The 2′-Phosphate of NADP Is Responsible for Proper Orientation of the Nicotinamide Ring in the Oxidative Decarboxylation Reaction Catalyzed by Sheep Liver 6-Phosphogluconate Dehydrogenase

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Sheep liver 6-phosphogluconate dehydrogenase shows a high specificity for NADP, with a much lower affinity for NAD. Discrimination between NADP and NAD suggests that the interactions between the 2′-phosphate and 6-phosphogluconate dehydrogenase contribute most of the binding energy for NADP. There are three active site residues, Asn-32, Arg-33, and Thr-34, that hydrogen-bond to the 2′-phosphate of NADP according to the crystal structure of the E-Nbr8ADP complex. In this study alanine mutagenesis was used to probe the contribution of each of the three residues to binding the cofactor and to catalysis. All mutant enzymes exhibit no significant change in V/E or K_{APG} but an increase in K_{NADP} that ranges from 6- to 80-fold. All mutant enzymes also exhibit at least a 7-fold increase in the primary kinetic 13C-isotope effect —1, indicating that the decarboxylation step has become more rate-limiting. Data are consistent with significant roles for Asn-32, Arg-33, and Thr-34 in providing binding energy for NADP, and more importantly, the 2′-phosphate of NADP is required for proper orientation of the cofactor to allow rotation about the N-glycosidic bond as it is reduced in the hydride transfer step.

6-Phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.44) catalyzes the reversible oxidative decarboxylation of 6-phosphogluconate (6PG), producing ribulose-5-phosphate and CO₂ with the concomitant reduction of NADP to NADPH. The kinetic mechanism is rapid equilibrium random on the basis of a complete kinetic characterization of the enzymes from sheep liver 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 studies suggest that Lys-183 and Glu-190 are likely the general base and the general acid, respectively (3, 4). 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 rotating 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 ribulose-5-phosphate with the general base used to protonate the carbonyl oxygen. A general acid (Glu-190) is needed to facilitate the tautomerization of the enediol to ribulose-5-phosphate to the keto product (Fig. 1).

6PGDH is specific for the substrate 6PG; the best alternative substrate is 6-phosphomannionate, which has a V/E_i 10^5-fold lower and a V/K_{APG}E_i 10^5-fold lower than values obtained with 6PG (6). The enzyme also has a very high specificity toward its cofactor NADP; the V/K_{NADP}E_i is 10^5-fold lower than that obtained with NADP (7). Most of the direct interactions between the protein and the cofactor are made to the 2′-phosphate (8). Removal of the 2′-phosphate of NADP decreases the affinity more than 1000-fold (7). In the structure of the E-Nbr8ADP (the active NADP analog) binary complex, the 2′-phosphate interacts with three active site residues that are within hydrogen-bond distance: Asn-32, Arg-33, and Thr-34 (6). Multiple sequence alignment of 6PGDH shows that Asn-32 is completely conserved, whereas Arg-33 is replaced by tyrosine in the Bacillus licheniformis and Bacillus subtilis sequences, which can use NAD as a cofactor, and Thr-34 is replaced by serine in some species (Fig. 2). Data indicate that identical hydrogen-bond potential can be retained at residues 32 and 34, whereas the ionic interaction at residue 33 is likely important for binding the 2′-phosphate.

At least four hydrogen bonds have been proposed between the 2′-phosphate and the three residues discussed above, two of which are contributed by Arg-33; Fig. 3 shows these residues in the protein environment. Mutation of Arg-34 in the 6PGDH from Lactococcus lactis (the homolog is Arg-33 in sheep liver 6PGDH) to tyrosine gave a mutant enzyme that lost most of its affinity for NADP (700-fold decrease) and used NAD as a cofactor but very poorly (9). In addition to the three residues discussed above, a helix dipole (ab) is positioned 5 Å away from the 2′-phosphate and is proposed to provide an additional electrostatic interaction for the 2′-phosphate.
In this paper we investigate the role of each of the residues that interact with the 2'-phosphate of NADP. Site-directed mutagenesis was used to change Asn-32, Arg-33, and Thr-34 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 2'-phosphate of NADP. They also play a role in catalysis in the 6PGDH reaction as a result of properly orienting the cofactor as well as 6PG.

**MATERIALS AND METHODS**

**Chemicals, Reagents, Bacterial Strains, and Plasmids**—Oligonucleotide primers, chemicals, and reagents were obtained from sources described previously (10). The XL1-Blue strain of *Escherichia coli* was used to transform the mutated plasmid, and M15[pREP4] was the host strain for expression of the mutant proteins.

**Site-directed Mutagenesis**—Mutagenesis was carried out using the QuikChange® site-directed mutagenesis. Double-stranded DNA prepared from recombinant plasmid pPGDH.LC4 (11) 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.

Growth and Purification Conditions—Bacterial growth, expression, and purification of the mutant enzymes as was described previously (10). Protein concentrations were measured for all fractions using the method of Bradford (12). The wild type and mutant proteins were purified in an identical manner, and all enzymes were stored at 4 °C in the same buffer.

Synthesis of 3-d-6PG—3-deutero-Glucose was prepared and purified as described previously (10). Briefly, 3-deutero-glucose 6-phosphate was prepared via the hexokinase reaction using wild type and mutant proteins were purified in an identical manner, and all enzymes were stored at 4 °C in the same buffer.

Initial Velocity Studies—Initial rates were measured with a Beckman DU640 UV/visible spectrophotometer monitoring the appearance of NADPH ($e_{340} = 6.22 \text{ mm}^{-1} \text{ cm}^{-1}$). Temperature was maintained at 25 °C using a Beckman temperature controller. For all enzymes, the initial rate was measured as a function of 6PG (15–500 μM) at different fixed levels of NADP (1–3000 μM) dependent on the mutant enzyme. All assays were measured in 100 mM Hepes, pH 7.5. Initial velocity studies were also performed with NAD as the cofactor at a saturating concentration of 6PG (40 $K_m$).

Primary Deuteration Isotope Effects—Concentrations of 3-h-6PG and 3-d-6PG were determined enzymatically in triplicate via end-point assay using wild type 6PGDH. The concentrations from three determinations were in agreement within 2%. Primary deuteration isotope effects, $D^{12}$V and $D^{13}$V/$K_{NADP}$, were obtained by direct comparison of initial velocities in triplicate varying 3-h-6PG or 3-d-6PG at saturating concentrations of NADP (14).

pH Studies—The pH dependence of $V$ and $V/K_{NADP}$ was measured at saturating 6PG (40 $K_m$). The pH was maintained using the following buffers at 100 mM concentrations: Bis-Tris, 5–6.5; Hepes, 6.5–8.5; Ches, 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.

$^{13}$C Kinetic Isotope Effect—Isotope effects were measured as described previously (10) using the natural abundance of $^{13}$C at the C-1 position of 6PG (15). High conversion (100% reaction, which represents $^{12}$C/$^{13}$C in the substrate 6PG) and low conversion samples were used to measure the $^{13}$C/$^{12}$C isotope ratios in the CO$_2$ produced from the reaction of 3-h-6PG or 3-d-6PG (16). From these ratios, the $^{13}$C kinetic isotope effect was calculated (17).

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 (18).

Data Processing—Double reciprocal plots of the data were visually evaluated, and all plots and replots were linear. Data were fitted using the appropriate rate equations and programs developed by Cleland (19). Data for substrate saturation curves obtained at a fixed concentration of the second substrate were fitted using Equation 1. Initial velocity patterns were fitted using Equation 2. Deuterium kinetic isotope effect data were fitted using Equation 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 $K_a$ is the dissociation constant for E-NADP. In Equation 3, $F$ is the fraction of deuterium label in
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TABLE 2
Summary of the kinetic parameters for 6PGDH wild type and mutant enzymes

|                 | WT         | N32A       | R33A       | T34A       |
|-----------------|------------|------------|------------|------------|
| $V/E_V$ $(s^{-1})$ | 9.4 ± 0.4  | 9.3 ± 0.8  | 10.4 ± 0.1 | 9.9 ± 0.3  |
| $K_{smp}$ (μM)   | 5 ± 1      | 310 ± 60   | 383 ± 9    | 300 ± 0.3  |
| Fold increase    |            |            | 62         | 77         |
| $K_{rec}$ (μM)   |            |            | 28 ± 4     | 57 ± 1     |
| Fold increase    |            |            | 2          | 1          |
| $V/K_{NADP}E$ (M$^{-1}$ s$^{-1}$) | $1.9 \times 10^6$ | $3 \times 10^4$ | $2.7 \times 10^4$ | $3.3 \times 10^5$ |
| Fold decrease    |            |            | 63         | 70         |
| $\Delta G^\circ$ (kcal mol$^{-1}$) | $-7.2$ | $-4.8$ | $-4.7$ | $-6.2$ |
| $\Delta \Delta G^\circ$ (kcal mol$^{-1}$) | $2.4$ | $2.5$ | $2.5$ | $1.0$ |

*Values are calculated from $K_{NADP}$, using $\Delta G^\circ = -RT\ln(1/K_{NADP})$. Units for $1/K$ are M$^{-1}$.

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 not shown). In addition, identical tryptophan fluorescence emission spectra were obtained for all mutant and wild type enzymes upon excitation at 298 nm, indicating that the microenvironment of tryptophan residues of the proteins is the same for all enzymes (data not shown). There are 16 tryptophan residues in 6PGDH spread throughout the protein structure. 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 (15–500 μM) and NADP (1–1000 μM) dependent on the mutant enzyme. Data are summarized in Table 2. No significant change was observed in $K_{6PG}$ or in the $V/E_V$ for all three mutant enzymes with respect to the values of the wild type enzyme. $K_{NADP}$ increased from 6- to 80-fold for all mutations, resulting in a 6–80-fold decrease in $V/K_{NADP}E_p$. Data indicate elimination of either asparagine or arginine impairs cofactor binding more than it does when the threonine is replaced.

With NAD as the cofactor, $K_{NAD}$ increases by more than 1000-fold compared with $K_{NADP}$ in fact saturation by NAD cannot be achieved, and only $V/K_{NAD}E_p$ can be estimated. For the wild type enzyme, a value of $1.1 \times 10^{-7}$ M$^{-1}$ s$^{-1}$ was obtained for $V/K_{NAD}E_p$, whereas for the N32A, R33A, and T34A mutant enzymes, values of $5 \times 10^{-7}$ M$^{-1}$ s$^{-1}$, $5 \times 10^{-9}$ M$^{-1}$ s$^{-1}$, and $3.7 \times 10^{-8}$ M$^{-1}$ s$^{-1}$, respectively, were estimated. Data suggest that substitution of the three residues by alanine do not improve the ability of the enzyme to use NAD as a cofactor.

Kinetic Primary Deuterium Isotope Effects—The kinetic isotope effects on $V$ and $V/K_{6PG}$ were measured at saturating NADP (40 $K_m$) (Table 3). The 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 3-6PG and 3-6PG are shown in Table 3. For all of the mutant enzymes, the value of $^{13}(V/K_{6PG})_H - 1$ is at least 7-fold higher than that of the wild type protein. Deuteration of 6PG decreases the observed $^{13}$C kinetic isotope effects in all cases, and the equality for a stepwise mechanism with oxidation preceding decarboxylation is satisfied within error (16).

**pH Dependence of Kinetic Parameters**—The pH dependence of the kinetic parameters was measured by varying NADP at saturating levels of 6PG (40 $K_m$). All of the mutant and the wild type enzymes are stable from pH 5.5 to 9.5. For all of the mutant enzymes, a bell shaped pH-rate profile with limiting slopes of 1 and $-1$ was obtained for $V/K_{6PG}$. The pH-rate profile for $V$ is bell-shaped in the case of the N32A and R33A mutant enzymes, whereas that of the wild type and T34A mutant enzymes.
decreases only at low pH. The pKa values are summarized in Table 3. The pH-rate profiles for the wild type and R33A mutant enzyme are shown in Fig. 4 and Fig. 5.

DISCUSSION

The main aim of this research was to determine the importance of residues Asn-32, Arg-33, and Thr-34, which interact with the 2′-phosphate of NADP, and their roles in providing binding energy. Site-directed mutagenesis was used to change Asn-32, Arg-33, and Thr-34 to alanine one at a time to eliminate the interaction between each of the residues and the 2′-phosphate of NADP. Steady-state kinetic parameters and isotope effects were measured to determine the effect of the substitutions on the ability of 6PGDH to use NADP as a cofactor.

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, 13). The oxidative decarboxylation reaction catalyzed by 6PGDH is stepwise with oxidation preceding decarboxylation as suggested by multiple deuterium/13C kinetic isotope effect studies (13). Multiple solvent deuterium/13C kinetic isotope effect and proton inventory studies indicate the presence of an isomerization of the enzyme complex before hydride transfer and decarboxylation (20). The kinetic mechanism of 6PGDH can be written,

\[
\begin{align*}
E & \xrightarrow{k_1} E_{\text{ADP}} & \xrightarrow{k_{-1}} E \\
E_{\text{ADP}} & \xrightarrow{k_2} E_{\text{ATP}} & \xrightarrow{k_{-2}} E \\
E_{\text{ATP}} & \xrightarrow{k_3} E_{\text{A}} & \xrightarrow{k_{-3}} E \\
E & \xrightarrow{k_4} E_{\text{Q}} & \xrightarrow{k_{-4}} E
\end{align*}
\]

(Eq. 8)

where A and B represent NADP and 6PG, respectively, and X, Q, and R represent 3-keto-6PG, ribulose 5-phosphate, and
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NADPH, respectively. The rate constants $k_4$ and $k_5$ 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 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 release of CO$_2$. Given the rapid equilibrium nature of the mechanism, central complex interconversion is rate-limiting (steps included from EAB to E’QR) and, thus, $k_4$ and $k_5$, $k_7$, $k_8$.

The following expressions have been developed.

$$V = \frac{k_7}{1 + c_f + c_r}$$
(Eq. 9)

$$V = \frac{k_7 k_5 k_3}{k_5 k_4 k_3}$$
(Eq. 10)

$$\frac{V}{K_{NADP}} = \frac{k_3 k_5 k_7}{k_7 k_6 + k_6}$$
(Eq. 11)

$$\frac{V}{K_{6PG}} = \frac{k_7 k_5 k_3}{k_5 k_4 k_3}$$

where

$$c_f = \frac{k_7}{k_6}$$
(Eq. 12)

$\text{and}$

$$c_r = \frac{k_6}{k_9}$$
(Eq. 14)

A kinetic deuterium isotope effect is observed on the hydride transfer step, depicted by $Dk_7$ and $Dk_8$, which can be related by the equilibrium isotope effect, $D^2K_{eq} = Dk_7/Dk_8$. Expressions for the primary kinetic deuterium isotope effects are given in Equations 15 and 16.

$$\frac{D}{V} = \frac{D(V/K)}{K_{6PG}} = \frac{k_7 + c_f + (D^2K_{eq})c_r}{1 + c_f + c_r}$$
(Eq. 15)

Because $D^2V = D(V/K)$ (Table 3), $c_f = c_{vp}$ and the equation for the isotope effect on $V$ (Equation 15) is equal to that on $V/K$, i.e. Equation 16. The rate constant $k_6$ will reflect a $^{13}$C kinetic isotope effect given by $^{13}k_6$. Expressions for the primary kinetic $^{13}$C kinetic isotope and the multiple $^{13}$C/D multiple isotope effect, i.e. the $^{13}$C kinetic isotope effect with 3-d-6PG, are given in Equations 17 and 18. Commitment factors assume the hydride transfer step and not the decarboxylation step is isotope-sensitive.

\[
\frac{13^V}{K_{6PG}} = \frac{13k_7 + \frac{1}{c_f}}{1 + \frac{1}{c_f}}
\]
(Eq. 17)

\[
\frac{13^V}{K_{6PG}} = \frac{13k_7 + \left(\frac{Dk_7}{c^2_{vp}}\right)\left(\frac{1}{1 + \frac{1}{c_f}}\right)}{1 + \left(\frac{Dk_7}{c^2_{vp}}\right)\left(\frac{1}{1 + \frac{1}{c_f}}\right)}
\]
(Eq. 18)

where $Dk_7$ is the intrinsic deuterium isotope effect on the hydride transfer step, and $D^2K_{eq}$ is the equilibrium isotope effect on hydride transfer (1.18 for oxidation of a secondary alcohol (21)).

$K_{6PG}$ 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 $c_f = c_{vp}$, which requires $k_6 \gg k_5$ and $k_6 \gg k_8$.

$$K_{6PG} = \left(\frac{k_4}{k_3}\right) = K_{d-6PG}
\]
(Eq. 19)

$$K_{NADP} = \left(\frac{k_4}{k_3}\right) = K_{d-NADP}
\]
(Eq. 20)

Therefore, $K_{6PG}$ is the equilibrium constant for dissociation of 6PG from E-NADP-6PG, and $K_{NADP}$ is the equilibrium constant for dissociation of NADP from E-NADP-6PG. Using the above rate equations, we discuss the results for each of the mutant enzyme.

The key to interpretation of the data obtained for the mutant enzymes lies in changes in $K_{NADP}$, $D^2(V/K_{6PG})$ and $^{13}(V/K_{6PG})$. The decrease in $K_{NADP}$, likely results from an increase in the net off-rate for NADP from E-NADP-6PG.

Kinetic Parameters

A significant increase in $K_{NADP}$ has been obtained for all three mutant enzymes. The positively charged arginine side chain is thought to help to neutralize the charge on the 2’-phosphate; it is also responsible for all the contacts to one face of the adenine ring and, thus, forms one side of its binding pocket, shielding it from solvent (8). Changing arginine to alanine not surprisingly decreases the affinity of the mutant enzyme for NADP by >70-fold. A similar phenomenon has been observed for glutathione reductase (22), indicating that this interaction may be one of the solutions to the problem of determining the specificity of the enzyme for NADP.

In the case of Asn-32, the amide side chain donates a hydrogen bond to the 2’-phosphate, and it also interacts with the 3’-hydroxyl of the adenine ribose. The amide of Asn-32 bridges two turns between B-a-a and B-b-a (Fig. 3) and is also within hydrogen-bonding distance of the main chain nitrogen of Leu-10 and the main chain carboxyl of Thr-34, forming a hydrogen-bond network (8). Substitution of asparagine with alanine eliminates all of the above interactions, and thus, a 60-fold decrease in the affinity to NADP is not unexpected.
Similar binding energy is provided by residues Asn-32 and Arg-33 (2.4 kcal/mol and 2.5 kcal/mol, respectively). T34A exhibits the smallest change in $K_{\text{NADP}}$, indicating this residue does not contribute as much as Asn-32 and Arg-33 in providing the binding energy to NADP. If a simple sum of $\Delta G^\circ$ values could accurately be used to give the overall $\Delta G^\circ$ of NADP-binding, the three residues together contribute about 78% of the total binding energy to NADP, with about 68% derived from Asn-32 and Arg-33. Although the simple sum is not valid, data are consistent with significant roles of Asn-32, Arg-33, and Thr-34 in providing the binding energy of NADP.

**pH Studies**

The pH dependence of the kinetic parameters measured for the wild type enzyme in this study is slightly different from the data reported previously. The maximum velocity exhibits a single pK of 6.4, whereas two pK values of 5.8 and 8.8 were suggested in an earlier study, although the decrease at high pH was slight (1, 3). The difference may reflect the difference in stability of the group with the pK of 6.4, whereas two pK values are attributed to Glu-190 (acid side) and Lys-183 (basic side), which serve as general acid and general base, respectively; that is, the two residues are closer together in the case of the mutant enzymes, largely a result of an increase in the observed pK of the group with the pK on the acid side of the profile. The pK values are attributed to Glu-190 (acid side) and Lys-183 (basic side), which serve as general acid and general base, respectively; that is, the two groups exist in reverse protonation states. It is suggested that the local environment of the general acid and the general base in the mutant enzymes is more hydrophobic than they are in the wild type enzyme. It is difficult to explain the change in the hydrophilicity of the local environment unless the structures of the mutant enzymes are available.

**Kinetic Isotope Effects**

**Qualitative Analysis**—A decrease in $D^V$ and $D(V/K)$ was obtained, whereas $^{13}(V/K)_{\text{K260A}}$ is increased about 6-fold compared with the WT enzyme (Table 3). Data suggest an increase in the reverse rate of the hydride transfer step and a decrease in the rate of the decarboxylation step. Using Equations 15–17, to have decreased $D^V$ and $D(V/K)$ and an increased $^{13}(V/K)$, the reverse commitment to catalysis ($c_v / k_v$) has to increase; that is, the partition ratio of the 3-keto-6-phosphogluconate changes to favor 6PG. Given the increase in $c_v$ and in order that $V$ for the mutant enzymes remain unchanged compared with that of the WT enzyme, another rate process must increase to compensate. The obvious choice is $k_r$ since $k_r / k_s$ determines the equilibrium constant for the hydride transfer step, which is unlikely to change as a result of any of the mutations.

**Quantitative Analysis**—A quantitative analysis of the data supports the above conclusions and extends the interpretation of the isotope effects. The largest primary $^{13}$C kinetic isotope effects were observed for enzymes with mutations in the 6-phosphate site, and the largest of these was 1.0397, measured for the K260A mutant enzyme (10). Pre-steady-state kinetic studies of the K260A mutant enzyme give a full stoichiometric burst of NADPH production, and thus, $^{13}k \approx 1.04$. A primary deuterium kinetic isotope effect of 2.5 was observed for the M131 mutation in 6PGDH (5). In addition, a quantitative analysis of multiple $^{13}$C/D isotope effects for the wild type enzyme published previously (13) allowed estimates of the value of commitment factors and intrinsic isotope effects. For values of $^{13}k$ from 1.025–1.1, the value of $^{13}k$ varied over a narrow range, 2.8–3.4, with a value of about 3 estimated for a $^{13}k$ value of 1.04. Using these estimates of $^{13}k$ and $D_k$, estimates of $c_j$, $c_v$, and $c_{ij}$ should be obtained that satisfy Equations 15–17 and generate the observed isotope effects for all three mutant enzymes, assuming the transition states for hydride transfer and decarboxylation do not change significantly. Because $D^V = D^V(V/K)$, $c_j = c_{ij}$; thus, only $c_j$ will be discussed. Values of the parameters of interest are given in Table 4, and all satisfy Equations 15–17.

As can be seen from Table 4, the reverse commitment does indeed increase consistent with partitioning of the 3-keto-6PG intermediate in favor of 6PG. In addition, $c_j \leq c_v$; a value of almost 4 is obtained for $c_j / c_v$ for the WT enzyme, whereas values range from 0.4 to 1 for the mutant enzymes. Overall, this results in a decrease in the commitment for the primary $^{13}$C kinetic isotope effect, $(1 + c_j / c_v)$, from 6 for WT to 0.9–1.7 for the mutant enzymes.

**TABLE 4**

| Parameter | WT | N32A | R33A | T34A |
|-----------|----|------|------|------|
| $c_j$     | 3  | 1.5  | 1.6  | 0.85 |
| $c_r$     | 0.7| 2.3  | 1.5  | 2.1  |
| $(1 + c_j)/c_r$ | 5.7| 1.1  | 1.7  | 0.88 |
| $c_j/c_r$ | 4.3| 0.7  | 1    | 0.4  |

**FIGURE 6.** Free energy diagram for WT and mutant 6PGDHs. Energy levels of the intermediates are arbitrary, whereas those for the conformational change ($EAB$ to $E'AB$) and the decarboxylation step ($E'XR$ to $E'QR$) are relative to the hydride transfer step ($E'AB$ to $E'XR$). The solid line represents WT enzyme, whereas 1, 2, and 3 represent the profiles for N32A, R33A, and T34A, respectively.
enzymes. This can be shown in terms of a free energy diagram (Fig. 6). Note that for each of the mutant enzymes the barrier, relative to hydride transfer, decreases for the conformational change and increases for decarboxylation. However, the overall barrier height for the reaction does not change significantly as required given the lack of change in \( V/E \), (Table 2). In fact, the highest barrier is slightly lower than that for the WT enzyme in the case of the R33A and T34A mutant enzymes, in agreement with their slightly higher \( V/E \) values. Thus, it is the change in \( c_f/c_r \) not just the increase in \( c \), that generates the large increase in \( 15^V(V/K) \) with a smaller decrease in \( D^V(V/K) \). The change in \( c_f \) is almost certainly attenuated as a result of an increase in \( k_T \) as suggested above.

**Conclusions**

We have recently provided evidence that reduction of the nicotinamide is accompanied by rotation of the ring about the \( N \)-glycosidic bond to a position that was occupied by the 1-carboxylate of 6PG (Fig. 1). The end result would be elimination of the hydrogen-bonding interaction between Glu-190 and the 1-carboxylate, facilitating decarboxylation and resulting in the \( re \) face of the nicotinamide ring being exposed to the active site (the stereochemistry of hydride transfer is to the \( si \) face). It is likely that changing the interaction with the 2'-phosphate by eliminating the side chain of Asn-32, Arg-33, or Thr-34 decreases the ability of the nicotinamide ring to rotate. This would result in a stabilization of the nicotinamide ring in the optimum position for hydride transfer, increasing the partitioning of the 3-keto-6PG toward 6PG compared with ribulose 5-phosphate. This aspect will require further experimentation.

Results obtained for the effects of the mutations on 6PG binding are not surprising; the three residues mutated provide most of the binding energy for NADP. However, these same changes at the 2'-phosphate site, which is remote from the active site (~15 Å), change the relative rates of hydride transfer and decarboxylation. The effect on partitioning of the intermediate provides additional, albeit indirect, evidence in support of the rotation of nicotinamide ring to facilitate decarboxylation. Data suggest that removal of any of the three residues may result in a positional change of the bound NADP, which may reorientate the nicotinamide ring of NADP to a position that disfavors ring rotation, but favors hydride transfer. This is not to say that the equilibrium constant for hydride transfer changes; rather, a change occurs in the amount of the enzyme form that undergoes the hydride transfer step.

In conclusion, the 2'-phosphate of NADP is critical for making critical interactions between the protein and the cofactor, helping position the cofactor for hydride transfer and participating in the rotational isomerization of the cofactor after reduction, probably by holding the cofactor in position so that the nicotinamide ring can flip into the right location. Destroying the interactions between the 2'-phosphate and the protein results in a slower or less efficient rotation. Attempts are now being made to obtain structures of one or more of the mutant enzymes in complex with NADP.

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**REFERENCES**

1. Price, N., and Cook, P. F. (1996) *Arch. Biochem. Biophys.* **336**, 215–223
2. Berdis, A. J., and Cook, P. F. (1993) *Biochemistry* **32**, 2036–2040
3. Karsten, W. E., Chooback, L., and Cook, P. F. (1998) *Biochemistry* **37**, 15691–15697
4. Zhang, L., Chooback, L., and Cook, P. F. (1999) *Biochemistry* **38**, 11231–11238
5. Cervellati, C., Dallocchio, F., Bergamini, C. M., and Cook, P. F. (2005) *Biochemistry* **44**, 2432–2440
6. Berdis, A. J., and Cook, P. F. (1993) *Biochemistry* **32**, 2041–2046
7. Berdis, A. J., and Cook, P. F. (1993) *Arch. Biochem. Biophys.* **305**, 551–558
8. Adams, J. M., Grant, H. E., Gover, S., Naylor, C. E., and Philips, C. (1994) *Structure* **2**, 661–666
9. Tetaud, E., Hanau, S., Wells, J. M., Page, R. W. F., Adams, J. M., Arkinson, S., and Barrett, M. P. (1999) *Biochem. J.* **338**, 55–60
10. Li, L., Dworzkowski, F. S. K., and Cook, P. F. (2006) *J. Biol. Chem.* **281**, 25568–25576
11. Chooback, L., Price, N. E., Karsten, W. E., Nelson, J., Sundstrom, P., and Cook, P. F. (1998) *Protein Expression Purif.* **13**, 251–258
12. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
13. Hwang, C. C., Berdis, A. J., Karsten, W. E., Cleland, W. W., and Cook, P. F. (1998) *Biochemistry* **37**, 12596–12602
14. Parkin, D. W. (1991) *Enzyme Mechanism from Isotope Effects* (Cook, P. F., ed) pp. 284–285, CRC Press, Inc., Boca Raton, FL
15. O’Leary, M. H. (1980) *Methods Enzymol.* **64**, 83–110
16. Weiss, P. M. (1991) *Enzyme Mechanism from Isotope Effects* (Cook, P. F., ed) pp. 291–311, CRC Press, Inc., Boca Raton, FL
17. Hermes, J. D., Roeske, C. A., O’Leary, M. H., and Cleland, W. W. (1998) *Biochemistry* **37**, 5106–5114
18. Craig, N. (1957) *Geochim. Cosmochim. Acta* **12**, 133–149
19. Cleland, W. W. (1979) *Methods Enzymol.* **63**, 103–108
20. Hwang, C. C., and Cook, P. F. (1998) *Biochemistry* **37**, 15698–15702
21. Cook, P. F., Blanchard, J. S., and Cleland, W. W. (1980) *Biochemistry* **19**, 4853–4858
22. Karplus, P., and Schulz, G. (1989) *J. Mol. Biol.* **210**, 163–180