Articles

Lysine 183 Is the General Base in the 6-Phosphogluconate Dehydrogenase-Catalyzed Reaction†

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ABSTRACT: Site-directed mutagenesis was used to change K183 of sheep liver 6-phosphogluconate dehydrogenase to A, E, H, C, Q, R, and M to probe its possible role as a general base catalyst. Each of the mutant proteins was characterized with respect to its kinetic parameters at pH 7 and the pH dependence of kinetic parameters for the K183R mutant enzyme. The only mutant enzyme that gives a significant amount of catalysis is the K183R mutant, and the extent of catalysis is decreased by about 3 orders of magnitude; the general base p*K* is perturbed to a pH value of >9. All other mutant enzymes exhibit rates that are decreased by about 4 orders of magnitude compared to that of the wild-type enzyme. Data are consistent with the general base function of K183.

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

Data have shown that the oxidative decarboxylation of 6PG by 6PGDH to the 1,2-enediol of ribulose 5-phosphate proceeds via a stepwise mechanism, with hydride transfer preceding decarboxylation under all conditions (*3*, *4*). The enediol is then tautomerized to give ribulose 5-phosphate. A general base−general acid mechanism has been suggested (Scheme 1) on the basis of the pH dependence of kinetic parameters (*5*, *6*). The three-dimensional structure of the 6PGDH from sheep liver has been determined as the apoenzyme, and in the presence of 6PG, NADP, and NADPH (*7*, *8*). Recent evidence from site-directed mutagenesis (*9*) strongly indicates that glutamate 190, which is completely conserved in all species for which sequences are now available, is the general acid. Data are consistent with its location in the binary E−6PG complex (*8*), within hydrogen-bonding distance of the substrate carboxylate, and its change in location is dependent on whether NADP or NADPH is bound.

The general base accepts a proton from the 3-hydroxyl of 6PG concomitant with hydride transfer, and then shuttles the proton between itself and the sugar oxygen throughout the reaction, ultimately accepting it as ribulose is formed. The most reasonable candidate for the general base is lysine 183, suggested to be present in the neutral form for optimum binding of 6PG (*6*) and within hydrogen-bonding distance of the 3-hydroxyl of 6PG (Figure 1; *8*). Lysine 183 is completely conserved in all species for which sequences are now available (Table 1).

In this paper, site-directed mutagenesis is used to change lysine 183 to a number of other amino acids so that either it could no longer serve as a general base or its p*K* would be significantly perturbed. Data support the assignment of the lysine side chain at position 183 in the sheep liver 6PGDH as the general base. Implications of the assignment are discussed.

MATERIALS AND METHODS

Chemicals and Reagents. Mutagenesis and sequencing primers were from either Biosynthesis or Gibco-BRL. The Altered Sites II in vitro mutagenesis system and the fmol DNA cycle sequencing system were purchased from Promega. The Geneclean II Kit was from Bio 101, Inc. The DNA molecular weight ladder was from New England Biolabs. Restriction endonucleases, Taq DNA polymerase, T_4 DNA ligase, and IPTG were purchased from Gibco-BRL. PFU polymerase was from Stratagene, and deoxynucleoside triphosphates were from Perkin-Elmer. T_4 kinase, protein molecular mass markers, and *Escherichia coli* strain JM 109 were from Promega. The QIAexpress type IV kit was purchased from QIAGEN. The Bio-Rad protein assay kit was used to determine protein concentrations. Ampicillin, kanamycin, and 6-phosphogluconic acid were from Sigma, and NADP was from U.S. Biochemicals. Hepes, Bis-Tris, and Ches buffers were from Research Organics Inc. All other

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[1] Abbreviations: IPTG, isopropyl β-D-thiogalactopyranoside; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; 6PG, 6-phosphogluconate; 6PGDH, 6-phosphogluconate dehydrogenase; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; BTP, [bis(2-hydroxyethyl)-amino]tris(hydroxymethyl)propane; Ches, 2-(*N*-cyclohexylamino)-ethanesulfonic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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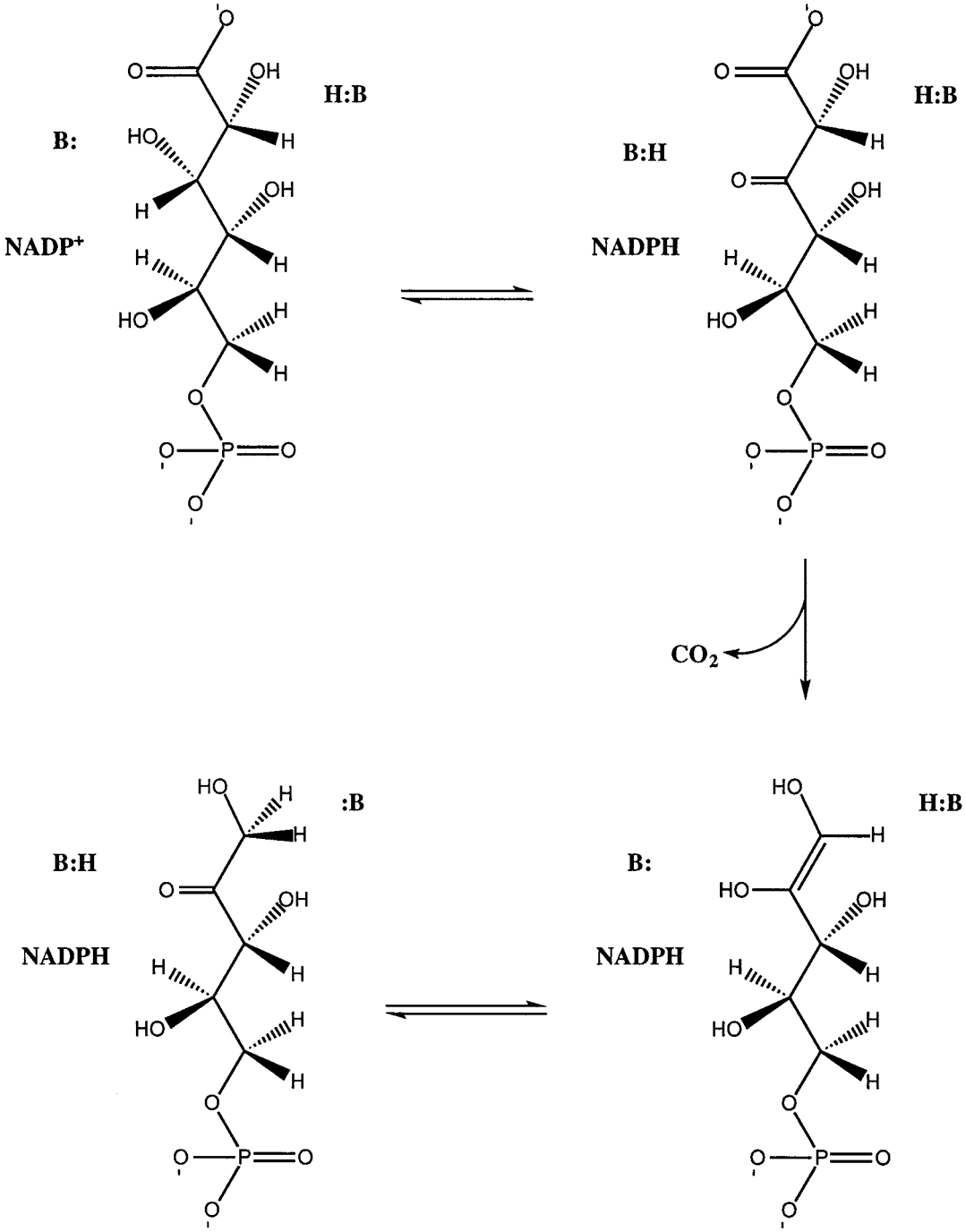
Scheme 1: Proposed Chemical Mechanism for 6-Phosphogluconate Dehydrogenase



chemicals were the highest quality available.

Bacterial Strains and Plasmids. The *E. coli* strain JM 109 was used as the host strain for plasmids containing the 6PGDH cDNA with amino acid changes at lysine 183, while M15[pREP4] was the host strain for expression of the mutant proteins. The plasmid pAlter-1 was used as the mutagenesis vector, and plasmid pQE-30 was the expression vector.

Cloning of 6-PGDH into the pAlter-1 Vector. Cloning of the wild-type 6-PGDH cDNA into the mutagenesis vector pAlter-1 was carried out as previously described (*9*). The resulting construct, pPGDH.LC5, was used as the template of the site-directed mutagenesis.

Site-Directed Mutagenesis. Site-directed mutagenesis was performed using the Altered Site II in vitro mutagenesis system. Site-directed mutagenesis was performed on single-stranded DNA prepared from the above clone using the Altered Sites II in vitro mutagenesis system and the synthetic oligonucleotide primers listed in Table 2. Newly synthesized DNA was then recovered from the recipient strain ES1301 *mutS* and subsequently transformed into JM109. Mutations were identified by sequencing through the mutation site. The mutated plasmids were designated as K183A.PAlter, K183E.PAlter, K183H.PAlter, K183R.PAlter, K183C.PAlter, K183M.PAlter, and K183Q.PAlter.

Subcloning of the Mutants into the pQE-30 Expression Vector. Two synthesized oligonucleotides containing the desired restriction sites were constructed. One primer, 5′-ACTATAGGGCGCATGCATGGCCCAAG-3′, creates a *Sph*I

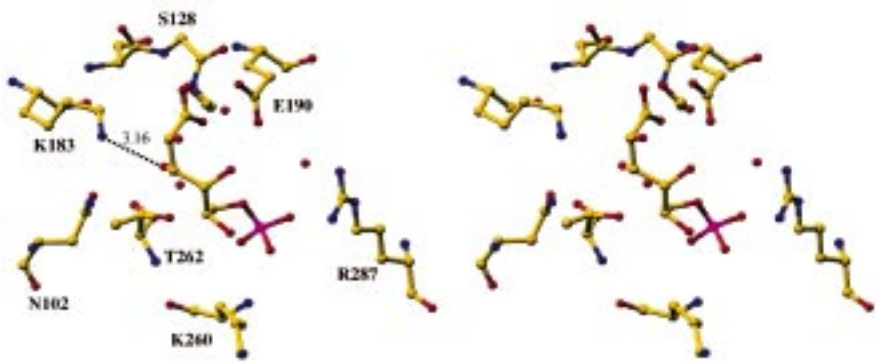


FIGURE 1: Stereopair of a portion of the 6PG binding site of sheep liver 6-phosphogluconate dehydrogenase. The proposed acid−base catalytic groups are labeled. 6-Phosphogluconate is shown with its carboxylate to the right and its 6-phosphate to the right. Serine 128 is shown immediately above the 1-carboxylate of the substrate, while K183 is shown hydrogen-bonded to the 3-hydroxyl.

Table 1: Alignment of 6PGDH Sequences at the Active Site, Including K183 and E190

Species	Sequence around K183 and E190	Ref.
Citobacter amalonaticus	HYV**K**MVHNGI **E**YGDMQLIAE	9
Citobacter diversus	HYV**K**MVHNGI **E**YGDMQLIAE	9
Citobacter freundii	HYV**K**MVHNGI **E**YGDMQLIAE	9
Escherichia vulneris	HYV**K**MVHNGI **E**YGDMQLIAE	9
Shigella boydii	HYV**K**MVHNGI **E**YGDMQLIAE	9
Shigella dysenteriae	HYV**K**MVHNGI **E**YGDMQLIAE	9
Shigella sonnei	HYV**K**MVHNGI **E**YGDMQLIAE	10
Shigella flexneri	HYV**K**MVHNGI **E**YGDMQLIAE	11
Escherichia coli	HYV**K**MVHNGI **E**YGDMQLIAE	12
Salmonella typhimurium	HYV**K**MVHNGI **E**YGDMQLIAE	13
Klebsiella planticola	HYV**K**MVHNGI **E**YGDMQLIAE	10
Klebsiella terrigena	HYV**K**MVHNGI **E**YGDMQLIAE	10
Klebsiella pneumoniae	HYV**K**MVHNGI **E**YGDMQLIAE	14
Escherichia coli	HYV**K**MVHNGI **E**YGDMQLIAE	15
Bacillus subtilis	HYV**K**MVHNGI **E**YGDMQLISE	16
Bacillus licheniformis	HFV**K**MVHNGI **E**YADMQLIAE	17
Bacillus subtilis	HFT**K**MVHNGI **E**YADMQLIAE	18
Actinomycetemcomitans	HFV**K**MVHNGI **E**YGDMQLICE	19
Haemophilus influenzae	HFV**K**MVHNGI **E**YGDMQLICE	20
Saccaromyces cervisiae	HYV**K**MVHNGI **E**YGDMQLICE	21
Ceratitis capitata	HFV**K**MVHNGI **E**YGDMQLICE	22
Drosophila melanogaster	HFV**K**MVHNGI **E**YGDMQLICE	23
Drosophila simulans	HFV**K**MVHNGI **E**YGDMQLICE	24
Homo sapiens (Human)	HFV**K**MVHNGI **E**YGDMQLICE	25
Ovis aries (Sheep)	HFV**K**MVHNGI **E**YGDMQLICE	26
Synechococcus Sp.	HYV**K**MVHNGI **E**YGDMQLIAE	27
Synechocystis Sp.	HYV**K**MVHNGI **E**YGDMQLIAE	28
Trypanosoma brucei brucei	SCV**K**MYHNSG **E**YAILQIWGE	29
Bacillus subtilis	HFL**K**MIHNGI **E**YGMMAAIGE	16
consensus	HYV**K**MVHNGI **E**YGDMQLIAE	

restriction site at the start of the gene, and the other one, 5′-TGTAGAGTTGAAGCTTGGAACAGAAG-3′, contains a *Hin*dIII site at the end of the gene. The two sites were introduced into the gene containing the desired mutation using polymerase chain reaction. The PCR product was subcloned into the pQE30 vector through the corresponding sites, with the recombinant DNA transformed into bacterial strain M15. Transformants were selected on LB/Amp/Kan plates. The resulting plasmids were designated as K183A.pQE30, K183E.pQE30, K183H.pQE30, K183R.pQE30, K183C.pQE30, K183M.pQE30, and K183Q.pQE30. The entire gene containing the mutation was sequenced to ensure the integrity of the cDNA. The pQE-30 vector adds a six-His tag to the N-terminus of the 6PGDH, which has

Table 2: Sequence of Mutagenic Oligonucleotides[a]

WT	ACACTTTGTGAAGATGGTGCACA
K183A	ACACTTTGTGGCGATGGTGCACA
K183E	ACACTTTGTGGAGATGGTGCACA
K183H	ACACTTTGTGCATATGGTGCACA
K183C	ACACTTTGTGTGTATGGTGCACA
K183R	ACACTTTGTGCGGATGGTGCACA
K183M	ACACTTTGTGATGATGGTGCACA
K183Q	ACACTTTGTGCAGATGGTGCACA

[a] The mutation site is underlined.

no effect on the enzyme's activity (*30*).

Expression and Purification of the Mutant Proteins. The expression and purification were performed using the QIAexpress kit. The bacterial strains containing the correct mutated plasmid were grown at 30 °C in 20 L of LB/Amp/Kan medium, and then induced with IPTG to a final concentration of 0.5 mM when the A_{600} reached 0.7−0.9. After growing for an additional 4.5 h, the cells were harvested by centrifugation at 7000 rpm for 15 min. The cell paste was resuspended in 3 volumes of sonication buffer [50 mM sodium phosphate (pH 8.0), 300 mM NaCl, and 10 mM β-mercaptoethanol], sonicated for 2 min on ice, and centrifuged at 13000*g* to pellet cell debris. The sonication supernatant was then bound to Ni−NTA resin, washed with sonication buffer, and finally eluted with an imidazole gradient (0 to 0.4 M). Protein concentrations were measured for all fractions (*31*), and fractions corresponding to the peak were collected. The mutant enzymes were then precipitated by 75% ammonium sulfate and stored at 4 °C. All mutant enzymes were analyzed via SDS−PAGE, and a purity of 95% was obtained.

Initial Velocity Studies. Initial velocity studies were performed using a HP 8453 diode array spectrophotometer, with the appearance of NADPH ($\epsilon_{340} = 6220\ M^{-1}\ cm^{-1}$) at 340 nm being monitored. Reactions were carried out in 1 mL volumes using 1 cm path length cuvettes. An initial velocity pattern was obtained at 100 mM Hepes (pH 7.0) using variable concentrations of 6PG (50−500 μM) and NADP (25−250 μM).

pH Studies. Initial velocity patterns for K183R were measured at pH 5.5 and 10.5 to obtain estimates of the kinetic parameters at the extremes of pH. The pH dependencies of V and V/K_{6PG} for the K183R mutant were carried out by measuring the initial velocity at 6PG concentrations around its K_m and a saturating (1 mM) concentration of NADP, as a function of pH over the range of 6−10. The pH was maintained using the following buffers at concentrations of 100 mM: BTP, pH 5.5−6.5; Hepes, pH 6.5−8.5; and Ches, pH 8.5−10.5. Sufficient overlap was obtained as buffers were changed to rule out any nonspecific effects. The pH value was recorded before and after the initial velocity was measured.

Data Processing. Reciprocal initial velocities were plotted against reciprocal substrate concentrations. Data were fitted to the appropriate rate equations [using Basic versions of the computer programs developed by Cleland (*32*)]. Initial velocity data were fitted using eq 1. For pH studies, substrate saturation curves were fitted using eq 2. Data for pH profiles with one ionization on the acid side were fitted using eq 3.

$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB) \quad (1)$$

$$v = VA/(K_a + A) \quad (2)$$

$$\log y = \log[C/(1 + H/K_1)] \quad (3)$$

$$\log y = \log[C/(1 + H/K_1 + K_2/H)] \quad (4)$$

In eqs 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 Michaelis constants for NADP and 6PG, respectively, and K_{ia} is the dissociation constant for NADP. In eqs 3 and 4, y is the value of the parameter of interest, C is the pH-independent value of y, H is the hydrogen ion concentration, and K_1 and K_2 are the acid dissociation constants for enzyme or substrate functional groups that are important in a given protonation state for optimal binding and/or catalysis.

RESULTS

Gross Structure of the Mutant Enzymes. To determine whether the mutation of K183 resulted in a loss of overall structural integrity, far-UV CD spectra were measured using an Aviv 62 DS spectropolarimeter. Additionally, the far-UV spectra could be superimposed once corrected to the same protein concentration. In all cases, the mutant proteins were expressed at a level equal to that of the wild-type (WT) enzyme. Thus, changes in structure, if any, must be localized to the active site.

Kinetic Parameters of the Mutant Proteins. Initial velocity patterns were obtained at pH 7 by measuring the initial rate as a function of NADP concentration at a fixed 6PG concentration, and then repeating the experiment at several different fixed 6PG concentrations. The activity was very low for some of the mutant enzymes, and there was not enough enzyme available to determine complete initial velocity patterns; in these cases, the 6PG saturation curve was obtained at saturating NADP concentrations. The kinetic parameter expected to show the most dramatic effect of the mutation is V/K_{6PG} since K183 is thought to interact with the 3-hydroxyl of 6PG. To ensure that NADP was saturating, individual rates were repeated at twice the concentration of NADP, with no change observed. Data are summarized in Table 3.

The value of V/E_t is decreased by 10^3−10^4-fold for all of the mutant proteins compared to that of the wild-type enzyme. In all cases except K183A, K183R, and K183M, K_{6PG} is within error identical to the value measured for the WT enzyme. Of the K_{NADP} values that were measured, only that for K183R increases significantly compared to that of the wild type. Decreases in V/K_{6PG} and V/K_{NADP} are either similar to or larger than changes in V. In the case of the K183M and K183R mutant proteins, the increase in K_{6PG} likely suggests a decreased affinity for 6PG as a result of steric interference by the bulky guanidinium group of the arginine side chain or the sulfur of the methionine side chain. All of the other mutations, with the exception of alanine, will potentially allow a hydrogen bond to the 3-hydroxyl that is similar to that of the WT enzyme.

pH Dependence of Kinetic Parameters. The pH dependence of kinetic parameters should provide the best indicator of the general base capability of K183, since they give a direct measure of the pK value for the general base

Table 3: Kinetic Parameters for K183 Mutant 6-Phosphogluconate Dehydrogenases[a]

	K_{6PG} (μM)	K_{NADP} (μM)	V/E_t (s^{-1})	$V/K_{6PG}/E_t$ (M^{-1} s^{-1})	$V/K_{NADP}/E_t$ (M^{-1} s^{-1})
WT[b]	36 ± 15	2 ± 1	3.5 ± 0.1	$(1 \pm 0.4) \times 10^5$	$(1.8 \pm 0.6) \times 10^6$
K183A	150 ± 30 (4 ± 2)	−	$(2.5 \pm 0.3) \times 10^{-4}$ ($14\,000 \pm 1700$)	1.7 ± 4 ($59\,000 \pm 27\,000$)	−
K183E	26 ± 3	8 ± 4 (4 ± 3)	$(4.7 \pm 0.1) \times 10^{-4}$ (7500 ± 300)	18 ± 2 (5600 ± 2300)	70 ± 40 ($25\,000 \pm 17\,000$)
K183H	80 ± 40 (2 ± 1)	9 ± 4 (4 ± 2)	$(6.2 \pm 0.8) \times 10^{-4}$ (5600 ± 750)	7 ± 3 ($14\,300 \pm 8400$)	100 ± 40 ($17\,500 \pm 9300$)
K183C	45 ± 2	−	$(3.9 \pm 0.05) \times 10^{-4}$ (9000 ± 300)	8.8 ± 0.4 ($11\,400 \pm 4600$)	−
K183Q	27 ± 5	−	$(7.2 \pm 0.3) \times 10^{-4}$ (4900 ± 250)	27 ± 4 (3700 ± 1600)	−
K183R	220 ± 30 (6 ± 3)	42 ± 8 (20 ± 11)	$(4.4 \pm 0.3) \times 10^{-3}$ (800 ± 60)	19 ± 1 (5300 ± 2100)	100 ± 10 ($17\,500 \pm 6300$)
K183M	300 ± 100 (8)	−	$(4.1 \pm 0.6) \times 10^{-4}$ (8500 ± 1250)	1.4 ± 0.4 ($70\,000 \pm 35\,000$)	−

[a] Values in parentheses represent the *x*-fold increase in K_m or *x*-fold decrease in *V* and *V*/*K* compared to those of the wild-type enzyme. [b] From ref *30*.

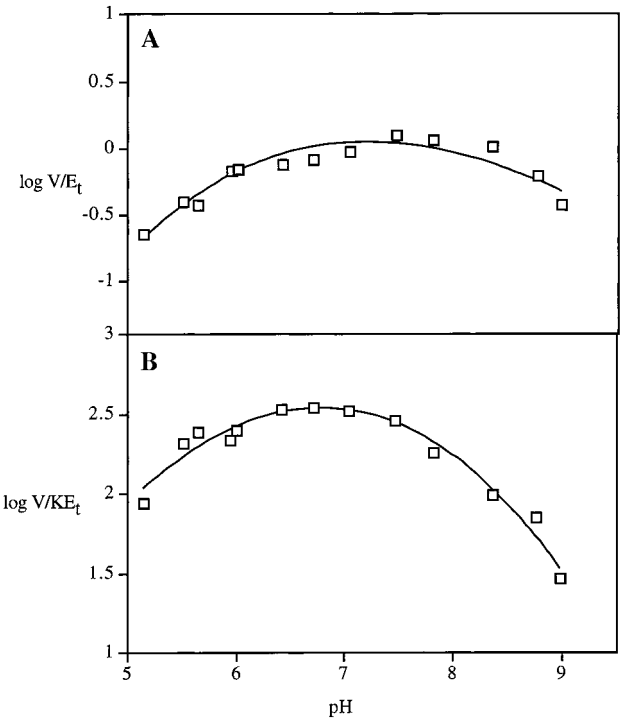


FIGURE 2: pH dependence of kinetic parameters for wild-type 6-PGDH. Data were obtained for *V* (A) and V/K_{6PG} (B). The points shown are the experimentally determined values, and the curves are from a fit of the data using eq 4.

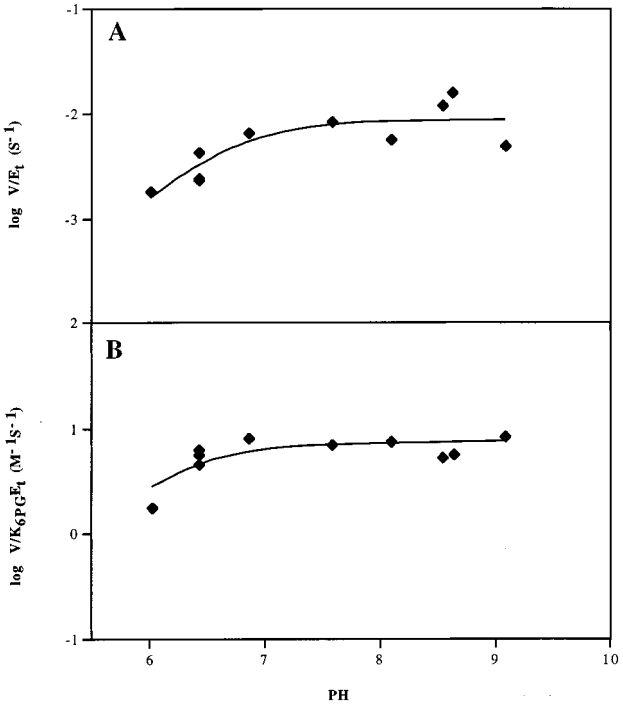


FIGURE 3: pH dependence of kinetic parameters for the K183R mutant of 6-PGDH. Data were obtained for V/E_t (A) and $V/K_{6PG}E_t$ (B). The points shown are the experimentally determined values, and the curves are from a fit of the data using eq 3.

functionality of 6PGDH (*5*, *6*). Because of the very low activity of most of the mutant proteins, the pH dependence of *V* and V/K_{6PG} was measured for only the K183R mutant, which has an 800-fold lower V/E_t value. The pH−rate profiles for K183R are shown in Figure 2. The *V* and V/K_{6PG} decrease below p*K* values of 6.2 ± 0.2 and 6.8 ± 0.2, respectively. In comparison, the pH−rate profiles for the WT enzyme are bell-shaped (*9*). The *V* profile exhibits p*K* values of 5.8 ± 0.1 and 8.8 ± 0.1, while the V/K_{6PG} profile exhibits p*K* values of 5.6 ± 0.1 and 8.0 ± 0.1. pH-independent values of V/E_t and $V/K_{6PG}E_t$ for K183R are $(9 \pm 2) \times 10^{-3}$ s^{-1} and 7.4 ± 0.9 M^{-1} s^{-1}, respectively.

Kinetic Deuterium Isotope Effect. To determine which step is impaired in the case of the K183R mutant, the kinetic deuteriun isotope effect was assessed using the method of direct comparison of initial velocities, varying the concentration of 6PG-3-(h,d). Data were only obtained for the R mutant because its activity is higher than those of other K183 mutant enzymes. The kinetic deuterium isotope effects on *V* and V/K_{6PG} are within error equal to 1.

DISCUSSION

As shown in Table 1, K183 is completely conserved in 6PGDH from all species for which a primary sequence has been determined. It is likely then that the lysine side chain is important to the mechanism of 6PGDH, especially since it is found in the active site within hydrogen-bonding distance of the 3-hydroxyl of 6PG (Figure 1) (*8*). An overlay of the three-dimensional structures of the E and E−NADP, E−NADPH, and E−6PG complexes (*7*, *8*) indicates no gross conformational changes throughout the structure, and only very slight changes in side chain positions within the active site (data not shown). Thus, the stereo representation shown in Figure 1 is a reasonable view of the interactions of K183 with the 3-hydroxyl of 6PG at the active site. Finally, since there is also no change in the position of K183 with NADPH bound, it is likely that the position of the side chain is fixed (with the possible exception of small changes in the K183 N^{ϵ} to 6PG O^{β} hydrogen-bonding distances) in all intermediates formed along the reaction pathway.

Interpretation of Kinetic Data. A minimal kinetic mechanism for 6PGDH (*4*−*6*, *33*), including 6PG binding, and

all three of the catalytic steps is given in eq 5

$$EA \underset{k_4}{\overset{k_3B}{\rightleftharpoons}} EAB \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E^*AB \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E^*RX \xrightarrow{k_9} E^*RY \xrightarrow{k_{11}} ER \xrightarrow{k_{13}} E \quad (5)$$

where A, B, R, X, and Y represent NADP, 6PG, NADPH, 3-keto-6PG, and the 1,2-enediol of ribulose 5-phosphate, respectively, the E to E* interconversion represents a conformational change prior to catalysis,[2] k_3 and k_4 are binding and dissociation constants for 6PG, respectively, k_5 and k_6 are rate constants for the enzyme isomerization, k_7 and k_8 represent the rates of forward and reverse hydride transfer, respectively, k_9 represents the rate of decarboxylation and release of CO_2, k_{11} represents the rate of either tautomerization and release of ribulose 5-phosphate or release of the enediol intermediate, and k_{13} represents the rate of release of NADPH. On the basis of the assigned rapid equilibrium random kinetic mechanism (*6*), and the proposed rapid release of CO_2, eqs 6−8 are obtained (*9*).

$$V = [k_7/(1 + k_6/k_5)]/\{1 + [k_7(1/k_5 + 1/k_{11})]/(1 + k_6/k_5)\} \quad (6)$$

$$V/K_{6PG} = (k_3k_5k_7/k_4k_6)/[1 + (k_7/k_6)] \quad (7)$$

$$K_{6PG} = K_d(1 + k_7/k_6)/[1 + k_5/k_6 + k_7(1/k_5 + 1/k_{11})] \quad (8)$$

The effect of changing K183 is easily understood via eqs 6−8. Two classes of mutant enzymes are obtained (Table 3). The first group is composed of the E, H, C, and Q mutants, which exhibit little significant change in the K_{6PG}, and thus equal decreases in V/E_t and $V/K_{6PG}E_t$. None of the mutant side chains likely function in a general base capacity at pH 7, given the low p*K* values of the side chains as compared to the much higher p*K* of the 3-hydroxyl of 6PG. Hwang et al. (*3*) have measured deuterium and ^{13}C kinetic isotope effects for the sheep liver enzyme, and on the basis of the value of observed and intrinsic deuterium isotope effects, one can estimate that the hydride transfer step is 20−30% limiting overall, while decarboxylation limits the overall rate by about 15−20%. Since the general base is required to accept a proton from the 3-hydroxyl in the oxidation step, and is also utilized to polarize the carbonyl and as a general acid in the decarboxylation step, it is of interest to determine whether hydride transfer or decarboxylation is impaired. The lack of a significant deuterium isotope effect on V and V/K_{6PG} suggests that it is a decrease in the rate of the decarboxylation step, k_9, that is reflected by the several-fold decreases in V and V/K shown in parentheses in Table 3; that is, both kinetic parameters (V and V/K) are dominated by k_9 in the mutant enzymes. The several-fold decrease in V/E_t for the E, H, C, and Q mutants is about the same for all, with a weighted average value of 6800 ± 800 estimated from the V/E_t values with the lowest standard error. As suggested above, this value is likely closer to 35000−45000. The decrease thus represents an estimate of the catalytic advantage realized from the general acid−electrostatic properties of K183, a $\Delta\Delta G^{\ddagger}$ of about 6 kcal/mol.

On the basis of the identity within error of K_{6PG} for the E, H, C, and Q mutant enzymes, it is likely that the hydrogen-bonding interaction between the 3-hydroxyl and the mutant side chains is allowed in all four of the above mutant enzymes. This conclusion is not unexpected, despite the apparent differences in the length of the mutant side chains, especially for cysteine and glutamate, compared to a fully extended lysine side chain. A glance at Figure 1 shows the side chain of K183 is not fully extended, but folded such that a fully extended cysteine or glutamate side chain could easily be within hydrogen-bonding distance of the 3-hydroxyl of 6PG. Thus, the carbonyl oxygen of glutamine, an unprotonated imidazole,[3] ionized thiol, and glutamate side chains may be able to accept a hydrogen bond from the 3-hydroxyl of 6PG.

A second class of K183 changes includes the A, M, and R mutants. This class exhibits an increase in K_{6PG} and a decrease in V/E_t, yielding a greater decrease in $V/K_{6PG}E_t$ than that observed in V/E_t. For these mutant enzymes, a decrease in the affinity for 6PG (an increase in the K_d term in eq 7 shown above, reflecting the dissociation constant for dissociation of 6PG from the E−NADP−6PG ternary complex) is observed in addition to a decrease in k_9. Each of the three mutations, however, derives its effect differently. In the case of the M mutant, the bulky sulfur likely causes a local disruption in the 6PG binding pocket in the vicinity of the 3-hydroxyl, and this along with the hydrophobicity of the thiomethyl group gives the observed approximately 8-fold decrease in 6PG affinity. The R mutant also presents a bulky side chain that likely interferes slightly with 6PG binding, but the guanidinium functional group can still function, albeit weakly, as a general base as discussed further below. Finally, the A mutation eliminates the side chain for K183, and any real possibility of acid−base catalysis. A possible exception could be a bound water molecule occupying the site of the missing side chain, but the water would have a very weak basicity. In addition, the decrease in V/E_t is larger, not smaller, than the average value of 6800-fold estimated above. (This would include the glutamine side chain, which presumably occupies a space similar to that of lysine.)

The A mutant provides an opportunity to estimate the contribution of the ϵ-amine of K183 to 6PG binding affinity. The overall ΔG° for the binding energy of 6PG in the combined E−NADP−6PG and E*−NADP−6PG complexes, based on the measured dissociation constant of 36 μM, is 5.8 kcal/mol. The decrease in the binding affinity of 2−4-fold, based on the data in Table 3, and assuming no effect of the mutation on the isomerization equilibrium represented by k_5 and k_6, gives a $\Delta\Delta G^{\circ}$ of 0.4−1 kcal/mol, with an average value of 0.8 kcal/mol for the contribution of K183 to 6PG binding. Thus, although the amine contributes to 6PG binding, the contribution is relatively modest.

Prior to a discussion of the pH−rate data, it is worth noting that substantial changes are also observed in $V/K_{NADP}E_t$ (for all those measured) as expected in a rapid equilibrium random kinetic mechanism. However, one would expect changes in k_7 to be identically expressed in both V/K values,

[2] Data from isotope effect studies indicate the presence of a kinetically significant conformation change prior to catalysis (*4*).

[3] The observed p*K* of K183 is about 8, compared to a p*K* of 10.5 expected for a lysine side chain in solution (*35*). Given the 2.5 pH unit decrease in the p*K* of K183, it is likely that the imidazole side chain of H183 is neutral at pH 7.

and they are not, as shown in Table 3. Differences in the decrease in the $V/K_{6PG}E_t$ and $V/K_{NADP}E_t$ values compared to those of the WT enzyme must reflect differences in the change in affinity for NADP and 6PG for mutant and WT enzymes. Although the errors are substantial, there is evidence that significant changes in the K_{NADP} occur that are larger, at least in the case of the R mutant, than the change in K_{6PG}. This result is not surprising given the close juxtaposition of the 6PG and NADP binding sites. Indeed, changes in the nicotinamide position are thought to occur as reduction of the ring takes place, resulting in a displacement of the 1-carboxyl of 6PG (*8*).

Interpretation of the pH−Rate Profiles. On the basis of the likely identity of the general base (K183) and the general acid (E190) from structural studies (*8*), previously determined pH−rate profiles have been interpreted in terms of reverse protonation states between the two groups. That is, although K183 is the general base, its p*K* is observed on the basic side of the pH profiles, while that of E190, although it is the general acid, is observed on the acidic side of the pH profile (*6*). The two groups exist in protonation states in the E−6PG and E−6PG−NADP complexes that are opposite from that expected on the basis of the p*K*s of Lys and Glu in solution (*34*). Thus, in the WT enzyme, the p*K* of 5.6 in the V/K_{6PG} profile is thought to be that of E190, while the p*K* of 8 is thought to reflect K183 (see above). The lysine and glutamate p*K*s must then be perturbed to lower and higher pH values as a result of the hydrophobic nature of the active site.

As stated above, only the R mutant was active enough to yield pH−rate profiles. The $V-$ and $V/K_{6PG}-$pH profiles are similar for the R mutant, with an acid p*K* of 6.2−6.8 observed on the acid side, and no decrease on the basic side up to pH 9.[4] Thus, although the p*K* for E190 is increased slightly, it is the p*K* for R183 that is not observed, likely because it is above pH 9. The latter is not unexpected since the p*K* of the arginine guanidinium is about 12.5 in solution (*35*), and would, by analogy with K183 (which has an observed p*K* of 8 compared to a solution p*K* of 10.5), be expected to be decreased by 2.5 pH units to a p*K* of around 10. Data are thus fully consistent with the predicted general base nature of K183.

Consistent with the above explanation is a quantitative analysis of the data. In the case of reverse protonation states between two groups, the fraction of active enzyme, which is with the base unprotonated and the acid protonated, is equal to the antilog of the difference in the p*K* values as long as the chemical step(s) is rate-determining. The 6PGDH reaction meets these criteria. The result of changing the general base from K to R would be a perturbation in the p*K* of around 3 pH units (8 to 11), giving a decrease in $V/K_{6PG}E_t$ of about 1000-fold, consistent with the data of Table 3.

Mechanism. Data, as discussed above, are those expected for the changes made to the general base catalyst, and thus, K183 is most probably the general base. The binding of 6PG is such that the 1-carboxyl is hydrogen-bonded to S128 to E190 via the intermediacy of a water molecule. Thus, in Scheme 1, the oxidation of the 3-hydroxyl of 6PG is facilitated by K183, which accepts a proton from the 3-hydroxyl as the hydride is transferred to the *si* face of the nicotinamide ring. The nicotinamide ring is thought to be held in position by an electrostatic interaction between the positively charged N1 of the nicotinamide and the negatively charged pyrophosphate moiety of NADP (*8*). Once the hydride has been transferred, the nicotinamide ring rotates by about 180° and presumably displaces the 1-carboxyl from its hydrogen-bonded position in preparation for decarboxylation. The positively charged K183 is now set to polarize the carbonyl of 3-keto-6PG in the decarboxylation step. This is likely why the 6PGDH has evolved to have reverse protonation states between E190 and K183, the general acid and general base, respectively. There is no divalent metal ion required for the 6PGDH reaction, unlike other β-hydroxyacid oxidative decarboxylases, e.g., malic enzyme. Thus, an enzyme residue must be present to polarize the carbonyl. In the decarboxylation step, K183 donates a proton to give the 1,2-enediol of ribulose 5-phosphate. It is the decarboxylation step that is defective in the K183R, and likely all other K183 mutant proteins, likely because of the dual role K183 must play in this step. CO_2 is then released, and tautomerization of the enediol occurs with acid−base catalysis by E190 and K183.

[4] Above pH 9, the enzyme is unstable (*6*).

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