Hydride Transfer Catalyzed by Glycerol Phosphate Dehydrogenase: Recruitment of an Acidic Amino Acid Side Chain to Rescue a Damaged Enzyme

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ABSTRACT: K120 of glycerol 3-phosphate dehydrogenase (GPDH) lies close to the carbonyl group of the bound dihydroxyacetone phosphate (DHAP) dianion. pH rate (pH 4.6−9.0) profiles are reported for $k_{\text{cat}}$ and $(k_{\text{cat}}/K_m)_{\text{dianion}}$ for wild type and K120A GPDH-catalyzed reduction of DHAP by NADH, and for $(k_{\text{cat}}/K_m)_{\text{dianion}}$ for activation of the variant-catalyzed reduction by CH$_3$CH$_2$NH$_3^+$, where $K_m$ and $K_d$ are apparent dissociation constants for CH$_3$CH$_2$NH$_3^+$ and DHAP, respectively. These profiles provide evidence that the K120 side chain cation, which is stabilized by an ion-pairing interaction with the D260 side chain, remains protonated between pH 4.6 and 9.0. The profiles for wild type and K120A variant GPDH show downward breaks at a similar pH value (7.6) that are attributed to protonation of the K204 side chain, which also lies close to the substrate carbonyl oxygen. The pH profiles for $(k_{\text{cat}}/K_m)_{\text{dianion}}$ and $(k_{\text{cat}}/K_dK_m)$ for the K120A variant show that the monoprotonated form of the variant is active toward reduction of enzyme-bound DHAP, because of activation by a ligand-driven conformational change. Upward breaks in the pH profiles for $k_{\text{cat}}$ and $(k_{\text{cat}}/K_m)_{\text{dianion}}$ for K120A GPDH are attributed to protonation of D260. These breaks are consistent with the functional replacement of K120 by D260, and a plasticity in the catalytic roles of the active site side chains.

Glycerol 3-phosphate dehydrogenase (GPDH) catalyzes the reduction of dihydroxyacetone phosphate (DHAP) by NADH to form glycerol 3-phosphate (G3P (Scheme 1A)), a reaction that links the metabolism of glucose to form DHAP and the biosynthesis of phosphoglycerides from G3P. Our interest in GPDH dates to the fortuitous observation that the activity of this enzyme for reduction of DHAP is largely retained during catalysis of the reduction of the substrate pieces glycolaldehyde and phosphite dianion (Scheme 1B), and that the binding energy of the DHAP phosphodianion or the phosphite piece is utilized to transform the floppy inactive open form of GPDH into a stiff catalytically active protein cage.

The structure of GPDH from human liver (hlGPDH) provides insight into the mechanism for enzyme-catalyzed hydride transfer from NADH to DHAP, and for the formation of G3P. The following side chains line the enzyme active site (Figure 1) and form a continuous chain of hydrogen bonds that stretch from the cofactor to the carbonyl group of DHAP: Q295, R269, N270, T264, N205, K204, D260, and K120. Most of these side chains are completely conserved across 11 organisms; N205 and T264 are 91% conserved, while Q295 and E295 occur with nearly equal frequency. Two of these side chains play a direct role in stabilizing the hydride transfer transition state. (1) The R269 side chain forms an ion pair with the substrate dianion.
The R269A substitution results in a 10^4-fold decrease in \( k_{cat}/K_m \) for hydride transfer to DHAP.\(^8\) (2) The K120 side chain cation is positioned to stabilize negative charge at the C-2 substrate oxygen of DHAP, which develops at the transition state for enzyme-catalyzed hydride transfer from NADH.\(^5\) The K120A substitution results in a 10^4-fold decrease in \( k_{cat}/K_m \) for hydride transfer.\(^5,9\)

The efficient rescue of the activity of the impaired K120A/R269A double variant by the combined action of ethylammonium and guanidinium cations\(^9\) is consistent with a high degree of organization of the K120 and R269 side chains at the active site of hlGPDH. The K120 side chain is immobilized in an ion pair to the D260 side chain; the loss of this ion pair at the D260G variant results in a 6.5 kcal/mol increase in \( \Delta G^\ddagger \) for \( k_{cat}/K_m \) for reduction of DHAP.\(^8\) The R269 side chain is immobilized by interactions with the DHAP phosphodianion and the Q295 side chain; Q295 substitutions result in a ≤3.0 kcal/mol increase in \( \Delta G^\ddagger \) for \( k_{cat}/K_m \) for reduction of DHAP.\(^11\) The K204 side chain cation also lies close to the carbonyl group of bound DHAP, but the effect of K204 substitutions on enzyme activity is not yet known.

The determination of kinetic parameters for wild type and variant enzymes over a broad range of pH reports on the effect of changing the ionization state of active site side chains on enzymatic activity.\(^12\) For example, the observation of breaks in pH-rate profiles shows the effect of changing side chain protonation or deprotonation on enzyme activity and gives rise to hypotheses for the specific side chains responsible for these breaks. These hypotheses may then be examined by comparing pH-rate profiles for wild type and variant enzymes. We report here the pH-rate profiles for \( k_{cat}/K_m \) and \( k_{cat} \) for reduction of DHAP by NADH catalyzed by wild type and K120A variant hlGPDH, and for \( k_{cat}/K_m \) for rescue of the K120A variant by \( \text{CH}_3\text{CH}_2\text{NH}_3^+ \), where \( K_m \) and \( K_p \) are apparent dissociation constants for \( \text{CH}_3\text{CH}_2\text{NH}_3^+ \) and DHAP, respectively. The effects of the K120A substitution on these pH-rate profiles provide strong evidence for a pH-dependent change in the favored reaction pathway, from a reaction at high pH through a transition state that is stabilized by exogenous ethylammonium cation to a reaction at low pH through a transition state that shows no detectable stabilizing interaction with \( \text{CH}_3\text{CH}_2\text{NH}_3^+ \), which is governed by protonation of an active site side chain with a \( pK_a \) of 5. We propose that protonation of the carboxylate side chain of D260 at the K120A variant provides an acid to substitute for the excised K120 side chain in stabilizing negative charge at O-2 of DHAP, which develops at the hydride transfer transition state. These profiles show that protonation of a second side chain, with \( pK_a \approx 8 \), is required to observe full activity. We propose that this is the alkyl amine side chain of K204.

### EXPERIMENTAL SECTION

The sources of chemical and biochemical reagents and most of the methods for the experiments reported herein were described in a recent publication.\(^5\) This includes the methods for preparation of solutions used in enzyme kinetic studies and for the preparation of the K120A variant of hlGPDH. Stock solutions of DHAP were prepared by dissolving the lithium salt of DHAP in water. The pH was adjusted to the desired final pH using 1.0 N NaOH or 1 N HCl, and the concentration of DHAP was determined as the concentration of NADH consumed during quantitative hlGPDH-catalyzed reduction. Published procedures were used to prepare stock solutions of the ethylammonium cation,\(^16\) and the pH was adjusted to the desired final pH using 1.0 N NaOH or 1 N HCl. The following reagent grade buffers were purchased from Sigma-Aldrich: sodium acetate, 2-(N-morpholino)ethanesulfonic acid (MES), 3-morpholinopropanesulfonic acid (MOPS), triethanolamine-HCl (TEA), [tris(hydroxymethyl)methylamino]-propanesulfonic acid (TAPS), and N-cyclohexyl-2-aminoisobutyric acid (CHES).

**hlGPDH-Catalyzed Reduction of DHAP.** The hlGPDH-catalyzed reduction of DHAP by NADH was assayed in solutions containing the appropriate buffer (20 mM), 0.1 mg/mL BSA, 200 μM NADH, and 0.04–8 mM DHAP at an ionic strength (\( I \)) of 0.12 (NaCl). The following buffers were used for these experiments: acetate buffer, 40% and 60% basic form at pH 4.6 and 4.9, respectively; MES buffer, 15%, 40%, 70%, and 80% basic form at pH 5.4, 6.0, 6.5, and 6.8, respectively; MOPS buffer, 40% and 50% basic form at pH 7.0 and 7.25, respectively; TEA buffer, 30% basic form at pH 7.5; TAPS buffer, 25% and 55% basic form at pH 8.0 and 8.5, respectively; and CHES buffer, 35% basic form at pH 9.0. The initial velocity (\( v \)) for the reduction of DHAP was determined from the change in absorbance at 340 nm over a 5–10 min reaction time. The kinetic parameters \( k_{cat} \) and \( K_m \) for hlGPDH-catalyzed reactions were determined from the nonlinear least-squares fit of plots of \( v/\left[E\right] \) against [DHAP] to the Michaelis–Menten equation (eq 1), where [DHAP] is the concentration of the carbonyl form of DHAP that is present as 55% of total DHAP.\(^17\)

\[
\frac{v}{[E]} = \frac{k_{cat}[DHAP]}{K_m + [DHAP]}
\]

The K120A variant hlGPDH-catalyzed reduction of DHAP by NADH in the presence of \( \text{CH}_3\text{CH}_2\text{NH}_3^+ \) was monitored in solutions containing 0.1 mg/mL BSA, 200 μM NADH, 0.5–5 mM DHAP, and 20–80 mM \( \text{CH}_3\text{CH}_2\text{NH}_3^+ \) at \( I = 0.12 \) (NaCl),\(^9\) and using the same buffers as given above for...
The ordered reaction mechanism, with NADH (in Table S1). In several cases, we determined separate sets of the enzyme is better than [DHAP] for reactions at pH 6.5 (Scheme 2). Values of $k_{cat}/K_m$ were previously obtained from Michaelis–Menten plots of $v/[E]$ against [DHAP] for wild type and K120A variant hGPDH-catalyzed reduction of this substrate by NADH (200 μM), at numerous pH values between 4.6 and 9.0 $[I = 0.12 \text{ (NaCl)}]$. We show here (Figures S1 and S2) that identical ($\pm 10\%$) values of $k_{cat}$ and $k_{cat}/K_m$ are obtained for wild type and K120A variant hGPDH-catalyzed reduction of DHAP by 100 and 200 μM NADH at pH 7.5. We concluded that these forms of hGPDH are saturated at pH 7.5 for reactions at 100 μM NADH. We show here (Figures S1 and S2) that identical ($\pm 10\%$) values of $k_{cat}$ and $k_{cat}/K_m$ are obtained for wild type and K120A variant hGPDH-catalyzed reduction of DHAP by 100 and 200 μM NADH at the pH extremes of 4.9 and 9.0.

The kinetic parameters $k_{cat}$ and $k_{cat}/K_m$ for hGPDH-catalyzed reactions, determined from the nonlinear least-squares fit of plots of $v/[E]$ against [DHAP] to eq 1, are listed in Table S1. In several cases, we determined separate sets of kinetic parameters at the same pH, but with separately purified preparations of hGPDH. The agreement between kinetic parameters from different batches of enzyme is better than $\pm 10\%$.

Figure S3 shows plots of $v/[E]$ against $[\text{CH}_3\text{CH}_2\text{NH}_3^+]$, determined at pH 4.9 and 6.0, 25 °C, and $I = 0.12 \text{ (NaCl)}$, for reduction of DHAP by NADH (200 μM) catalyzed by the K120A variant at several different fixed DHAP concentrations. These plots show that $v/[E]$ is independent of $[\text{CH}_3\text{CH}_2\text{NH}_3^+]$ for reactions at low pH, in contrast to the efficient rescue of the K120A variant observed for reactions at pH 7.5. Figure S4 shows plots of $v/[E]$ against [DHAP] for K120A variant hGPDH-catalyzed reduction of DHAP by NADH (200 μM) at 25 °C, $I = 0.12 \text{ (NaCl)}$, and different fixed concentrations of $\text{CH}_3\text{CH}_2\text{NH}_3^+$, for reactions at numerous pH values between 6.5 and 9.0. The values of $(k_{cat}/K_m)_{obs} = \frac{k_{cat}[\text{RNH}_3^+]}{k_{cat[K_m]}}$ for reactions in the presence of different fixed $\text{CH}_3\text{CH}_2\text{NH}_3^+$ concentrations (Figure S4) were determined as the slopes of linear correlations of $v/[E]$ against [DHAP] for reactions at pH 6.5–9.0 (eq 2, derived for Scheme 2). Values of $k_{cat}/k_{cat[K_m]}$ for activation of the K120A variant by $\text{CH}_3\text{CH}_2\text{NH}_3^+$ were determined as the slopes of plots of $(k_{cat}/K_m)_{obs}$ against $[\text{CH}_3\text{CH}_2\text{NH}_3^+]$. In several cases, we determined values of $k_{cat}/k_{cat[K_m]}$ at a single pH, but with two separately prepared and purified samples of hGPDH. The agreement between kinetic parameters from different batches of the enzyme is better than $\pm 10\%$.

Scheme 2. Rescue of the Catalytic Activity of K120A hGPDH by $\text{CH}_3\text{CH}_2\text{NH}_3^+$

$$
\frac{v}{[E]}_{obs} = \frac{v}{[E]}_o + \frac{k_{cat}[RNH_3^+][DHAP]}{k_{cat[K_m]}K_{am}}
$$

$$(2)$$

The pH profiles of second-order rate constants ($k_{cat}/K_m$) are shown for the wild type (●) and K120A variant (▲) hGPDH-catalyzed reduction of the DHAP dianion by NADH and for third-order rate constants ($k_{cat[K_m]}$) (●) for rescue of the K120A variant by $\text{CH}_3\text{CH}_2\text{NH}_3^+$.

Figure 2 and 3 show pH–rate profiles constructed using the kinetic parameters $k_{cat}/k_{cat[K_m]}$ (Scheme 2), and $k_{cat}$ reported in Table S1. hGPDH shows a high specificity for catalysis of the reaction of the DHAP phosphodianion, compared with the monooanion. This arises from the tight ion pair interaction with the cationic side chain of R269, which is estimated to stabilize the hydride transfer transition state by 9 kcal/mol. The values of $(k_{cat}/K_m)_{dianion}$ reported in Figure 2 are calculated from $(k_{cat}/K_m)_{obs}$ (Table S1) as $k_{cat}$/...
\( K_m^{\text{dianion}} = (k_{\text{cat}}/K_m)_{\text{obs}}/f_{\text{dianion}} \) where \( f_{\text{dianion}} \) is determined from the reaction pH and a \( pK_a \) of 6.0 for ionization of the DHAP monoanion to form the dianion.\(^{24} \)

Figure 2 also shows the pH profiles for second-order rate constants \( (k_{\text{cat}}/K_m)_{\text{dianion}} \) for K120A hGlPDH-catalyzed reduction of DHAP by NADH, and the observed third-order rate constants \( (k_{\text{cat}}/K_mK_d)_{\text{obs}} \) for activation of the K120A variant by CH\(_3\)CH\(_2\)NH\(_3^+\). Figure 3 shows the pH profiles for observed first-order rate constants \( (k_{\text{cat}}/K_m)_{\text{obs}} \) for wild type and K120A hGlPDH-catalyzed reduction of DHAP by NADH. The uncertainty in these kinetic parameters, estimated as the average of values determined in separate experiments and using different batches of enzyme, is generally smaller than the symbol in the figure: the exception is data for rescue of the activity of the K120A variant by CH\(_3\)CH\(_2\)NH\(_3^+\) [Figure 2 (●)].

**DISCUSSION**

We note the following unusual features of the pH–rate profiles for the kinetic parameters \( k_{\text{cat}}/K_m \) and \( k_{\text{cat}}/K_mK_d \) (Figure 2) and \( k_{\text{cat}} \) (Figure 3).

1. In three of four pH profiles for \( k_{\text{cat}}/K_m \) and \( k_{\text{cat}} \) for wild type and K120A variant, the pH-dependent kinetic parameter is observed to increase at low pH, in contrast to the expected pH optima at physiologically neutral pH. The exception is the profile for \( k_{\text{cat}} \) for the wild type enzyme, but these values show only a modest change as the pH is decreased from 9.0 to 4.6, and a maximum at pH 8.

2. A value of \( \Delta \log (k_{\text{cat}}/K_m)/(\Delta \text{pH}) = \{ \log[(840 \text{ M}^{-1} \text{ s}^{-1})/(4.7 \text{ M}^{-1} \text{ s}^{-1})]/1.5 \} = 1.5 \) can be calculated from data for K120A hGlPDH-catalyzed reactions of DHAP at pH 9.0 and 7.5 (Figure 2). This is consistent with a slope of >1.0 over this pH range and with the requirement for addition of more than one proton to the K120A variant as the pH is changed from pH 9 to 6, and the variant hGlPDH is converted to the catalytically active form (see below for the fit of these data from Figure 2).

3. The pH–rate profiles from Figure 2 show increasing values of \( \log(k_{\text{cat}}/K_m)_{\text{dianion}} \) for the K120A variant-catalyzed reduction of DHAP, with a decrease in pH, relative to the pH-independent values of \( \log(k_{\text{cat}}/K_mK_d) \) for the rescue of this variant by CH\(_3\)CH\(_2\)NH\(_3^+\). At pH <6.5, where \( (k_{\text{cat}}/K_m)_{\text{dianion}} \gg (k_{\text{cat}}/K_mK_d)[\text{CH}_3\text{CH}_2\text{NH}_3^+] \), rescue is no longer detected. These results require a change, with the changing protonation state of hGlPDH, in the dominant pathway for the K120A variant-catalyzed reduction of DHAP, from a hydride transfer reaction at high pH through a transition state stabilized by exogenous CH\(_3\)CH\(_2\)NH\(_3^+\), to a reaction at pH ≤6.5 through a transition state that shows no detectable stabilization by this cation.

**Modeling the pH–Rate Profiles**. We speculate about the identity of the catalytic side chains that give rise to the breaks in the pH–rate profiles shown in Figures 2 and 3 but focus on the qualitative insight that these profiles provide into the roles of these side chains in stabilization of the hydride transfer transition state. Figure 2 shows the fit of the values of \( \log(k_{\text{cat}}/K_mK_d) \) to eq 2, derived for Scheme 3 for the rescue of the variant by CH\(_3\)CH\(_2\)NH\(_3^+\), using the following values: \( pK_a = 7.7 \) and \( k_{\text{cat}}/K_mK_d = (1.2 \pm 0.17) \times 10^5 \text{ M}^{-2} \text{ s}^{-1} \). By comparison, a \( k_{\text{cat}}/K_mK_d \) value of \( 8.5 \times 10^5 \text{ M}^{-2} \text{ s}^{-1} \) was reported in an earlier study at pH 7.5.\(^5 \) We propose that a \( pK_a \) of 7.7 is for deprotonation of the K204 side chain cation, which lies close to the bound substrate (Figure 1).

![Scheme 3. Kinetic Scheme for Activation of K120A hGlPDH](image)

The pH–rate profiles for \( \log(k_{\text{cat}}/K_m)_{\text{dianion}} \) (Figure 2) and \( k_{\text{cat}} \) (Figure 3) were fit to equations derived for Schemes 4,

![Scheme 4. Kinetic Schemes for hGlPDH-Catalyzed Reduction of DHAP](image)

in which hGlPDH exists largely in the inactive form E at high pH and is converted to EH and E\(_h\) by protonation of side chains with pK\(_a\) and pK\(_b\), respectively. Figure 2 shows the nonlinear least-squares fit of values of \( \log(k_{\text{cat}}/K_m)_{\text{dianion}} \) to eq 3, derived for Scheme 4A, for reactions catalyzed by wild type hGlPDH. This fit gives the following values: \( \text{pK}_a \approx 4.4 \), \( \text{pK}_b = 7.6 \), \( (k_{\text{cat}}/K_m)_{\text{dianion}} = 5.7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1} \), and \( (k_{\text{cat}}/K_m) = 4.7 \times 10^6 \text{ M}^{-1} \text{ s}^{-1} \). We propose that a \( pK_a \) of 7.6 is for deprotonation of the K204 side chain cation and the K120 side chain with pK\(_b\), because a similar downward break is observed for the pH profile for values of \( k_{\text{cat}}/K_mK_d \) for the K120A variant. The poorly defined break in the profile for \( (k_{\text{cat}}/K_m)_{\text{dianion}} \) observed at low pH is due to either (1) protonation of an essential side
chain with a pK_a of \( \approx 4.4 \) or (2) a change in the rate-determining step for the enzyme-catalyzed hydride transfer, from reduction of DHAP to rate-determining formation of the Michaelis complex to DHAP. The value of \( \left( k_{cat}/K_m \right)_{dianion} \) of \( 5.7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1} \) obtained from this fit lies within the range of values for second-order-rate constants determined for rate-determining substrate binding in other enzymatic reactions.\(^{25,26}\)

\[
\left( k_{cat}/K_m \right)_{dianion} = \left( k_{cat}/K_m \right) \frac{1}{1 + \frac{k_a}{[H^+]} + \frac{k_a k_b}{[H^+]} + \frac{k_a k_b}{[H^+]}}
\]

(3)

\[
\left( k_{cat}/K_m \right)_{obs} = \left( k_{cat} \right) \frac{1}{1 + \frac{k_a}{[H^+]} + \frac{k_a k_b}{[H^+]} + \frac{k_a k_b}{[H^+]} + k_{cat}}
\]

(4)

The fit of the values of log \( k_{cat}/K_m \) to eq 3', derived for Scheme 4B, for reactions catalyzed by the K120A variant of hGPDH is shown in Figure 2, where EH shows no detectable activity toward catalysis of reduction of DHAP. The theoretical line through these data was drawn for the following values: pK_a = 5.0, pK_b = 7.6, and \( \left( k_{cat}/K_m \right) = 5.6 \times 10^7 \text{ M}^{-1} \text{ s}^{-1} \). The pK_a value of 7.6 is in agreement with the pK_a of 7.7 determined from the fit of the data for the rescue of the K120A variant by CH_3CH_2NH_3^+ (Scheme 3). This is required because the unactivated and CH_3CH_2NH_3^+-activated reactions of K120A variant DHAP involve monoprotonated hGPDH [EH (Schemes 3 and 4A\')] We propose that the pK_a of 7.6 is for the K204 side chain cation and that the pK_a of 5.0 is for protonation of the D260 side chain to form EH_2, which is discussed below.

Figure 3 shows the fit to eq 4, derived for Scheme 4B, of values of log \( k_{cat} \) for the reaction catalyzed by wild type hGPDH. This fit gives the following values: pK_a = 8.1, pK_b = 8.0, \( k_{cat} = 720 \text{ s}^{-1} \), and \( k_{cat} = 32 \text{ s}^{-1} \). Figure 3 shows that the Michaelis complex of DHAP with wild type hGPDH maintains robust catalytic activity throughout the entire pH range. The pH maximum is more hump- than bell-shaped, because only small decreases in log \( k_{cat} \) were observed on both sides of the maximum. These data may also be fit by co-dependent values of pK_a, pK_b, \( k_{cat} \), and \( k_{cat} \) for a "reverse protonation" reaction mechanism,\(^{27}\) where the essential proton at the monoprotonated enzyme sits at the less basic of two ionizable side chains (pK_a > pK_b), so that the active enzyme EH-S (Scheme 4B) is never the major form. We are unable to rigorously exclude "reverse protonation" for the reaction of these side chains but propose a relatively simple model in which (1) the pK_a of 8.1 is for deprotonation of the K204 side chain, which gives a functional active site, (2) the K120 side chain, which is stabilized by an ion pair to D260, remains protonated throughout the entire pH range for Figure 3, and (3) there is a third unidentified side chain that provides a modest stabilization of the hydride transfer transition state when protonated. This could be one of several second-shell ionizable active site side chains.

Figure 3 shows the fit of values of log \( k_{cat} \) to eq 4', derived for Scheme 4B, where K_a \( \approx [H^+] \), for the reaction catalyzed by the K120A variant of hGPDH. This fit gives the following values: pK_a = 8.1, \( k_{cat} = 0.57 \text{ s}^{-1} \), and \( k_{cat}/K_a = 2.9 \times 10^5 \text{ M}^{-1} \text{ s}^{-1} \). We propose that the pK_a of 8.1 is for deprotonation of the K204 side chain. We suggest that the pK_a of <4.6 is for the carboxylic acid side chain of D260, and that this side chain substitutes for the cationic K120 side chain for the variant-catalyzed hydride transfer reaction at low pH.

**Enzymatic Reaction Mechanism.** Three ionizable amino acid side chains lie close to DHAP bound to wild type hGPDH (Figure 1); K120, K204, and D260 (Scheme 5). We have proposed that the K120 side chain cation acts to stabilize negative charge at the C-2 oxygen, which develops at the hydride transfer transition state, and have provided support for this proposal in studies of the K120A variant.\(^{29}\) The pH-rate profiles from Figure 2 \( \left( k_{cat}/K_m \right)_{dianion} \) and Figure 3 \( k_{cat} \) for wild type hGPDH show that this enzyme is activated by protonation of a side chain with a pK_a of 7.6–8.0. This side chain is not K120, because the pH-rate profiles for the K120A variant from Figure 2 \( k_{cat}/K_m \) and Figure 3 \( k_{cat} \) show the same requirement for a protonated side chain with a pK_a of 8.0.

**Scheme 5. Assignments of Basic Amino Acid Side Chains Whose Protonation States Are Proposed to Control the pH–Rate Profiles Shown in Figures 2 and 3**

![Scheme 5](image-url)

WT Glycerol Phosphate Dehydrogenase

K120A Variant
The second step of proton transfer forming neutral and enzyme-bound G3P is not shown.

**Scheme 6. Hypothetical GPDH-Catalyzed Hydride Transfer from NADH to DHAP to Form an Alkoxide Anion Intermediate Stabilized by the K120 and K204 Side Chain Cations**

![Scheme 6](https://dx.doi.org/10.1021/acs.biochem.0c00801)

**Bronsed Catalysis at the Carbonyl Oxygen.** The Ne atom of K120 is nearly in the plane defined by the trigonal C==O bond of DHAP bound to hlgPDPH (Figure 1). It is well-positioned to protonate this oxygen, while the Ne atom of K204 lies well below this plane and was judged to be less likely to participate directly in protonation of the carbonyl oxygen. Our results are consistent with a model in which protonated side chains of K120 and K204 act together in the stabilization of the transition state of hlgPDPH-catalyzed hydride transfer. The proximity of these two cationic side chains to O-2 favors a “late” transition state, with the nearly complete hydride transfer to the carbonyl carbon providing for optimal stabilizing electrostatic interactions between the side chain cations and negative charge at O-2. There are at least two advantages for the fully stepwise pathway shown in Scheme 6, where the transfer of a hydride to carbon and the transfer of a proton to oxygen occur as separate steps.

1. Immobilization of the K120 side chain in an ion pair with D260 provides for the unusually efficient electrostatic rescue of the K120A variant by CH3CH2NH3+. By comparison, the formation of the stable K120-D260 ion pair should result in a decrease in the acidity of the K120 side chain, or the rescue agent, for deprotonation to form an amine that eliminates the stable ion pair. This decrease in acidity will reduce the driving force for a concerted hydride transfer-reaction mechanism, where there is formal proton transfer from either the K120 side chain or CH3CH2NH3+ rescue agent to O-2 of DHAP.

2. Any transition state stabilization obtained from the concerted transfer of a proton to the developing O-2 oxyanion will be balanced by a weakening of stabilizing electrostatic interactions with the K120 and K204 side chains, which accompanies neutralization of negative charge from proton transfer to O-2. We suggest that the stepwise pathway, with no formal proton transfer to oxygen, provides for optimal electrostatic interactions between the protein catalyst and reaction transition state.

The robust activity for the K120A variant at low pH (Figures 2 and 3) is rationalized by the recruitment of the neutral protonated D260 side chain, to serve in place of cationic K120, in stabilization of negative charge at O-2 at the hydride transfer transition state. This is consistent with a plasticity in side chain function at the active site of hlgPDPH. We suggest that the change in the side chain that participates in protonation of O-2 from the weakly acidic and cationic K120 side chain for wild type hlgPDPH to the strongly acidic and neutral protonated D260 of the K120A variant might be accompanied by a change...
to a concerted reaction mechanism due to the increase in the driving force for protonation of O-2 by the acidic D260 side chain.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at [https://pubs.acs.org/doi/10.1021/acs.biochem.0c00801](https://pubs.acs.org/doi/10.1021/acs.biochem.0c00801).

Kinetic parameters $k_{cat}$ and ($k_{cat}/K_m$)$_{DHAP}$ ($k_{cat}/K_m$)$_{dianion}$ and $k_{cat}/K_m$ for wild type and K120A variant $h$GPDH reactions, respectively (Table S1); Michaelis–Menten plots for wild type and the K120A variant $h$GPDH-catalyzed reduction of DHAP by NADH at pH 4.6–9.0 (Figures S1 and S2, respectively); effect of increasing concentrations of CH$_3$CH$_2$NH$_2^+$ on $v/[E]$ for K120A variant $h$GPDH-catalyzed reduction of DHAP determined at several different fixed concentrations of DHAP at pH 4.9 and 6.0 (Figure S3); effect of increasing concentrations of DHAP on $v/[E]$ for K120A variant $h$GPDH-catalyzed reduction of DHAP determined at several different fixed concentrations of CH$_3$CH$_2$NH$_2^+$ at pH 6.5–9.0 (Figure S4A); and effect of increasing concentrations of CH$_3$CH$_2$NH$_2^+$ on the apparent second-order rate constant for K120A $h$GPDH-catalyzed reduction of DHAP determined at pH 6.5–9.0 (Figure S4B) (PDF)

### Accession Codes

Human glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic, P21695.

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## ABBREVIATIONS

GPDH, glycerol-3-phosphate dehydrogenase; $h$GPDH, glycerol-3-phosphate dehydrogenase from human liver; DHAP, dihydroyacetone phosphate; NADH, nicotinamide adenine dinucleotide, reduced form; NAD, nicotinamide adenine dinucleotide, oxidized form; MES, 2-(N-morpholino)-ethanesulfonic acid; MOPS, 3-morpholinopropane-1-sulfonic acid; TEA, triethanolamine; TAPS, [(tri(hydroxymethyl)-methylamino)propanesulfonic acid; CHES, N-cyclohexyl-2-aminoethanesulfonic acid; BSA, bovine serum albumin.

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