Glutamate 190 Is a General Acid Catalyst in the 6-Phosphogluconate-Dehydrogenase-Catalyzed Reaction†

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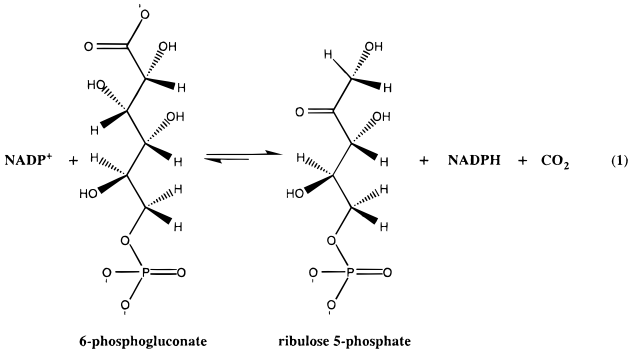
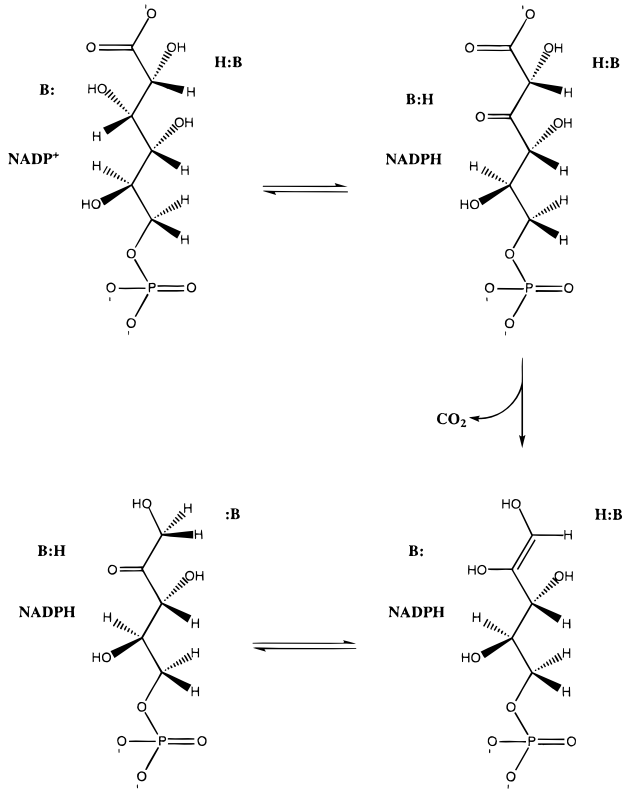
ABSTRACT: Site-directed mutagenesis was used to change E190 of sheep liver 6-phosphogluconate dehydrogenase to A, D, H, K, Q, and R to probe its possible role as a general acid catalyst. Each of the mutant proteins was characterized with respect to the pH dependence of kinetic parameters. Mutations that eliminate a titrable group at position 190, result in pH-rate profiles with no observable p*K* on the basic side of the V/K_{6PG} profile. Mutations that change the p*K* of the group at position 190 result in the expected p*K* perturbations in the V/K_{6PG} profile. Kinetic parameters obtained at the pH optimum in the pH-rate profiles are consistent with a rate-limiting tautomerization of the 1,2-enediol of ribulose 5-phosphate consistent with the proposed role of E190. Data are also consistent with some participation of E190 in an isomerization required to form the active Michaelis complex.

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*).



Recent isotope effect data have shown that the oxidative decarboxylation of 6PG to the 1,2-enediol of ribulose 5-phosphate proceed via a stepwise mechanism with hydride transfer preceding decarboxylation for both *cu*6PGDH and *sl*6PGDH (*3*). A general base/general acid mechanism has been suggested (Scheme 1) based on the pH dependence of kinetic parameters (*4*, *5*). 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 general acid presumably plays a role in only the last of three steps, viz. the tautomerization of the enediol of ribulose 5-phosphate to the keto product.

Scheme 1: Proposed Chemical Mechanism for 6-Phosphogluconate Dehydrogenase



The three-dimensional structure of the 6PGDH from sheep liver has been solved as the apoenzyme and in the presence of 6PG, NADP, and NADPH (*6*, *7*). However, the identity of the general acid is not certain based on an inspection of the E:6PG structure. Indeed, a water molecule has been suggested to play the role by Adams (1994), while glutamate 190 has been implicated by Price and Cook (*5*). The glutamate is completely conserved in all species for which

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[1] Abbreviations: IPTG, Isopropyl-*β*-D-thiogalactopyranoside; NADP, nicotinamide adenine dinucleotide phosphate; NADPH-reduced nicotinamide adenine dinucleotide phosphate; *cu*6PGDH, *Candida utilis* 6-phosphogluconate dehydrogenase; *sl*6PGDH, sheep liver 6-phosphogluconate dehydrogenase.

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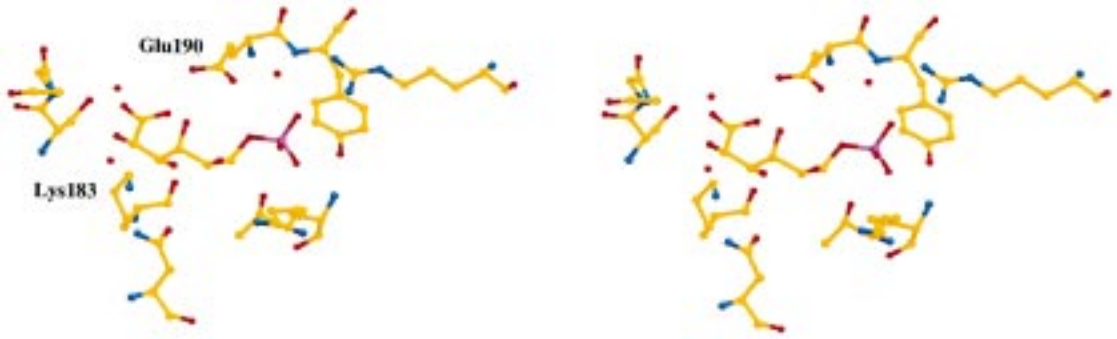

FIGURE 1: Stereopair of a portion of the 6PG binding site of sheep liver 6-phosphogluconate dehydrogenase. The proposed acid−base catalytic groups are labeled. 6-Phosphogluconate is shown with its carboxylate to the left and its 6-phosphate to the right. Serine 128 is shown immediately to the left of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

Table 1: Aligment of Consensus Sequence at the Active Site

species	sequence around E190	ref
Citobacter amalonaticus	HYVKMVHNGI**E**YGDMQLIAE	*15*
Citobacter diversus	HYVKMVHNGI**E**YGDMQLIAE	*15*
Citobacter freundii	HYVKMVHNGI**E**YGDMQLIAE	*15*
Escherichia vulneris	HYVKMVHNGI**E**YGDMQLIAE	*15*
Shigella boydii	HYVKMVHNGI**E**YGDMQLIAE	*15*
Shigella dysenteriae	HYVKMVHNGI**E**YGDMQLIAE	*15*
Shigella sonnei	HYVKMVHNGI**E**YGDMQLIAE	*15*
Shigella flexneri	HYVKMVHNGI**E**YGDMQLIAE	*16*
Escherichia coli	HYVKMVHNGI**E**YGDMQLIAE	*17*
Salmonella typhimurium	HYVKMVHNGI**E**YGDMQLIAE	*18*
Klebsiella planticola	HYVKMVHNGI**E**YGDMQLIAE	*15*
Klebsiella terrigena	HYVKMVHNGI**E**YGDMQLIAE	*15*
Klebsiella pneumoniae	HYVKMVHNGI**E**YGDMQLIAE	*19*
Escherichia coli	HYVKMVHNGI**E**YGDMQLIAE	*20*
Bacillus subtilis	HYVKMVHNGI**E**YGDMQLISE	*21*
Bacillus licheniformis	HFVKMVHNGI**E**YADMQLIAE	*22*
Bacillus subtilis	HFTKMVHNGI**E**YADMQLIAE	*23*
Actinobacillus actinomycetemcomitans	HFVKMVHNGI**E**YGDMQLICE	*24*
Haemophilus influenzae	HFVKMVHNGI**E**YGDMQLICE	*25*
Saccharomyces cervisiae	HYVKMVHNGI**E**YGDMQLICE	*26*
Saccaromyces cervisiae	HYVKMVHNGI**E**YGDMQLICE	*27*
Ceratitis capitata	HFVKMVHNGI**E**YGDMQLICE	*28*
Drosophila melanogaster	HFVKMVHNGI**E**YGDMQLICE	*29*
Drosophila simulans	HFVKMVHNGI**E**YGDMQLICE	*30*
Homo sapiens	HFVKMVHNGI**E**YGDMQLICE	*31*
Ovis aries	HFVKMVHNGI**E**YGDMQLICE	*32*
Synechococcus Sp.	HYVKMVHNGI**E**YGDMQLIAE	*33*
Synechocystis Sp.	HYVKMVHNGI**E**YGDMQLIAE	*34*
Trypanosoma brucei brucei	SCVKMYHNSG**E**YAILQIWGE	*35*
Bacillus subtilis	HFLKMIHNGI **E**YGMMAAIGE	*21*
consensus	HYVKMVHNGI**E**YGDMQLIAE	

sequences are now available, Table 1. In addition, the glutamate is close to the desired position (*7*), although within hydrogen-bonding distance to the substrate carboxylate in the E:6PG binary complex, Figure 1, and its location changes dependent on whether NADP or NADPH is bound (*7*).

In this manuscript, site-directed mutagenesis is used to change glutamate 190 to a number of other residues, such that it could either no longer serve as a general acid or its p*K* would be significantly perturbed. Data support the assignment of the glutamate side chain at position 190 in the sheep liver 6PGDH as a general acid. Implication of the assignment is discussed.

MATERIALS AND METHODS

Chemicals and Reagents. Deoxynucleotide triphosphates were from Perkin-Elmer, and Taq Pfu was from Stratagene. The fmol^R DNA cycle sequencing kit, T_4 DNA ligase, T_4 kinase, protein molecular mass markers, and *Escherichia coli* strain JM109 were from Promega. Restriction endonucleases, Taq Plus DNA polymerase, and IPTG were from Life Technologies (Gibco-BRL). The ampicillin, kanamycin, 6-phosphogluconic acid, and NADP were from Sigma or U.S. Biochemicals. Hepes, Bis-Tris, and Ches buffers were from Research Organics Inc. Glycerol was purchased from Fisher. The DNA molecular weight ladder was purchased from New England Biolabs. Mutagenesis and sequencing primers were purchased from either Biosynthesis or Gibco-BRL. Site-directed mutagenesis was performed using the Altered Sites II in vitro Mutagenesis System from Promega. The QIA express type IV kit which contained the pQE-30 vector, *E. coli* strain M15 and the Ni-NTA matrix was from QIAGEN. The Bio101 Kit was from Bio101, Inc. All other chemicals used were the highest quality available and were used without further purification.

Protein concentrations were determined according to Bradford (*8*) using the Bio-Rad protein assay kit with bovine serum albumin as a standard.

Bacterial Strains and Pasmids. The *E. coli* strain JM109 (*9*) was the host strain for plasmids containing glutamic acid 190 mutations, and M15[pREP4] (*10*) was used as a host for expression. The plasmid pAlter-1 was used as the mutagenesis vector and the plasmid pQE-30 was used as an expression vector.

Cloning of 6-PGDH into the pAlter-1 Vector. The plasmid pPGDH.LC2 (*11*) was digested with restriction endonuclases *Eco*RI and *Hin*dIII to excise the 6-PGDH cDNA. The 6-PGDH cDNA fragment was eluted from agarose gel using the Bio101 Kit. The fragment was then subcloned into the pALTER-1 vector previously digested with the corresponding enzymes. Competent cells of *E. coli* strain JM109 were transformed using an EC100 electroporator according to the manufacturer's specifications. The resulting strain was named pPGDH.LC5. Frozen stocks of the strain harboring plasmid were stored in LB/Amp medium containing 15% glycerol at −70 °C.

Site-Directed Mutagenesis. 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 sequence analysis. The mutated plasmids were designated as pAlt.E190A, pAlt.E190D, pAlt.E190H, pAlt.E190K, pAlt.E190R, and pAlt.E190Q.

Table 2: Sequence of Mutagenic Oligonucleotides

E190A	CAACGGCATAGCGTACGGGGACA
E190D	CAACGGCATAGATTACGGGGACA
E190H	CAACGGCATACATTACGGGGACA
E190K	CACAACGGCATAAAAGTACGGGGACA
E190R	CACAACGGCATAAGGGTACGGGGACATG
E190Q	CACAACGGCATACAGTACGGGGACATG

Subcloning of the Mutants into the pQE-30 Expression Vector. The 6-PGDH gene containing the desired mutation was amplified using primer pairs 5′ ACTATAGGGCGCATGCATGGCCCAAG 3′ which creates an *Sph*I restriction site at the start of the gene and 5′ TGTAGAGTTGAAGCTTGGAACAGAAG 3′ primer containing a *Hin*dIII restriction site. The 1.49 kb cDNA fragment containing the 6-PGDH cDNA was subcloned into the pQE-30 expression vector at the corresponding sites. The resulting plasmids were designated as pE190A, pE190D, pE190H, pE190K, pE190R, and pE190Q. Competent cells of *E. coli* M15 were transformed using the QIAGEN protocol. Frozen stocks of strains harboring plasmid were stored in LB/Amp/Kan medium containing 15% glycerol at −70 °C. The entire gene in the expression vector was sequenced to ensure the integrity of the cDNA.

Growth and Purification Conditions. The bacterial strains containing each mutant was grown in 10 L of LB/Amp/Kan medium until an absorbance of 0.7 at 600 nm was reached, at which time IPTG was added to a final concentration of 0.5 mM and growth was continued for another 4 h. The bacterial cell paste was collected by centrifugation at 7000 rpm for 5 min, resuspended in 3× volume sonication buffer (50 mM sodium phosphate, pH 8.0, and 300 mM NaCl) and stored at −20 °C.

Protein purification conditions were as previously described (*11*). All mutant enzymes purified in a manner identical to wild-type enzyme.

pH Studies. Enzyme assays were carried out using an HP 8453 diode array spectrophotometer. The appearance of NADPH was monitored at 340 nm ($\epsilon_{340} = 6220\ M^{-1}\ cm^{-1}$). Reactions were carried out in 1 mL volumes using 1 cm path-length cuvettes. Initial velocity data were obtained at different pH values varying 6PG and maintaining NADP at a fixed saturating concentration of 0.4 mM. The pH was maintained using single buffers at low pH (Bis-Tris, pH ∼6), intermediate pH (Hepes, pH 7−8), and high pH (Ches, pH ∼9). Mixed buffers were used to span the pH values between the pH of the stock buffers. The total buffer concentration was 100 mM in all cases. The buffers were checked for any nonspecific enzyme inhibition with no inhibition found. The pH of the assay mix was determined after the initial velocity measurement. Each of the assays using mutant enzymes contained between 16 and 100 μg of enzyme. Initial experiments revealed, at high mutant enzyme concentrations in the absence or presence of NADP, a background nonspecific absorbance change in the 340 nm region. Consequently, a three point drop line background correction was used with 290 and 410 nm background wavelengths and 340 nm as the reference wavelength. It was subsequently determined that the background absorbance change could be reduced or eliminated if 10% glycerol was included in the assays. Thus, all pH profiles except those for the mutants E190K and E190H were obtained in the presence of 10% glycerol. In separate experiments, it was determined that the presence of 10% glycerol resulted in no change in the kinetic parameters for wild type 6PGDH compared to those obtained in the absence of glycerol.

Table 3: Summary of Kinetic Parameters for E190 Mutants of 6PGDH

	K_{6PG} (μM)	V/E_t (s^{-1})	fold decrease	$V/K_{6PG}E_t$ ($M^{-1}\ s^{-1}$)	fold decrease
WT	30 ± 10	5		1.7×10^5	
E190A	13 ± 3	0.007	714	551	310
E190D	22 ± 7	0.007	735	310	555
E190H	10 ± 3	0.014	357	1450	120
E190K	4 ± 1	0.012	417	2860	60
E190Q	3.1 ± 0.2	0.043	116	1.4×10^4	12
E190R	1800 ± 100	0.006	877	3	5.4×10^4

Data Processing. All plots of reciprocal initial velocities versus reciprocal substrate concentrations were linear. Data were fitted to the appropriate rate equations using BASIC versions of the computer programs developed by Cleland (*12*). Substrate saturation curves were fitted using eq 2. Data for pH profiles in which the log of the kinetic parameter decreases at low and high pH with unit slopes were fitted using eq 3. Data for pH profiles with one ionization on the acid side were fitted using eq 4 and profiles with one ionization on the basic side were fitted using eq 5.

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

$$\log y = \log[C/(1 + H/K_1 + K_2/H)] \quad (3)$$

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

$$\log y = \log[C/(1 + K_2/H)] \quad (5)$$

In eq 2, v is the initial velocity, V is maximum velocity, A is substrate concentration, and K_m is the Michaelis constant for A. In eqs 3−5, 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 important in a given protonation state for optimum binding and/or catalysis.

RESULTS

Kinetic Parameters of the Mutant Proteins. Saturation curves were obtained for 6PG at saturating NADP, at the optimum pH for each of the mutant proteins. 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 at least 2 orders of magnitude for all of the mutant proteins compared to the wild-type enzyme. In all cases except that of E190R, K_{6PG} is either identical or decreases compared to the value measured for the WT enzyme. Note that with one exception, E190R, the fold decrease in the second-order rate constant, $V/K_{6PG}E_t$, is less than that observed on the first-order rate constant, V/E_t. In the case of the E190R mutant protein, K_{6PG} has increased 60-fold, suggesting a decreased affinity for 6PG, likely a result of steric interference by the bulky guanidinium group of the arginine side chain. All of the other mutations, with the exception of lysine, result in shorter

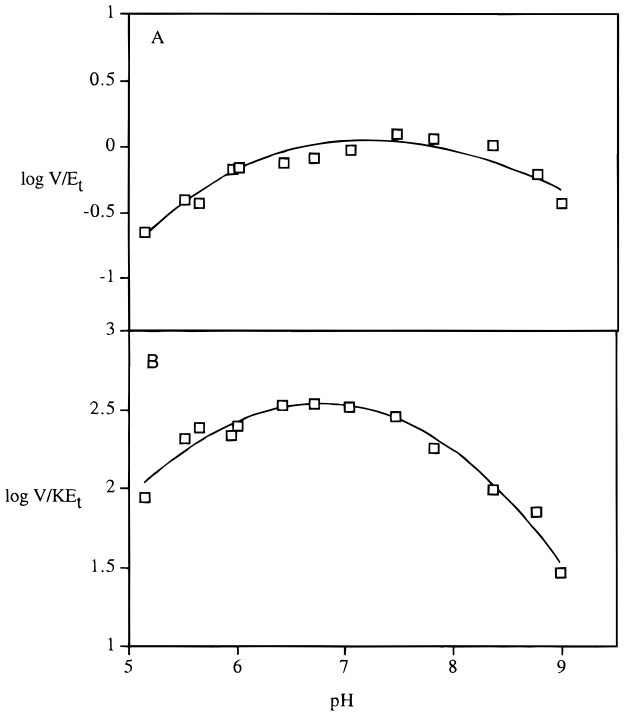

FIGURE 2: pH dependence of kinetic parameters for wild type 6-PGDH. Data were obtained for V (A) and V/K_{6PG} (B). The points shown are the experimentally determined values and the curves are from a fit of the data using eq 3.

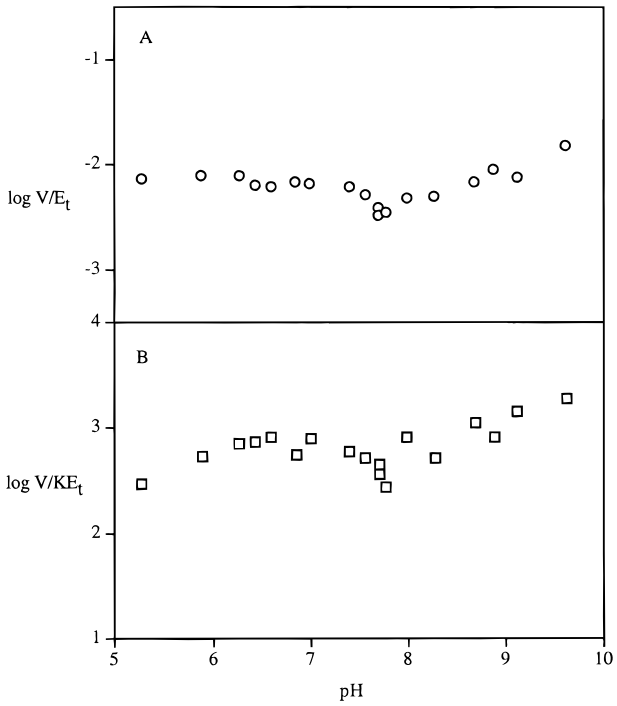

FIGURE 3: pH dependence of kinetic parameters for the E190A mutant of 6-PGDH. Data were obtained for V (A) and V/K_{6PG} (B).

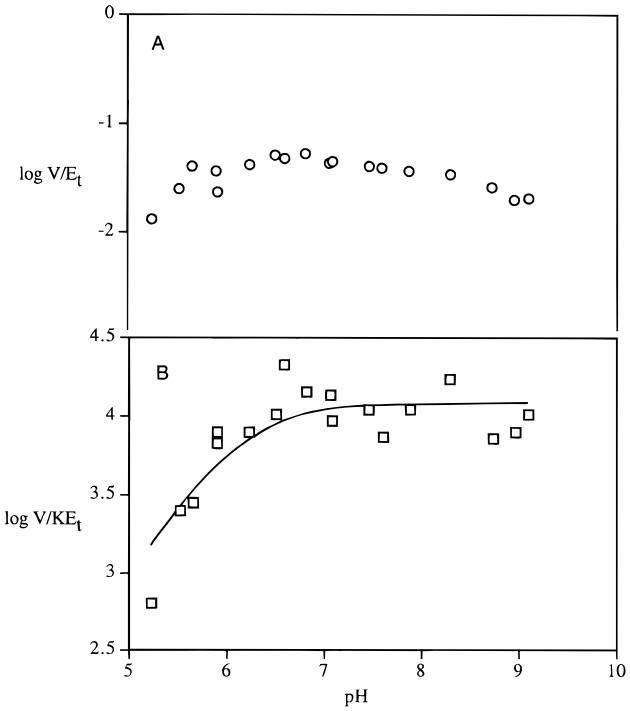

FIGURE 4: pH dependence of kinetic parameters for the E190Q mutant of 6-PGDH. Data were obtained for V (A) and V/K_{6PG} (B). The points shown in panel B are the experimentally determined values and the curve is a fit of the data using eq 4.

side chains compared to that of the WT glutamate, and the ϵ-amine of lysine is not as bulky as that of the D-guanidino of arginine.

pH Dependence of Kinetic Parameters. The pH dependence of kinetic parameters should provide the best indicator as to the general acid capability of E190, since they give a direct measure of the pK value for the general acid functionality of 6PGDH (*4*, *5*). The pH-rate profiles have been obtained for the WT 6PGDH as a frame of reference[2] for the mutant pH profiles, Figure 2. In addition, the pH-rate profiles have been measured for all mutant proteins, Figures 3−8. A summary of estimated pK values for the WT and mutant proteins is given in Table 4.

Unlike the WT 6PGDH, which exhibits a pH dependence to V/E_t, all of the mutant enzymes show no pH dependence to the first-order rate constant over the pH range studied. Generally, above and below the pH range indicated, the mutant proteins were unstable. The pH independence of V/E_t suggests that the mutant enzyme, unlike the WT enzyme, selectively binds substrate only when catalytic/binding groups are in their optimum protonation state (*13*). With the possible exception of the E190H mutant, the $V/K_{6PG}E_t$ pH-rate profiles have been altered considerably, compared to those of the WT enzyme, consistent with E190 playing a catalytic role.

DISCUSSION

As shown in Table 1, E190 is completely conserved for 6PGDH from all species for which a primary sequence has been determined. It is likely then, that the glutamate side chain is important to the mechanism of 6PGDH, especially since it is found in the active site within hydrogen-bonding distance to the carboxylate of 6PG, Figure 1. In the binary complex with N(8-BrA)DP, E190 is in van der Waals contact with the amide side chain of the nicotinamide ring, as is K183 (*7*). Rearrangent takes place upon binding of 6PG (*7*), and E190 and S128 are observed hydrogen bonded to the carboxylate of 6PG, Figure 1. (E190 is also hydrogen

[2] The pK on the acid side of the V/K profile is lower than that reported previously (*5*). The lower value is a result of an inhibitory effect by Mes buffer unrealized in the previous studies.

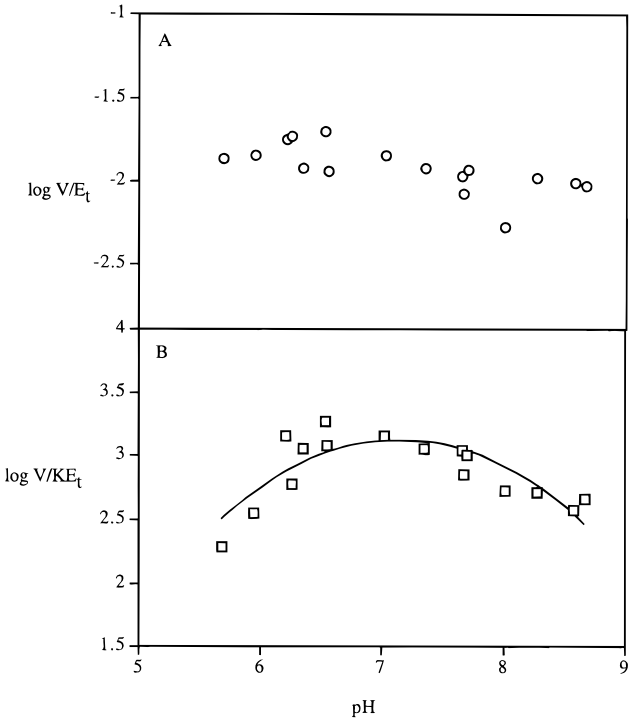

FIGURE 5: pH dependence of kinetic parameters for the E190H mutant of 6-PGDH. Data were obtained for V (A) and V/K_{6PG} (B). The points shown in panel B are the experimentally determined values and the curve is a fit of the data using eq 3.

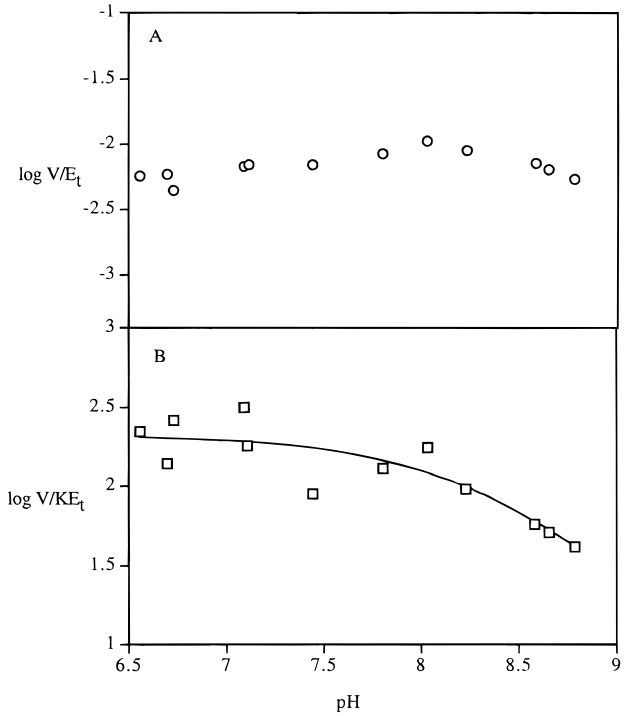

FIGURE 6: pH dependence of kinetic parameters for the E190D mutant of 6-PGDH. Data were obtained for V (A) and V/K_{6PG} (B). The points shown in panel B are the experimentally determined values and the curve is a fit of the data using eq 5.

bonded to a tightly bound water.) If E190 is the catalytic general acid, the hydrogen-bonding interactions are expected to change once the enzyme prepares to catalyze the decarboxylation, since the hydrogen bond would increase the difficulty of decarboxylation by opposing electron flow from the carboxylate toward the carbonyl carbon at C-3. The

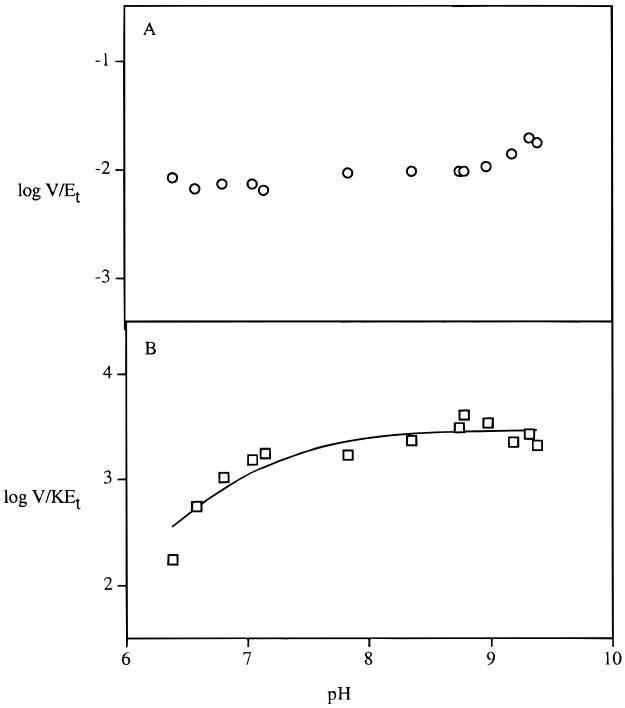

FIGURE 7: pH dependence of kinetic parameters for the E190K mutant of 6-PGDH. Data were obtained for V (A) and V/K_{6PG} (B). The points shown in panel B are the experimentally determined values and the curve is from a fit of the data using eq 4.

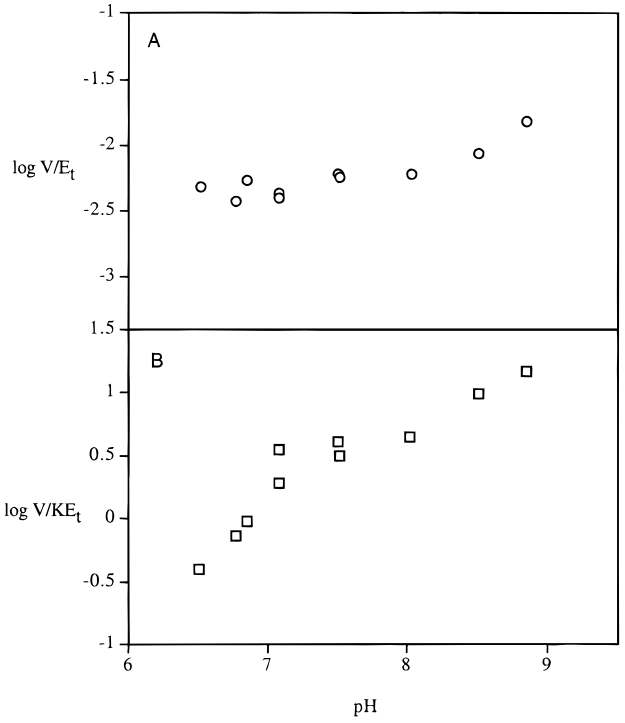

FIGURE 8: pH dependence of kinetic parameters for the E190R mutant of 6-PGDH. Data were obtained for V (A) and V/K_{6PG} (B).

binary complex with NADPH shows significant movement of the nicotinamide of the reduced cofactor such that it now occupies the position of the 1-carboxylate of 6PG with the NH of its carboxamide side chain hydrogen bonded to S128 and E190 hydrogen bonded to a water molecule (*7*). It is thus surmised that the hydrogen-bonding interaction of E190 to the carboxyl no longer exists once oxidation has occurred, and E190 could now be in proper position to act as the

general acid in the tautomerization step. There is likely to be additional change (although probably subtle) in the position of the 3-keto intermediate as well, resulting from the hybridization change at C-3. It thus appeared reasonable that E190 served as the general acid, required to protonate C-1 of the enediol of ribulose 5-phosphate as it is tautomerized to the keto form.

Interpretation of Kinetic Data. A minimal kinetic mechanism for 6PGDH, including 6PG binding, and all three of the catalytic steps are given in eq 6

$$\mathrm{EA} \underset{k_4}{\overset{k_3\mathrm{B}}{\rightleftharpoons}} \mathrm{EAB} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} \mathrm{E^*AB} \underset{k_8}{\overset{k_7}{\rightleftharpoons}} \mathrm{E^*RX} \xrightarrow{k_9} \mathrm{E^*RY} \xrightarrow{k_{11}} \mathrm{ER} \xrightarrow{k_{13}} \mathrm{E} \quad (6)$$

where the A, B, R, X, and Y represent NADP, 6PG, NADPH, 3-keto-6PG, and the 1,2-enediol of ribulose 5-phosphate, E* represents a conformational change prior to catalysis,[3] k_3 and k_4 are binding and dissociation constants for 6PG, k_5 and k_6 are rate constants for the enzyme isomerization, k_7 and k_8 represent forward and reverse hydride transfer, k_9 represents decarboxylation and release of CO_2, k_{11} represents either tautomerization and release of ribulose 5-phosphate or release of the enediol intermediate, and k_{13} represents release of NADPH.

Expressions for V, V/K_{6PG}, and K_{6PG}, based on the above mechanism, are given as eqs 7−9.

$$V = [k_7/(1 + k_6/k_5)]/\{1 + [k_7(1/k_5 + 1/k_9 + 1/k_{11} + 1/k_{13})]/(1 + k_6/k_5) + k_8/k_9\} \quad (7)$$

$$V/K_{6PG} = [k_3k_5k_7/k_4k_6]/[1 + (k_7/k_6)(1 + k_5/k_4) + k_8/k_9] \quad (8)$$

$$K_{6PG} = K_d[1 + (k_7/k_6)(1 + k_5/k_4) + k_8/k_9]/[(1 + k_5/k_6)(1 + k_8/k_9) + k_7(1/k_5 + 1/k_9 + 1/k_{11} + 1/k_{13})] \quad (9)$$

On the basis of the assigned rapid equilibrium random kinetic mechanism (*5*) and the proposed rapid release of CO_2, k_5/k_4 and k_8/k_9, k_7/k_9, and k_7/k_{13} are zero, and the above expressions reduce to those given in eqs 10−12.

$$V = [k_7/(1 + k_6/k_5)]/[1 + \{k_7(1/k_5 + 1/k_{11})\}/(1 + k_6/k_5)] \quad (10)$$

$$V/K_{6PG} = [k_3k_5k_7/k_4k_6]/[1 + (k_7/k_6)] \quad (11)$$

$$K_{6PG} = K_d[1 + (k_7/k_6)]/[1 + k_5/k_6 + k_7(1/k_5 + 1/k_{11})] \quad (12)$$

Thus, dependent on the values k_7/k_6 in the numerator of eq 12 and $k_5/k_6 + k_7(1/k_5 + 1/k_{11})$ in the denominator of the equation, the K_d (equal to k_4/k_3, the dissociation constant for 6PG from the E:NADP:6PG ternary complex) will differ from K_{6PG}. To further simplify, one can consider the chemical mechanism, noting that the general acid functions as a catalyst in the conversion of E*RY to ER, the step represented by k_{11}. The mutant proteins all have rates lower than those of the WT enzyme, and thus the denominator of eq 12 is likely to be larger than the numerator, resulting in a lower value of K_{6PG} for the mutant proteins than for the WT enzyme. Data in Table 3 are consistent with the analysis (with the exception of the R mutant discussed above) and support the identity of the general acid as E190.

[3] Data from isotope effect studies indicate the presence of a kinetically significant conformation change prior to catalysis (see following article in this issue).

Table 4: Summary of pK Values for 6PGDH WT and E190 Mutant Proteins

	pK_a ± SE	pK_b ± SE
WT		
V/E_t	5.8 ± 0.1	8.8 ± 0.1
$V/K_{6PG}E_t$	5.6 ± 0.1	8.0 ± 0.1
E190A $V/K_{6PG}E_t$	ca. 6	
E190D $V/K_{6PG}E_t$		8.2 ± 0.2
E190H $V/K_{6PG}E_t$	6.3 ± 0.2	8.2 ± 0.2
E190K $V/K_{6PG}E_t$	7.2 ± 0.1	
E190Q $V/K_{6PG}E_t$	6.1 ± 0.2	
E190R $V/K_{6PG}E_t$	ca. 8.2	

Regarding the differential changes in V and V/K, since k_7/k_{11} is present in the denominator of V, but not V/K, one would expect a decrease in the value of V, but not V/K. The data in Table 3 do give a greater decrease in V than in V/K, but there is also a significant decrease in V/K. The decrease in the second order rate constant likely reflects a decrease in the rate of isomerization of the ternary complex. However, to account for the lower K_{malate} values of the mutant enzymes, k_7/k_6 must be less than $k_5/k_6 + k_7(1/k_5 + 1/k_{11})$.

Interpretation of the pH-Rate Profiles. On the basis of the likely identity of the general base (K183) and general acid (E190) from structural studies (*7*), previously determined pH-rate profiles have been interpreted in terms of reverse protonation states between the two groups. That is, although K183 is the likely general base, its pK is observed on the basic side of the pH profiles, while that of E190, although it is the likely general acid, is observed on the acidic side of the pH profile (*5*). The two groups exist in protonation states in the E:6PG and E:6PG:NADP complexes that are opposite that expected based on the pKs of Lys and Glu in solution (*13*). Thus, the pK of 5.6 in the V/K_{6PG} profile is thought to be that of E190, while the pK of 8 is thought to reflect K183 (Table 4, Figure 2). A second possibility is that the acidic pK could be attributed to the 6-phosphate group of 6PG. However, the value of the pK is 5.6, considerably lower than the phosphate pK of 6.1 (*14*), and in addition, the pK is observed in the V pH profile, which effectively rules out this possibility. The lysine and glutamate pKs must then be perturbed to lower and higher pH values as a result of the hydrophobic nature of the active site.

The majority of the mutant proteins show little or no pH dependence to their kinetic parameters, and this is especially true for the V profiles. As stated above, the pH independence of V suggests a mechanism in which only the correctly protonated form of the E:NADP complex binds 6PG in preparation for catalysis, at least over the pH range accessed. On the basis of the limited studies presented, other mechanisms cannot be ruled out at this time and additional studies will be required to sort out the mechanistic possibilities. The pH-rate profiles of the mutant V/K pH profiles are dramatically changed when compared to those of the WT enzyme, consistent with the assignment of E190 as the general acid catalyst. The mutant V/K pH profiles will be interpreted

within the framework of the mechanism proposed for the WT enzyme.

The E190A and E190Q mutant protein *V*/K profiles decrease at low pH, but are pH independent at high pH. The acidic p*K* of about 6, in both cases, is likely that of the lysine which must be unprotonated for catalysis. The lysine p*K* has decreased by about 2 pH units as a result of elimination of the glutamate and may suggest that the two residues are hydrogen bonded to one another in the E:NADP complex.

The E190D and E190H mutant proteins likely behave in a manner similar to the WT enzyme. In the case of the D substitution, data could not be collected at low enough pH to observe the acidic p*K*, but it is expected to be 6 or below, reflecting the p*K* of the aspartyl side chain. The p*K* of 8 is observed, reflecting the lysine. In the case of the histidine substitution, both p*K*s are similar to those observed for the WT enzyme, but the p*K* of about 6.3 now reflects the imidazole side chain. The p*K* is higher than that observed for WT, consistent with the higher p*K* for imidazole vs a carboxylate. Although the imidazole side chain introduces another positive charge in the site, it does not influence the K183 p*K*, since the imidazole will be neutral over the pH range the lysine is titrated.

The E190K mutant *V*/K profile shows a decrease on the acidic side, likely reflecting the p*K* of K183 at a lower pH because of the additional positive charge introduced along with the lysine substitution. The second lysine p*K* is not observed and is presumably above pH 9.5, closer to a normal lysine p*K*, unperturbed by K183 which will be neutral over the pH range K190 is titrated.

The E190R pH profile differs from those of the other mutants in that it decreases on the acidic side with a p*K* of about 8−9. It is suggested that the observed p*K* is that of K183 which must be unprotonated for catalysis and that the pH profile will likely also decrease on the basic side with the p*K* of the arginine. Unfortunately, data cannot be collected to high enough pH to observe the other p*K*.

Data, as discussed above, are those expected for the changes made to a general acid catalyst. It is not possible at this point to determine the rate enhancement realized by E190, since the overall mechanism is multistep and E190 participates in at least two steps, the enzyme isomerization depicted in mechanism 6 and the tautomerization reaction. Additional research will be required to sort out the multistep process.

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