Enzyme Architecture: A Startling Role for Asn270 in Glycerol 3-Phosphate Dehydrogenase-Catalyzed Hydride Transfer

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Supporting Information

ABSTRACT: The side chains of R269 and N270 interact with the phosphodianiion of dihydroxyacetone phosphate (DHAP) bound to glycerol 3-phosphate dehydrogenase (GPDH). The R269A, N270A, and R269A/N270A mutations of GPDH result in 9.1, 5.6, and 11.5 kcal/mol destabilization, respectively, of the transition state for GPDH-catalyzed reduction of DHAP by the reduced form of nicotinamide adenine dinucleotide. The N270A mutation results in a 7.7 kcal/mol decrease in the intrinsic phosphodiianion binding energy, which is larger than the 5.6 kcal/mol effect of the mutation on the stability of the transition state for reduction of DHAP; a 2.2 kcal/mol stabilization of the transition state for unactivated hydride transfer to the truncated substrate glycolaldehyde (GA); and a change in the effect of phosphite diianion on GPDH-catalyzed reaction of GA, from strongly activating to inhibiting. The N270A mutation breaks the network of hydrogen bonding side chains, Asn270, Thr264, Asn205, Lys204, Asp260, and Lys120, which connect the diianion activation and catalytic sites of GPDH. We propose that this disruption dramatically alters the performance of GPDH at these sites.

Orotidine monophosphate decarboxylase (OMPDC),1 triosephosphate isomerase (TIM),2 and glycerol 3-phosphate dehydrogenase (GPDH)3 achieve a high specificity for expression of 6–8 kcal/mol of diianion binding energy at the transition states for the enzyme-catalyzed decarboxylation, proton transfer, and hydride transfer reactions, respectively (Figure 1).4–16 Previous studies of diianion activation of OMPDC and TIM provide support for the proposal that their active sites consist of functionally independent catalytic and diianion activation loci.4,5,7–9,12 Less is known about the mechanism for diianion activation of hydride transfer catalyzed by GPDH.17

The hydrogen bond between the amide side chain of N270 and the phosphodianiion of substrate dihydroxyacetone phosphate (DHAP) is shown in Figure 2.18 We predicted that the elimination of this hydrogen bond by the N270A mutation would result in small and similar decreases in the activation barriers (ΔG‡) for GPDH-catalyzed reactions of whole substrate DHAP, and the substrate pieces glycolaldehyde (GA) and phosphite diianion, as observed in a study of the effect of a Q215A mutation of a similar diianion-grpper residue at OMPDC.12,19 We report, instead, that the N270A mutation causes large and complex changes in the activation barriers for the GPDH-catalyzed reaction of the whole substrate DHAP and the substrate pieces glycolaldehyde (GA) and phosphite diianion. The results suggest that this amide side chain is a linchpin in an assembly of side chains (Figure 2) that connect the catalytic and diianion activation sites and is essential to the optimal functioning of these sites.

The N270A and R269A/N270A mutants of GPDH from human liver (hlGPDH) were prepared by standard methods, described in the Supporting Information, and their activity was assayed at 25 °C and pH 7.5 (triethanolamine buffer) by monitoring the reduction of DHAP by the reduced form of nicotinamide adenine dinucleotide (NADH).15 The Michaelis–Menten plots of initial velocity data, v/[E], against [DHAP] for N270A (Figure S1A) and R269A/N270A (Figure S1B) mutant hlGPDH-catalyzed reactions, conducted at 100 or 200 μM NADH, show excellent fits to single sets of kinetic parameters kcat/Km and Kcat/Km (Table 1), so that Km ≪ 100 μM for NADH.15,17 There is no significant rescue of the activity of the N270A mutant by 60 mM formamide.20

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A comparison of the values for $k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$) for the wild-type and mutant $h$GPDH-catalyzed reactions shows that the N270A and R269A/N270A mutations result in 5.6 and 11.5 kcal/mol destabilization, respectively, of the transition states for $h$GPDH-catalyzed reduction of DHAP by NADH. The latter is slightly larger than the 10.8 kcal/mol intrinsic phosphodianion binding energy for wild-type $h$GPDH, calculated from the ratio of second-order rate constants $k_{cat}/K_m$ for $h$GPDH-catalyzed reduction of the whole and phosphodianion-truncated substrates.17

The effect of R269A and N270A mutations of $h$GPDH on the stability of the transition state for enzyme-catalyzed reduction of DHAP depends upon their order (Scheme 1). The 5.6 kcal/mol effect of the N270A mutation of wild-type $h$GPDH is 3.2 kcal/mol larger than the effect of the N270A mutation at the R269A mutant. The 9.1 kcal/mol effect of the R269A mutation is, likewise, larger than the 5.9 kcal/mol effect of this mutation at the N270A mutant. These results do not reflect the elimination of a 3.2 kcal/mol direct stabilizing interaction between the side chains of R269 and N270,15 because the side chains are well-separated (Figure 2). We propose that the N270A mutation of wild-type $h$GPDH results in the loss of a 2.4 kcal/mol interaction between the amide side chain and the phosphodianion, plus an additional ~3.2 kcal/mol weakening of the 9.1 kcal/mol interaction between the phosphodianion and the side chain of R269 (Scheme 1). The remaining 5.9 kcal/mol interaction of this side chain cation is then eliminated by the R269A mutation of the N270A mutant. By comparison, the N270A mutation at R269A $h$GPDH results in the loss of the intrinsic ~2 kcal/mol side chain–dianion interaction.

The following observations show that the N270A mutation of $h$GPDH results in a dramatic structural reorganization at the active site of $h$GPDH.

(1) The N270A mutation results in a 7.7 kcal/mol decrease in the 10.8 kcal/mol intrinsic phosphodianion binding energy for wild-type $h$GPDG,15 to 3.1 kcal/mol (Table 1). This 7.7 kcal/mol effect is 2.1 kcal/mol larger than the 5.6 kcal/mol effect of the mutation on the stability of the transition state for reduction of DHAP. We conclude that the N270A mutation results in a reorganization of the active site side chains of $h$GPDH, which alters the binding conformation for DHAP. The result is that the large 7.7 kcal/mol reduction in transition state stabilization from phosphodianion binding interactions is offset by a 2.1 kcal/mol transition state stabilization from new interactions with the catalyst.

(2) The N270A mutation cripples $h$GPDH-catalyzed reduction of DHAP but results in a 40-fold increase in $(k_{cat}/K_m)_E$ for $h$GPDH-catalyzed reduction of the truncated substrate GA by NADH (Table 1). This corresponds to a 2.2 kcal/mol stabilization of the transition state for hydride transfer to the truncated substrate, in contrast to the 5.6 kcal/mol destabilization of the transition state for reduction of DHAP (Scheme 1). We suggest that similar enzyme–ligand interactions account for this stabilization of the transition state for reduction of GA, and for reduction of DHAP (see above).

(3) The N270A mutation results in a change in the effect of phosphite dianion on $(k_{cat}/K_m)_{obs}$ for $h$GPDH-catalyzed reduction of GA, from strongly activating15 to inhibiting (Figure 3). Figure 3 (inset) shows the nonlinear least-squares fit of data at 0–1.0 mM HPO$_3^{2-}$ to eq 1, derived for Scheme 2, obtained using a $(k_{cat}/K_m)_E$ of 2.0 M$^{-1}$ s$^{-1}$ (Table 1), a $(k_{cat}/K_m)_E$ of 18.7 M$^{-1}$ s$^{-1}$ (Table 1), and a $(k_{cat}/K_m)_E$ of 2.3 M$^{-1}$ s$^{-1}$ (Table 1).

Table 1. Kinetic Parameters for Wild-Type and Mutant $h$GPDH-Catalyzed Reactions of Whole Substrate DHAP and Truncated Substrate Glycolaldehyde at pH 7.5 (triethanolamine buffer) and an Ionic Strength of 0.12 (NaCl)$^a$

| enzyme         | $k_{cat}$ (s$^{-1}$)$^b$ | $K_m$ (M)$^b$ | $k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$)$^b$ | $(k_{cat}/K_m)_E$ (M$^{-1}$ s$^{-1}$)$^d$ |
|----------------|-------------------------|----------------|-------------------------------------|------------------------------------------|
| WT,14 10.8 kcal/mol$^c$ | 240 ± 10                | (5.2 ± 0.3) × 10$^{-3}$ | (4.6 ± 0.3) × 10$^6$ | (5.0 ± 0.6) × 10$^{-2}$ |
| R269A,9.1 kcal/mol$^c$ | 3(5.9 ± 0.4) × 10$^{-3}$ | (5.7 ± 0.5) × 10$^{-3}$ | 1.0 ± 0.1 | ≤0.003$^e$ |
| N270A, 3.1 kcal/mol$^c$ 5.6 kcal/mol$^d$ | 9.0 ± 0.5                | (2.5 ± 0.2) × 10$^{-3}$ | 360 ± 35 | 2.0 ± 0.2 |
| R269A/N270A, 11.5 kcal/mol$^d$ | (2.8 ± 0.1) × 10$^{-4}$ | (1.5 ± 0.1) × 10$^{-2}$ | (1.7 ± 0.1) × 10$^{-3}$ | ≤0.003$^e$ |

$^a$The uncertainty in the kinetic parameters is the standard error from least-squares fits of the kinetic data. $^b$Kinetic parameter for $h$GPDH-catalyzed reactions at saturating [NADH], calculated for the carbonyl form of DHAP.15 $^c$Calculated for the carbonyl form of GA at saturating [NADH].15 $^d$From ref 17. $^e$The intrinsic dianion binding energy, calculated from the ratio of $k_{cat}/K_m$ for $h$GPDH-catalyzed reduction of DHAP and GA.$^f$Effect of the mutation on the activation barrier $\Delta G^\ddagger$ for wild-type $h$GPDH (Scheme 1), determined as described in the text. $^g$Estimated upper limit for $(k_{cat}/K_m)_E$ calculated as described in the Supporting Information.
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Figure 3. Effect of increasing [HPO$_3^{2-}$] on the observed second-order rate constant for N270A hGPDH-catalyzed reduction of GA at 25 °C and pH 7.5 (10 mM triethanolamine buffer). The data from the inset show a good fit to eq 1 derived for Scheme 2.

Scheme 2

\[
\begin{align*}
\text{E + S} & \xrightarrow{\text{cat/K_m/E}} \text{E+HPO$_3^{2-}$ + S} \\
\text{Products} & \xrightarrow{\text{k_{cat}/K_m/E}} \text{Products}
\end{align*}
\]

\[
\frac{k_{\text{cat}}}{K_{\text{m,obs}}} = \frac{k_{\text{cat}}}{k_{\text{cat}}/K_{\text{m,obs}}} \cdot \frac{K_{\text{d}}}{K_{\text{d}} + [\text{HPO$_3^{2-}$}]} \cdot \frac{K_{\text{d}}}{K_{\text{d}} + [\text{HPO$_3^{2-}$}]} \cdot \frac{k_{\text{cat}}}{K_{\text{m,E}}/K_{\text{m,HIP}}}
\]

Scheme 1

\[
\begin{align*}
\text{E} & \xrightarrow{\text{cat/K_m/E}} \text{E+HPO$_3^{2-}$ + S} \\
\text{Products} & \xrightarrow{\text{k_{cat}/K_m/E}} \text{Products}
\end{align*}
\]

N270A mutation results in a 40-fold increase in \(k_{\text{cat}/K_{\text{m,E}}}\). hGPDH-catalyzed reduction of GA by NADH.

(3) The Q215A, Y217F, and R235A mutations each result in the same increase in activation barrier \(\Delta G^\ddagger\) for the catalyzed reactions of whole substrate OMP, and the substrate pieces (Figure 1).22 This supports the conclusion that the entire effect of each mutation results from the elimination of single side chain interactions with enzyme-bound phosphate dianion or the substrate phosphodianion. By contrast, we propose that the N270A mutation results in a dramatic structural reorganization at the active site of hGPDH that extensively alters ligand—side chain interactions.

(4) The side chain cations of K12, R235, and R269 from TIM,24 OMPDC,25 and hGPDH,18 respectively, form ion pairs to the substrate phosphodianion. The K12G, R235A, and R269A mutations result in 7.8, 5.8, and 9.1 kcal/mol destabilization of the respective enzymatic transition states.17,22,26 The binding of alkyl ammonium cations (K12G)27 or guanidine cation (R235A and R269A)17,22 at the site of the excised side chain results in a large rescue of enzymatic activity. The previous results were cited as evidence of a similar catalytic architecture for TIM, OMPDC, and GPDH that optimizes stabilizing ion pairing interactions. The results reported in this paper show that this similarity in architecture for GPDH does not extend beyond the cationic side chain of R269.

To summarize, the restructuring of the active site of N270A mutant hGPDH results in (a) a 7.7 kcal/mol reduction in the intrinsic phosphodianion binding energy, (b) a 2.2 kcal/mol stabilization of the transition state for reduction of GA, and (c) the elimination of the specificity for expression of dianion binding at the transition state for GPDH-catalyzed reduction of GA.8,15 These effects are consistent with the alteration of interactions between the site for catalysis of hydride transfer and for dianion activation. We note that the N270A mutation disrupts the following network of hydrogen-bonded side chains, Asn270, Thr264, Asn205, Lys204, Asp260, and Lys120 (Figure 2 and Figure S3), which forms a bridge between the Asn270 side chain, which interacts with the substrate phosphodianion, and Lys120, which interacts with the carbonyl of DHAP. The distances between the interacting side chains are listed in Table S1 of the Supporting Information. We propose that this bridge plays a key role in enabling the differential expression of dianion interactions at the Michaelis complex and the transition state for GPDH-catalyzed hydride transfer.

These results demonstrate a previously unrecognized plasticity in the active site of GPDH, which is reflected by the opposing effects of the N270A mutation on enzyme activity toward reduction of whole substrate DHAP and truncated substrate glycolaldehyde. We emphasize, on the one hand, that the nature of this plasticity is not understood while, on the other, such unexpected and dramatic results are often the driving force for new discoveries.

Finally, we note that wild-type GPDH shows a large intrinsic dianion binding energy, but a low activity for catalysis of reduction of GA,8,15 while the N270A mutation shows a dramatic reduction in the intrinsic dianion binding energy but an increase in the level of direct stabilization of the transition state for reduction of GA. Consequently, wild-type hGPDH compared with N270A hGPDH shows optimal transition state stabilization from dianion interactions, but this is obtained only at the expense of protein—ligand interactions, which stabilize the transition state for reduction of GA catalyzed by the N270A
mutant. This emphasizes the imperative for effective catalysis, through specificity in the expression of intrinsic dianion interactions at the transition state for GPDH-catalyzed hydride transfer.\textsuperscript{16,28}

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.6b00116.

Procedures for the preparation of mutant hlgPDHs, kinetic protocols, Michaelis–Menten plots for N270A and R269A/N270A mutant enzyme-catalyzed reactions, the dependence of the velocity of wild-type and N270A mutant enzyme-catalyzed reactions on $[\text{HPO}_4^{2-}]$, a representation of the enzyme active site, and a table that lists the distances between interacting side chains (PDF)

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**Notes**

The authors declare no competing financial interest.

**REFERENCES**

(1) Radzicka, A., and Wolfenden, R. (1995) *Science* 267, 90–93.
(2) Richard, J. P. (2012) *Biochemistry* 51, 2652–2661.
(3) Choe, J., Guerra, D., Michels, P. A. M., and Hol, W. G. J. (2003) *J. Mol. Biol.* 329, 335–349.
(4) Zhai, X., Amyes, T. L., and Richard, J. P. (2015) *J. Am. Chem. Soc.* 137, 15185–15197.
(5) Reyes, A. C., Zhai, X., Morgan, K. T., Reinhardt, C. J., Amyes, T. L., and Richard, J. P. (2015) *J. Am. Chem. Soc.* 137, 1372–1382.
(6) Zhai, X., Go, M. K., O’Donoghue, A. C., Amyes, T. L., Pegan, S. D., Wang, Y., Loria, J. P., Mesecar, A. D., and Richard, J. P. (2014) *Biochemistry* 53, 3486–3501.
(7) Zhai, X., Amyes, T. L., and Richard, J. P. (2014) *J. Am. Chem. Soc.* 136, 4145–4148.
(8) Richard, J. P., Amyes, T. L., Goryanova, B., and Zhai, X. (2014) *Curr. Opin. Chem. Biol.* 21, 1–10.
(9) Zhai, X., Amyes, T. L., Wierenga, R. K., Loria, J. P., and Richard, J. P. (2013) *Biochemistry* 52, 5928–5940.
(10) Malabanan, M. M., Nitsch-Velasquez, L., Amyes, T. L., and Richard, J. P. (2013) *J. Am. Chem. Soc.* 135, 5978–5981.
(11) Malabanan, M. M., Koudelka, A. P., Amyes, T. L., and Richard, J. P. (2012) *J. Am. Chem. Soc.* 134, 10286–10298.
(12) Goldman, L. M., Amyes, T. L., Goryanova, B., Gerlt, J. A., and Richard, J. P. (2014) *J. Am. Chem. Soc.* 136, 10156–10165.
(13) Goryanova, B., Spong, K., Amyes, T. L., and Richard, J. P. (2013) *Biochemistry* 52, 537–546.
(14) Goryanova, B., Goldman, L. M., Amyes, T. L., Gerlt, J. A., and Richard, J. P. (2013) *Biochemistry* 52, 7500–7511.
(15) Tsang, W.-Y., Amyes, T. L., and Richard, J. P. (2008) *Biochemistry* 47, 4575–4582.
(16) Amyes, T. L., and Richard, J. P. (2013) *Biochemistry* 52, 2021–2035.
(17) Reyes, A. C., Koudelka, A. P., Amyes, T. L., and Richard, J. P. (2015) *J. Am. Chem. Soc.* 137, 5312–5315.
(18) Ou, X., Ji, C., Han, X., Zhao, X., Li, X., Mao, Y., Wong, L.-L., Bartlam, M., and Rao, Z. (2006) *J. Mol. Biol.* 357, 858–869.