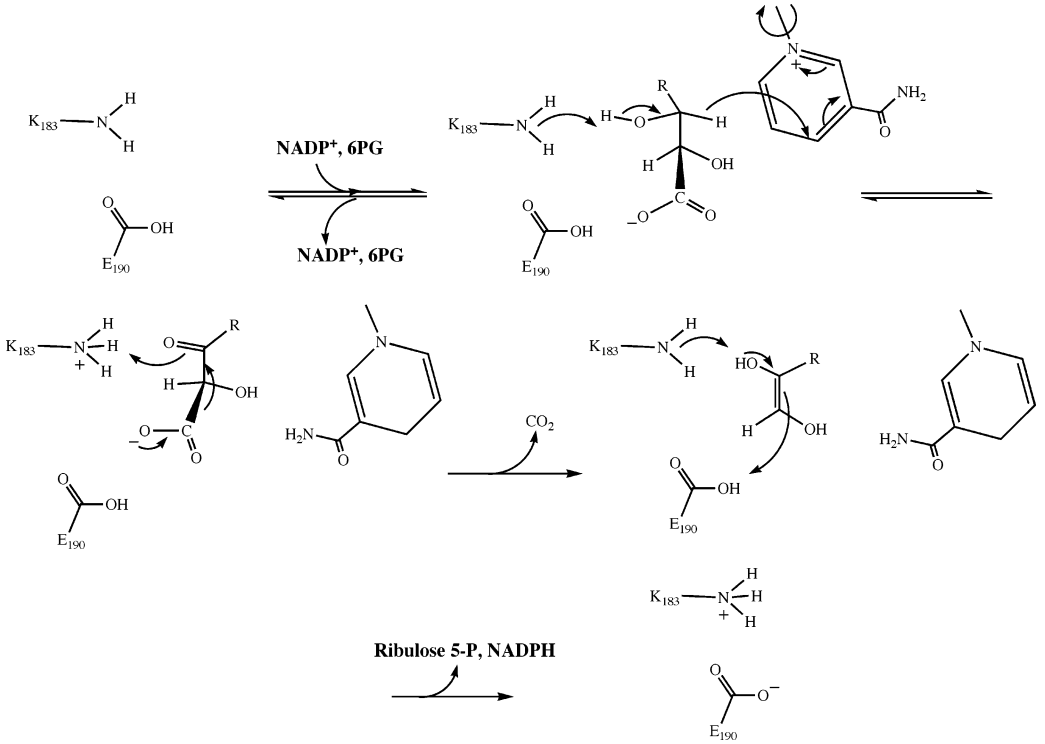


FIGURE 1: Proposed chemical mechanism for 6-phosphogluconate dehydrogenase.

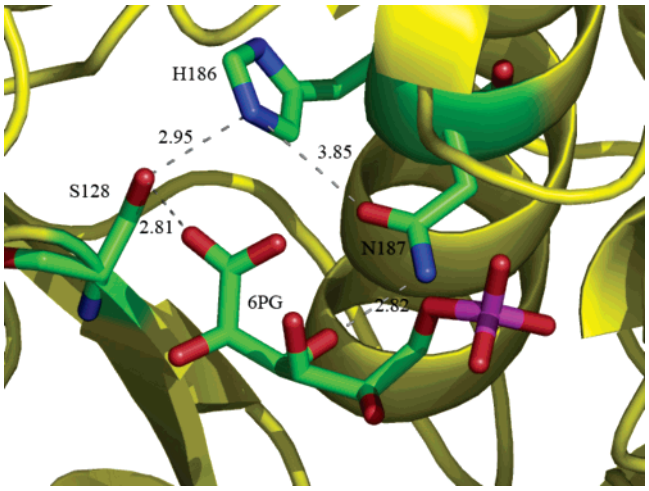


FIGURE 2: Close-up view of the active site highlighting the S128, H186, and N187 triad in the E:6PG binary complex. Residues of interest and 6PG are shown in stick representation: C, green; N, blue; O, red; P, magenta. The dashed lines represent potential hydrogen bonds between the residues of interest and the substrate. The numbers above the dashed lines are the hydrogen bond distances in Å. The figure was created using PyMOL from DeLano Scientific LLC (www.pymol.org). The accession number in the PDB is 1PGP for the E:6PG structure.

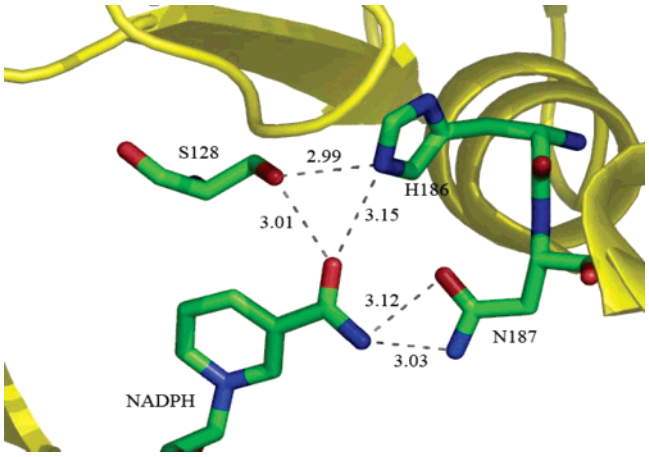


FIGURE 3: Close-up view of the active site highlighting the S128, H186, and N187 triad in the E:NADPH binary complex. Residues of interest and the nicotinamide ring of NADP are shown in stick representation, and atoms are colored as in Figure 2. The dashed lines represent potential hydrogen bonds between the residues of interest and the cofactor. The numbers above the dashed lines are the hydrogen bond distances in Å. The figure was created using PyMOL from DeLano Scientific LLC (www.pymol.org). The accession number in the PDB is 1PGO for the E:NADPH structure.

MATERIALS AND METHODS

Chemicals and Reagents. All chemicals were the highest quality available and were obtained from commercial sources as described previously (*13*). Oligonucleotide primers for mutagenesis and sequencing were from Biosynthesis, GibcoBRL, and Invitrogen.

Bacterial Strain and Plasmids. The *Escherichia coli* strain XL1-Blue was used to amplify 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 altered site II in vitro mutagenesis system from Promega was used to perform site-directed mutagenesis. Single-stranded DNA prepared from recombinant plasmid pPGDH.LC5 (*12*) was used as a template, and the synthetic oligonucleotide primers are as follows: S128A, GGGAGCGGAGTT**GCT**GGTGGAGAGGA, H186A, GTGAAGATGGTG**GCC**AACGGCATAGAG, and N187A, AAGATGGTGCAC**GCC**GGCATAGAGTAC. The mutated codon is given in bold type. Newly synthesized DNA was recovered from the recipient strain ES1301 *mutS* and subsequently transformed into JM109. The entire gene was sequenced 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 to that of the wild type 6PGDH using BLAST. Frozen stocks of strains harboring plasmid were stored in LB/ampicillin/kanamycin medium containing 15% glycerol at −80 °C.

Growth and Purification Conditions. Bacterial growth, protein expression, and purification of wild type and mutant enzymes were carried out as described previously (*13*). 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. Proteins were nearly homogeneous by SDS−PAGE.

Synthesis of 3-d-6PG. 3-*deutero*-Glucose (98 atom % D; Omicron Biochemicals, Inc.) was converted to 3-*deutero*-glucose 6-phosphate enzymatically using hexokinase as described previously (*14*). The 3-*deutero*-G6P was then oxidized with bromine to yield the final product 3-*d*-6PG, which was purified as described previously (*10*).

Initial Velocity. Initial velocity studies were performed for all mutant and wild type enzymes as described previously (*13*). Initial rates were measured at pH 7.5, 100 mM Hepes, as a function of 6PG ($0.5-5K_m$) at different fixed concentrations of NADP ($0.5-5K_m$).

The K_i for NADPH as an inhibitor competitive vs NADP was measured with 6PG equal to its K_m (E:NADP) and at a saturating ($20K_m$) concentration (E:NADP:6PG). Data were obtained for the wild type and mutant enzymes as above at pH 7.5.

Primary Deuterium Kinetic Isotope Effects. Isotope effects were measured via the direct comparison of initial velocities (*15*). Triplicate determinations of ^{D}V and $^{D}(V/K_{6PG})$ were measured varying 3-*h*-6PG or 3-*d*-6PG ($0.5-5K_m$) at saturating NADP ($40K_m$) as described previously (*25*).

^{13}C-Kinetic Isotope Effect. Effects were measured using the natural abundance of ^{13}C at the C-1 position of 6PG (*13*, *16*). High conversion (100% reaction, which represents ^{12}C/^{13}C in substrate) and low conversion samples were used to measure ^{12}C/^{13}C in the CO_2 produced from the reaction of 3-*h*-6PG or 3-*d*-6PG (*17*). From these ratios, the ^{13}C-kinetic isotope effect was calculated (*18*). 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 (*19*).

Nomenclature. Isotope effects are expressed using the nomenclature developed by Northrop (*20*) and Cook and Cleland (*21*). Deuterium and ^{13}C kinetic isotope effects are written with a leading superscript D, or 13, e.g., a primary deuterium isotope effect on V/K is written $^{D}(V/K)$. Multiple isotope effects are written with a leading superscript to depict the isotope varied, and a following subscript to depict the fixed isotope, e.g., a ^{13}C effect measured with deuterated 6PG would be written $^{13}(V/K)_D$.

Data Processing. Double reciprocal plots were used to visually inspect the data, and all plots and replots were linear. Data were fitted using the appropriate rate equations and programs developed by Cleland (*22*). Data for substrate saturation curves obtained at a fixed concentration of the second substrate were fitted using eq 1. Initial velocity patterns were fitted using eq 2. Deuterium kinetic isotope effect data were fitted using eq 3, which allows for independent isotope effects on V and V/K (*21*). In eqs 1 to 3, 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_{ia} is the dissociation constant for E:NADP. In eq 3, F_i is the fraction of deuterium label in the substrate or solvent, and E_V and $E_{V/K}$ are the isotope effects minus 1 on V and V/K.

Table 1: Summary of the Kinetic Parameters for 6PGDH Wild Type and Mutant Enzymes[a]

	wt	S128A	H186A	N187A
V/E_t (s^{-1})	9.4 ± 0.4	0.49 ± 0.05	1.40 ± 0.04	0.14 ± 0.04
fold decrease		19 ± 2	6.7 ± 0.3	67 ± 19
K_{NADP} (μM)	5 ± 1	5.4 ± 0.1	2.0 ± 0.1	29.8 ± 0.5
fold increase		1.1 ± 0.2	0.4 ± 0.2[b]	6 ± 1
K_{6PG} (μM)	28 ± 4	95 ± 8	12.1 ± 0.1	530 ± 1
fold increase		3.4 ± 0.6	0.43 ± 0.06[b]	19 ± 3
$V/K_{6PG}E_t$ (M^{-1} s^{-1})	3.4×10^5	5.2×10^3	1.2×10^5	260
fold decrease		65	3	1300
$\Delta G°$ (kcal mol^{-1})[c]	−6.2	−5.5	−6.7	−4.5
$\Delta\Delta G°$ (kcal mol^{-1})[c]		+0.7	−0.5	+1.7

[a] Values are ±SE. [b] The ratio is <1, indicating a decrease in the K_m values by 2.5-fold. [c] Values are calculated from K_{6PG}, using $\Delta G° = -RT \ln K_{6PG}$.

$$v = \frac{VA}{K_a + A} \quad (1)$$

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \quad (2)$$

$$v = \frac{VA}{K_a(1 + F_iE_V/K) + A(1 + F_iE_V)} \quad (3)$$

Calculation of ^{13}C-kinetic isotope effects was performed according to eq 4, where f is the fraction of completion of the reaction, and R_p and R_∞ are the ^{12}C/^{13}C isotopic ratios for CO_2 at partial and complete conversion, respectively. Isotope ratios, given as δ^{13}C, were calculated from eq 5, 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 with a ^{12}C/^{13}C of 0.011 237 2 (*19*).

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

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

RESULTS

Spectral Properties of Mutant Enzymes. Far UV CD spectra were recorded for all mutant and wild type enzymes, and all were identical after adjusting for protein concentration (data now shown). As a result, there are no major changes in the overall structure of the enzyme resulting from the mutation, and 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 and NADP. Data are summarized in Table 1. All three mutant enzymes exhibit a decreased V/E_t, ranging from 7- to 67-fold. No significant

Table 2: K_{iNADPH} for Wild Type and Mutant 6PGDHs[a]

	wt[d]	S128A	H186A	N187A
6PG (s)[b]	8 ± 2	19 ± 8	27 ± 6	9.3 ± 1.3
fold increase		2 ± 1	3 ± 1	
6PG (ns)[c]	1.8 ± 0.3	12 ± 1	9.5 ± 1.6	2.8 ± 0.1
fold increase		6 ± 1	5 ± 1	1.6 ± 0.3

[a] Values are ±SE. [b] s, saturating ($20K_m$). [c] ns, nonsaturating (K_m). [d] From Price and Cook (*1*).

Table 3: Summary of Isotope Effects for Wild Type and Mutant 6PGDH[a]

	wt	S128A	H186A	N187A
^{D}V	1.9 ± 0.1[b]	1.2 ± 0.1	1.5 ± 0.1	1.10 ± 0.04
$^{D}(V/K_{6PG})$	1.9 ± 0.1[b]	1.2 ± 0.2	1.4 ± 0.1	1.02 ± 0.02
$^{13}(V/K_{6PG})_H$	1.0059[b] (0.0001)[c]	1.0152 (0.0005)	1.0088 (0.0005)	1.0209 (0.0011)
$^{13}(V/K_{6PG})_D$	1.0036[b] (0.0008)	nd[d]	1.0083 (0.001)	nd

[a] Values are ±SE. [b] From ref *14*. [c] Values in parentheses are standard errors for the ^{13}C-kinetic isotope effects. [d] Not determined as a result of a deuterium isotope effect near 1.

change was observed in K_{NADP} of the S128A and H186A mutant enzymes. The K_{NADP} of the N187A mutant enzyme increases about 6-fold with respect to the value of the wild type enzyme, suggesting changes in the 6PG binding site somehow affect the binding of NADP, i.e., there is a linkage between the two sites. In contrast, K_{6PG} increases 3- and 19-fold for the S128A and N187A mutant enzymes, respectively, while it decreases about 2-fold for the H186A mutation, resulting in a decrease in $V/K_{6PG}E_t$ of 3- to 1300-fold.[2]

Inhibition Constants for NADPH. The inhibition constants for NADPH vs NADP with 6PG equal to its K_m and at $20K_m$ were measured and are given in Table 2. In all cases, K_{iNADPH} of all the mutant enzymes is greater than the value estimated for the wild type enzyme.

Kinetic Primary Deuterium Isotope Effects. The deuterium kinetic isotope effects on V and V/K_{6PG} were measured at saturating NADP ($40K_m$) (Table 3). The deuterium isotope effects are smaller than the values obtained with the wild type enzyme in all cases, and within error equal to each other, consistent with the proposed rapid equilibrium random kinetic mechanism (*1*).

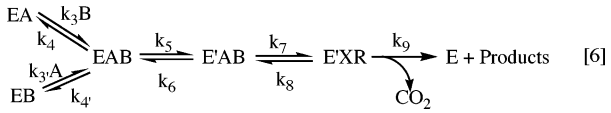
^{13}C-Kinetic Isotope Effects. Data for ^{13}C-kinetic isotope effects obtained with 3-*h*-6PG and 3-*d*-6PG for the H186A mutant enzyme are shown in Table 3. The effect with 3-*d*-6PG was not measured for the other two mutant enzymes because the deuterium isotope effects were, within error, close to 1. For all of the mutant enzymes, the value of $^{13}(V/K_{6PG})_H$ minus 1 is increased at least 3-fold compared to the value of the wild type enzyme, indicating that the decarboxylation step has become slower overall for the mutant enzymes. A stepwise mechanism with hydride transfer preceding decarboxylation predicts a $^{13}(V/K_{6PG})_D$ smaller than $^{13}(V/K_{6PG})_H$ in the case of H186A (*18*). The trend is in this direction, but the higher error on $^{13}(V/K_{6PG})_D$ makes analysis inconclusive.

[2] pH−rate profiles obtained for all three of the mutant enzymes are similar to those of the wild type enzyme with the exception that the observed pK_a values are slightly closer together. The reason for this difference is presently unknown.

DISCUSSION

The main aim of this research was to determine the importance of residues S128, H186, and N187, which interact with 6PG and NADPH, and their roles in providing ground state binding energy and proper orientation of substrates and intermediates. On the basis of the crystal structures of the E:6PG and E:NADPH binary complexes, direct and indirect interactions are suggested between the side chains of each of the three residues and the substrates. A multiple sequence alignment of 6PGDHs from a variety of species from bacteria to humans indicates a complete conservation of all of the residues considered (data not shown). Site-directed mutagenesis was used to change S128, H186, and N187 to alanine one at a time to eliminate the interaction between each of the residues and the substrates. Steady-state kinetic parameters and isotope effects were measured to determine the effect of the substitutions on the ability of 6PGDH to use 6PG and NADP as substrates.

The oxidative decarboxylation reaction catalyzed by 6PGDH is stepwise with oxidation preceding decarboxylation as suggested by multiple deuterium/^{13}C kinetic isotope effect studies (*23*). Multiple solvent deuterium/primary deuterium/primary ^{13}C kinetic isotope effects and proton inventory studies indicate the presence of an isomerization of the enzyme complex prior to hydride transfer and decarboxylation (*24*). No evidence has been found for slow steps after the decarboxylation step (*5*, *13*, *14*, *23*, *24*, *26*), and the release of CO_2 is rapid and considered concomitant with the decarboxylation step. As shown previously (*13*), the kinetic mechanism of the sheep liver 6PGDH is rapid equilibrium, the E:NADPH and E:Ru5P complexes do not build up in the steady state, and finite solvent deuterium, substrate deuterium, and ^{13}C isotope effects are observed for the native enzyme, i.e., central complex interconversion is rate-limiting (steps included from EAB to E'QR), and thus k_4 and $k_{4'}$ are both greater than k_5. Thus, once products are released the resulting free enzyme is rapidly converted to EA or EB, given saturating concentrations of NADP or 6PG. The kinetic mechanism of 6PGDH can thus be written



[6]

where A and B represent NADP and 6PG, respectively, while X and R represent 3-keto-6PG and NADPH, respectively. The rate constants k_3 and k_4 are for binding and dissociation of 6PG and $k_{3'}$ and $k_{4'}$ are for binding and dissociation of NADP, k_5 and k_6 are for an isomerization of the E:NADP: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 concomitant release of CO_2.

The equilibrium random kinetic mechanism proposed for the wt enzyme (*1*, *2*) does not change in the mutant enzymes. All of the mutant enzymes exhibit a decrease in V, and the only real possibility for a change in kinetic mechanism would be to an equilibrium ordered addition of reactants. This would have easily been observed when initial velocity patterns were performed for the mutant enzymes (the K_m for one of the reactants would be zero).

Theory for interpretation of kinetic parameters and isotope effects in the 6PGDH reaction has been developed previ-

ously, and modified forms of some of the equations are reproduced here to aid in data interpretation (*13*). In the following expressions, commitment factors assume that hydride transfer, and not decarboxylation, is the isotope sensitive step. The kinetic parameters are written in terms of commitments (*27*).

$$V = \frac{\dfrac{k_7}{1 + \dfrac{k_6}{k_5}}}{1 + c_{Vf} + c_r} \tag{7}$$

$$\frac{V}{K_{6PG}} = \frac{\dfrac{k_3 k_5 k_7}{k_4 k_6}}{1 + c_f + c_r} \tag{8}$$

$$\frac{V}{K_{NADP}} = \frac{\dfrac{k_{3'} k_5 k_7}{k_{4'} k_6}}{1 + c_f + c_r} \tag{9}$$

where

$$c_{Vf} = \frac{k_7\left(\dfrac{1}{k_5} + \dfrac{1}{k_9}\right)}{1 + \dfrac{k_6}{k_5}}, \qquad c_f = \frac{k_7}{k_6}, \qquad \text{and} \qquad c_r = \frac{k_8}{k_9}$$

A kinetic deuterium isotope effect is observed on the hydride transfer step, depicted by ${}^{D}k_7$ and ${}^{D}k_8$, which can be related by the equilibrium isotope effect, ${}^{D}K_{eq} = {}^{D}k_7 / {}^{D}k_8$ (1.18 for oxidation of a secondary alcohol (*25*)). Expressions for the primary kinetic deuterium isotope effects are given in eqs 10 and 11.

$${}^{D}V = \frac{{}^{D}k_7 + c_{Vf} + ({}^{D}K_{eq})c_r}{1 + c_{Vf} + c_r} \tag{10}$$

$${}^{D}\left(\frac{V}{K_{6PG}}\right) = \frac{{}^{D}k_7 + c_f + ({}^{D}K_{eq})c_r}{1 + c_f + c_r} \tag{11}$$

Since ${}^{D}V = {}^{D}(V/K)$ (Table 3), $c_f = c_{Vf}$, which requires that $k_9 \gg k_5$ and $k_6 \gg k_5$, i.e. the rate constant for the decarboxylation step and the reverse rate constant for the conformational change to close the site are greater than the forward rate constant for the conformational change. Thus, the expressions for ${}^{D}V$ and ${}^{D}(V/K)$ can be described by eq 11.[3] The intrinsic ^{13}C-kinetic isotope for the decarboxylation step is given by ${}^{13}k_9$. The expression for the primary ^{13}C kinetic isotope effect is given in eq 12.

$${}^{13}\left(\frac{V}{K_{6PG}}\right)_H = \frac{{}^{13}k_9 + \dfrac{1 + c_f}{c_r}}{1 + \dfrac{1 + c_f}{c_r}} \tag{12}$$

[3] The realization that $k_9 \gg k_5$ and $k_6 \gg k_5$ was not made in a previous study (*13*), but this did not affect interpretation of the data.

where ${}^{D}k_7$ is the intrinsic deuterium isotope effect on the hydride transfer step.

Reactant Binding. Since K_{6PG} is the ratio of V and V/K_{6PG}, and K_{NADP} is the ratio of V and V/K_{NADP} and taking into account $c_f = c_{Vf}$, $K_{6PG} = k_4/k_3$ and $K_{NADP} = k_{4'}/k_{3'}$. Using the above rate equations, we discuss the results for each of the mutant enzymes.

The mutant enzymes behave differently with respect to binding of 6PG, NADP, and NADPH. The S128A mutant enzyme exhibits a 3.5-fold increase in K_{6PG}, but gives no change in K_{NADP}, while the N187A mutant enzyme exhibits increases in both K_{6PG} and K_{NADP} with a 3-fold greater increase in K_{6PG}. Data suggest an increase in the off-rate constant for 6PG from E:NADP:6PG (k_4). Data for K_{6PG} are consistent with the structural data of Adams (*11*), which show that the side chains of S128 and N187 interact with 6PG in the E:6PG binary complex, Figure 2. The observed change in K_{NADP} for N187A indicates that replacement of this side chain also increases $k_{4'}$, albeit slightly. Data suggest a linkage between the two sites, which is expected since the nicotinamide ring must be close to C3, and N187 is within hydrogen-bonding distance to the C3-OH. Finally, the H186A mutant enzyme exhibits 2.5-fold decreases in the dissociation constants for E:6PG and E:NADP, and thus must decrease the off-rate constants for both NADP and 6PG. This may result from an increase in k_5, the forward rate constant for the conformational change.

Evidence for rotation of the nicotinamide ring about the N-glycosidic bond has been obtained previously from mutation of M13, which interacts with the carboxamide side chain of NADP (*5*). Each of the M13 mutant enzymes exhibited a 10-fold increase in K_{NADP}, with no change in K_{iNADPH}. As a result, the M13 mutant enzymes see the oxidized and reduced cofactor differently, in agreement with the proposed rotation of the nicotinamide ring. Values of K_{iNADPH} measured for the mutant enzymes characterized in this study are consistent with the structural data of Adams (*11*), which show that the side chains of S128 and N187 interact with 6PG in the E:6PG binary complex and the carboxamide side chain of NADPH in the E:NADPH binary complex, but do not interact with NADP in the E:NADP binary complex, Figure 3. The side chains of all of the residues studied, S128, H186, and N187, are within hydrogen-bonding distance of the carboxamide side chain of NADPH. It was thus anticipated that elimination of each of the residues would increase the K_i for NADPH. With the exception of N187A at nonsaturating 6PG, all of the mutant enzymes do exhibit an increase in K_{iNADPH} whether 6PG is saturating or nonsaturating, but the change is greater and about equal for the S128A and H186A mutant enzymes compared to N187A; 5- to 6-fold at nonsaturating and 2.5- to 3.5-fold at saturating 6PG. The N187A mutant enzyme gives no significant change unless 6PG is saturating, and then K_{iNADPH} is only increased by 1.5-fold. Thus, N187 does not appear to be as important as S128 and N187 in serving as an anchor for the carboxamide side chain of the reduced dinucleotide.

Qualitative Analysis of Kinetic and Isotope Effect Data. Both the S128A and the N187A mutant enzymes exhibit an increased K_{6PG}. V/E_t decreases by 19- and 67-fold, resulting in a $V/K_{6PG}E_t$ 65 and 1300 times lower than the wild type protein for the S128A and the N187A mutant enzymes, respectively. On the other hand, the H186A mutant enzyme

Table 4: Estimates of Commitment Factors for Wild Type and Mutant 6PGDHs

parameter	wt[a]	S128A	H186A	N187A
c_f	3	8.4	2.5	≥100
c_r	0.7	5.9	1	≥100
$(1 + c_f)/c_r$	5.7	1.6	3.5	0.9
c_f/c_r	4.3	1.4	2.5	∼1
$^{D}(V/K)^b$	1.5	1.2	1.4	1.1
$^{13}(V/K)^b$	1.006	1.016	1.009	1.021

[a] Standard errors are ≤20% for wt, ≤35% for S128A and N187A, and ≤20% for H186A. [b] Isotope effects calculated from the estimated intrinsic isotope effects ($^{D}k = 3$; $^{13}k = 1.04$) and the estimated commitment factors given in the table using eqs 11 and 12.

exhibits only a 7-fold decrease in V/E_t, resulting in a 3-fold decrease in $V/K_{6PG}E_t$ given the 2-fold decrease in K_{6PG}.

The value of $^{13}(V/K_{6PG})$ has increased from 1.0028 in the wild type enzyme to 1.0152 and 1.0209 for the S128A and N187A mutant enzymes, respectively, concomitant with a decrease in ^{D}V and $^{D}(V/K_{6PG})$ to small finite values. On the other hand, the ^{13}C-kinetic isotope effect minus 1 obtained for the H186A mutant enzyme is increased by only about 3-fold compared to the value of the wild type enzyme, while the primary deuterium isotope effect decreases to about 1.4 (a factor of about 2.5 on the isotope effect minus 1). Isotope effects for all of the mutant enzymes suggest a decrease in the rate of the decarboxylation step relative to hydride transfer with the smallest effect observed for H186A.

Quantitative Analysis of Isotope Effects for Mutant Enzymes. Estimates of ^{D}k and ^{13}k, the intrinsic isotope effects for wt 6PGDH, are 3 and 1.04 (*26*). Using these and the observed values of ^{D}V, $^{D}(V/K)$, and $^{13}(V/K)$ and eqs 11−13, estimates of the commitment factors, c_f, c_r, and c_{Vf}, were calculated and are shown in Table 4. The estimates are internally consistent, i.e., together with the estimated intrinsic isotope effects generate, within error, the observed values of the isotope effects given in Table 3. Note that, consistent with the increase in the observed values of $^{13}(V/K)$, $(1 + c_f)/c_r$ decreases from about 6 for the wt enzyme to 1.6 and 0.9 for S128A and N187A, a result of increasing forward and reverse commitments so that they are close in value to one another. This can be seen schematically in Figure 4. The barrier heights for the conformational change and the decarboxylation step are increased relative to those in the wt enzyme. The end result for S128A and N187A is (1) the closed form of the enzyme is favored, likely as a result of a decrease in the rate constant for opening the site to release reactants, k_6, and (2) reverse hydride transfer, k_8, is favored. This is much more evident in the case of the N187A mutant enzyme, which gives the largest estimated decrease in k_6 relative to k_7, ≈30-fold compared to the wt enzyme. Consistent with the estimate, the value of V/E_t is decreased by about 70-fold for this enzyme. The latter is likely a result of a decrease in the affinity of enzyme for the nicotinamide ring once it has been reduced and rotated into position to facilitate decarboxylation once C1 of 6PG has been displaced. Data are consistent with the proposed interaction of S128 and N187 with the carboxamide side chain of NADPH, Figure 3. Data are also consistent with the lower values of the deuterium isotope effects, with a value of essentially unity observed for V/K of N187A.[4]

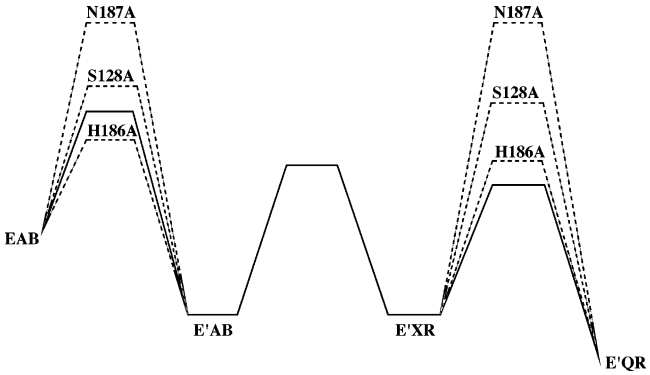


FIGURE 4: Free energy profiles. Arbitrary energy levels were chosen for the intermediates. Energy levels for the conformational change (EAB to E′AB) and the decarboxylation step (E′XR to E′QR) were made relative to the hydride transfer step (E′AB to E′XR). The solid line represents wt enzyme.

The situation is different, but no less interesting, in the case of the H186A mutant enzyme. The effect on reverse hydride transfer is less pronounced but still noticeable, consistent with its proposed interaction with the carboxamide side chain of NADPH, Figure 3. The barrier for decarboxylation has become about equal to that for hydride transfer, Figure 4. On the other hand, the relative rate constants for hydride transfer and the formation of the open conformation are opposite those observed for the other two mutations. The reverse conformational change appears more rapid and the closed form is disfavored in this case. The increase in the value of $^{13}(V/K)$ can thus be attributed to changes in the rate constants for the conformational change and reverse hydride transfer, but in a manner slightly different from that observed for the other two mutant enzymes.

Roles of the Three Residues. On the basis of the above data the S128, H186, and N187 triad are multifunctional in the 6PGDH reaction. (1) They participate in the precatalytic conformational change. Absence of the two residues that interact with 6PG, S128, and N187 causes the closed conformation to accumulate, while the absence of H186 has the opposite effect. It is thus likely that the three residues in concert help to control the equilibrium between the open and closed forms of the enzyme. How this occurs structurally is at present unknown, but attempts are now being made to obtain structural information for one or more of the mutant enzymes. (2) The triad provides ground state binding energy for 6PG and NADPH as predicted by the structural work (*11*); S128 and N187 participate in 6PG binding, and all three participate in NADPH binding. (3) The triad affects the rate of disappearance of the 3-keto-6PG intermediate such that it favors 6PG formation via reverse hydride transfer rather than decarboxylation to the 1,2-enediol of Ru5P. The third function is almost certainly coupled to the second. With the exception of the N187A mutant enzyme, which exhibits a 6-fold increase in K_{NADP}, the mutant enzymes exhibit a decreased affinity for NADPH, but no change in K_{NADP}, suggesting that the mutant enzymes treat the oxidized and reduced cofactor differently and they help to anchor the cofactor after hydride transfer accompanied by the rotation of the nicotinamide ring around the N-glycosidic bond, Figure 1. These data corroborate those obtained previously

[4] In agreement with the suggested increase in rate limitation of the conformational change, an inverse solvent deuterium isotope effect is observed for the mutant enzymes as discussed in ref *13*.

via site-directed mutagenesis at position M13, which interacts with NADP, but not NADPH (*5*).

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BI0613675