Enzyme Architecture: Self-Assembly of Enzyme and Substrate Pieces of Glycerol-3-Phosphate Dehydrogenase into a Robust Catalyst of Hydride Transfer

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ABSTRACT: The stabilization of the transition state for \( hGPDH \)-catalyzed reduction of DHAP due to the action of the phosphodianion of DHAP and the cationic side chain of R269 is between 12.4 and 17 kcal/mol. The R269A mutation of glycerol-3-phosphate dehydrogenase (\( hGPDH \)) results in a 9.1 kcal/mol destabilization of the transition state for enzyme-catalyzed reduction of dihydroxyacetone phosphate (DHAP) by NADH, and there is a 6.7 kcal/mol stabilization of this transition state by 1.0 M guanidine cation (\( \text{Gua}^+ \)) [\( \text{J. Am. Chem. Soc. 2015, 137, S312–S315} \)]. The R269A mutant shows no detectable activity toward reduction of glycolaldehyde (\( \text{GA} \)), or activation of this reaction by 30 mM HPO\(_4^{2-}\). We report the unprecedented self-assembly of R269A \( hGPDH \) dianions (\( X^2=-\text{FPO}_4^{2-}, \text{HPO}_4^{2-}, \) or \( \text{SO}_4^{2-}\)), \( \text{Gua}^+ \) and \( \text{GA} \) into a functioning catalyst of the reduction of \( \text{GA} \), and fourth-order reaction rate constants \( k_{cat}/K_{cat} \) for dianion activation of wildtype \( hGPDH \)-catalyzed reduction of \( \text{GA} \) and \( k_{cat}/K_{cat} \text{K}_{\text{Gua}} \) shows that the electrostatic interaction between exogenous dianions and the side chain of R269 is not significant perturbed by cutting \( hGPDH \) into R269A and \( \text{Gua}^+ \) pieces. The advantage for connection of \( hGPDH \) (R269A mutant + \( \text{Gua}^+ \)) and substrate pieces (\( \text{GA} + \text{HPi} \)) pieces, \( \Delta G^{\ddagger}_{\text{GA}+\text{HPi}+\text{Gua}} = 5.6 \text{ kcal/mol} \), is nearly equal to the sum of the advantage to connection of the substrate pieces, \( \Delta G^{\ddagger}_{\text{GA}+\text{HPi}} = 3.3 \text{ kcal/mol} \), for wildtype \( hGPDH \)-catalyzed reaction of \( \text{GA} + \text{HPi} \) and for connection of the enzyme pieces, \( \Delta G^{\ddagger}_{\text{Gua}} = 2.4 \text{ kcal/mol} \), for \( \text{Gua}^+ \) activation of the R269A \( hGPDH \)-catalyzed reaction of DHAP.

INTRODUCTION

The term self-assembly is used in biology, supramolecular chemistry, and nanotechnology to describe molecules that spontaneously combine to form defined structures, which carry out complex functions. Protein catalysts and their substrates may be deconstructed into two or more pieces by cleavage of covalent bonds, but there have been limited studies on the reassembly of these pieces into a catalytically active unit, because of the anticipated difficulties in overcoming the entropic barrier to reassembly. An important and under-appreciated exception is the rescue of mutant enzymes by small molecule analogues of the excised amino acid side chain. We have developed a protocol for comparing the reactivity of whole enzyme and substrate with the reactivity of the enzyme and substrate in pieces, and have applied this protocol to mutants of triosephosphate isomerase (TIM, K12G mutant), orotidine 5’-monophosphate decarboxylase (OMPDC, R235A mutant), and glycerol-3-phosphate dehydrogenase (GPDH, R269A mutant). In each case, the side chain cation of the essential amino acid sits at a cleft on the protein surface and interacts with the phosphodianion through a strong ion pair, as shown in Figure 1A for OMPDC and Figure 1B for GPDH from human liver (\( hGPDH \)). The efficient rescue of these mutant enzymes reflects the ease of transfer of the exogenous cation from water to the protein surface, and the high stability of the transition state cation-phosphodianion pair.

We now extend earlier work, and report the results of a kinetic study to characterize the unprecedented self-assembly of R269A mutant \( hGPDH \) dianions (\( X^2=-\text{FPO}_4^{2-}, \text{HPO}_4^{2-}, \) or \( \text{SO}_4^{2-}\)), \( \text{Gua}^+ \) and \( \text{GA} \) into a functioning catalyst of the reduction of \( \text{GA} \), and fourth-order reaction rate constants \( k_{cat}/K_{cat} \) for dianion activation of wildtype \( hGPDH \)-catalyzed reduction of \( \text{GA} \) and \( k_{cat}/K_{cat} \text{K}_{\text{Gua}} \) shows that the electrostatic interaction between exogenous dianions and the side chain of R269 is not significant perturbed by cutting \( hGPDH \) into R269A and \( \text{Gua}^+ \) pieces. The advantage for connection of \( hGPDH \) (R269A mutant + \( \text{Gua}^+ \)) and substrate pieces (\( \text{GA} + \text{HPi} \)) pieces, \( \Delta G^{\ddagger}_{\text{GA}+\text{HPi}+\text{Gua}} = 5.6 \text{ kcal/mol} \), is nearly equal to the sum of the advantage to connection of the substrate pieces, \( \Delta G^{\ddagger}_{\text{GA}+\text{HPi}} = 3.3 \text{ kcal/mol} \), for wildtype \( hGPDH \)-catalyzed reaction of \( \text{GA} + \text{HPi} \) and for connection of the enzyme pieces, \( \Delta G^{\ddagger}_{\text{Gua}} = 2.4 \text{ kcal/mol} \), for \( \text{Gua}^+ \) activation of the R269A \( hGPDH \)-catalyzed reaction of DHAP.

EXPERIMENTAL SECTION

Materials. Water was obtained from a Milli-Q Academic purification system. Q-Sepharose was purchased from GE Healthcare. Nicotinamide adenine dinucleotide reduced form (NADH, disodium salt), glycolaldehyde dimer, 2-(N-morpholino)ethanesulfonic acid

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Published procedures.5a Concentrated solutions of R269A mutant to give the desired acid/base ratio and were prepared by addition of 1 M NaOH or 1 M HCl and solid NaCl.

The concentration of the carbonyl form of GA is calculated from the absorbance at 340 nm (eq = 6200 M$^{-1}$ cm$^{-1}$) at 25 °C and in the presence of 20 μM GPDH and either 30 mM Gua$^+$ or 30 mM phosphite dianion (HPi). No attempt was made to rigorously demonstrate that the small difference between $\Delta A_{340}$ observed in the presence and absence of GPDH ($\Delta A_{340}$ ≈ 0.0042) represented the slow R269A $h$GPDH-catalyzed reduction of GA. Instead, we use this difference to obtain an upper limit for reaction velocity. This sets an upper limit of 0.16 μM for the decrease in [NADH] for the 60 min R269A $h$GPDH-catalyzed reduction of GA; and, the following upper limits for the rate constants for these $h$GPDH-catalyzed reactions (Scheme 2): $k_{cat}/k_{GA} < 0.003$ M$^{-1}$ s$^{-1}$ for R269A $h$GPDH-catalyzed reduction of GA; $k_{cat}/k_{GA}K_{HIPI} < 0.1$ M$^{-2}$.

Scheme 2

$$E = R269A\ AGPDH$$

$$\text{E-NADH} \leftrightarrow \text{E-NADH-GA}$$

pH meter equipped with a Radiometer pHC4006-9 combination electrode that was standardized at pH 4.00, 7.00, and 10.00 at 25 °C. The concentration of NADH in these solutions was determined at 340 nm. Assay mixtures for R269A $h$GPDH-catalyzed reduction of GA by NADH at pH 7.5 contained 10 mM triethanolamine (pH 7.5), 0.2 mM NADH, 1.8 or 3.6 mM of the carbonyl form of GA, from 0 to 30 mM guanidine hydrochloride and from 0 to 30 mM oxydianion at $I = 0.12$ (NaCl). The following enzyme concentrations were used in studies on activation by different dianions: [E] = 20 μm, HPO$_3^{2-}$; [E] = 5–10 μm, FPO$_3^{2-}$; [E] = 40 μm, SO$_4^{2-}$. The initial velocity was determined over a 10–40 min reaction time. The pH was determined at the end of each reaction, and in no case was a significant (>0.01 unit) change observed in the starting pH.

RESULTS

The R269A $h$GPDH-catalyzed reduction of GA (3.6 mM carbonyl form of GA) by NADH (0.2 mM $\gg K_m$) in the absence of GPDH, in the presence of 20 μM GPDH, and in the presence of 20 μM GPDH and either 30 mM Gua$^+$ or 30 mM phosphate dianion (HP$_2$) was monitored at 340 nm for a total of 60 min. A marginally larger decrease in $A_{340}$ was observed for the reaction in the presence of GPDH ($\Delta A_{340} $ ≈ 0.0102) than for the reaction in the absence of GPDH ($\Delta A_{340} $ ≈ 0.0080). This difference was not significantly affected by the addition of 30 μM Gua$^+$ or 30 mM phosphate dianion (HP$_2$). No attempt was made to rigorously demonstrate that the small difference between $\Delta A_{340}$ observed in the presence and absence of GPDH ($\Delta A_{340} $ ≈ 0.0042) represented the slow R269A $h$GPDH-catalyzed reduction of GA. Instead, we use this difference to obtain an upper limit for reaction velocity. This sets an upper limit of 0.16 μM for the decrease in [NADH] for the 60 min R269A $h$GPDH-catalyzed reduction of GA; and, the following upper limits for the rate constants for these $h$GPDH-catalyzed reactions (Scheme 2): $k_{cat}/k_{GA} < 0.003$ M$^{-1}$ s$^{-1}$ for R269A $h$GPDH-catalyzed reduction of GA; $k_{cat}/k_{GA}K_{HIPI} < 0.1$ M$^{-2}$.

Scheme 2

$$E = R269A\ AGPDH$$

$$E \text{-NADH} \leftrightarrow E \text{-NADH-GA}$$

Figure 1. A comparison of the protein surfaces from X-ray crystal structures of the following: (A) The complex between OMPDC from yeast and 6-hydroxymethyluridine 5’-monophosphate (PDB entry 1DQX). (B) The nonproductive ternary complex of dihydroxyacetone phosphate (DHAP) and NAD$^+$ with hGPDH (PDB entry 1WPQ). These structures show the loops that trap the ligand in a protein cage shaded red, and the guanidine side chains at the protein surface shaded black. The respective enzyme-bound ligands at the structures represented by A and B are buried in the protein, with the phosphodianion projecting toward the surface, and in a stable ion pair with the guanidine side chains of R235 (OMPDC) or R269 (hGPDH). Reproduced from ref 5a. Copyright 2015 American Chemical Society.
s⁻¹ for activation of R269A hGPDH-catalyzed reduction of GA by 30 mM HPi; and, $k_{cat}/K_{i_GA}\text{[Gua]} < 0.1 \text{ M}^{-2} \text{s}^{-1}$ for activation of R269A hGPDH-catalyzed reduction of GA by 30 mM Gua⁺.

Figure 2 shows the effect of increasing concentrations of phosphate (HPi) (Figure 2A) fluorophosphate (FPi) (Figure 2B) and sulfate (SO₄²⁻) (Figure 2C) dianions on the observed second-order rate constant $v/\langle E\rangle \text{[GA]} \text{ (M}^{-1} \text{s}^{-1})$ for R269A hGPDH-catalyzed reduction of the carbonyl form of GA by saturating 0.2 mM NADH at different fixed concentrations of Gua⁺. The effect of increasing [HPi] on $v/\langle E\rangle \text{[GA]} \text{ (M}^{-1} \text{s}^{-1})$ for reactions activated by 30 mM or 15 mM Gua⁺ at 60 mM (solid symbols), and at 30 mM (open symbols) total [carbonyl + hydrate] GA. These correspond to 3.6 mM and 1.8 mM, respectively, of the reactive carbonyl form of GA. In every case, the data obtained at the two concentrations of GA show a good fit to a single correlation line. This shows that reactions at total [GA] ≤ 60 mM are first-order in [GA], so that the apparent $K_{GA}$ for dissociation of GA from R269A mutant hGPDH is >60 mM (Scheme 2).

The apparent third-order rate constants $k_{cat}/K_{i_GA}\text{[Gua]} \text{[X]}$ (M⁻³ s⁻¹) for dianion activation of R269A hGPDH-catalyzed reduction of GA (carbonyl form) determined as the slopes of the linear correlations from Figure 2, are reported in Table 1. Figure 3 shows plots of $k_{cat}/K_{i_GA}\text{[Gua]} \text{[X]}$ for dianion activation of R269A hGPDH-catalyzed reduction of GA by the combined action of Gua⁺ and dianions X²⁻ (Scheme 2).

**DISCUSSION**

This work combines two protocols from studies on the reactions of substrates in pieces and on enzymes in pieces.¹² ¹³ (1) The reactivity of hGPDH in catalysis of the reaction of the substrate DHAP [(k_{cat}/K_{m})_{DHAP}] was compared with that for catalysis of the truncated substrate GA [(k_{cat}/K_{m})_{GA}] (Scheme 3) and for dianion activation of catalysis of GA [(k_{cat}/K_{m})_{GPDH\text{[Gua]}}, Scheme 3] and for dianion activation of catalysis of GA [(k_{cat}/K_{m})_{GPDH\text{[Gua]}}, Scheme 3]. These comparisons give changes in activation barriers ($\Delta \Delta G^{\ddagger}$) that correspond to (a) the stabilization of the transition state for hGPDH-catalyzed hydride transfer to DHAP by interaction with the phosphate phosphodianion ($\Delta G_{\text{Pi}}^{\ddagger}$) or (b) the stabilization of the transition state for hGPDH-catalyzed hydride transfer to GA by interaction with 1.0 M phosphate dianion ($\Delta G_{\text{phosphate}}^{\ddagger}$).

The advantage obtained from connecting the pieces GA and Gua⁺ by interaction with 1.0 M phosphite dianion ($\Delta G_{\text{Pi}}^{\ddagger} = 0.12$ (NaCl)). (A) The increase in $v/\langle E\rangle \text{[GA]} \text{ (M}^{-1} \text{s}^{-1})$, with increasing [HPi], for reactions in the presence of 30 mM (open symbols) or 60 mM (closed symbols) total GA [carbonyl + hydrate] at different fixed concentrations of Gua⁺.

The equilibrium constant for hydration of GA is $K_{eq} = \text{[carbonyl]/[hydrate]} = (6/94)$. Key: ([GA]_{carbonyl} = 2.8 mM) (▼, 30 mM Gua⁺; (●), 25 mM Gua⁺; (■), 20 mM Gua⁺; (▲), 10 mM Gua⁺; ([GA]_{carbonyl} = 1.8 mM) (□), 30 mM Gua⁺; (○), 15 mM Gua⁺. (B) The increase in $v/\langle E\rangle \text{[GA]} \text{ (M}^{-1} \text{s}^{-1})$, with increasing [FPi], for reactions at [GA]_{carbonyl} = 3.6 mM and at different fixed concentrations of Gua⁺. Key: (▼) 30 mM Gua⁺; (●), 25 mM Gua⁺; (■), 20 mM Gua⁺; (▲), 15 mM Gua⁺; (○), 10 mM Gua⁺. (C) The increase in $v/\langle E\rangle \text{[GA]} \text{ (M}^{-1} \text{s}^{-1})$, with increasing [SO₄²⁻], for reactions at 3.6 mM GA and at different fixed concentrations of Gua⁺. Key: (▼) 30 mM Gua⁺; (●), 25 mM Gua⁺; (■), 20 mM Gua⁺; (▲), 15 mM Gua⁺; (○), 10 mM Gua⁺. Figure 3 shows the linear plots of the slopes of these linear correlations, $k_{cat}/K_{i_GA}\text{[Gua]} \text{[X]}$ against [Gua⁺].

**Table 1. Kinetic Parameters for Activation of R269A hGPDH-Catalyzed Reduction of GA (1.8 mM or 3.6 mM) by the Combined Action of a Dianion and Gua⁺**

| dianion | Gua⁺ (mM) | $k_{cat}/K_{i_GA}\text{[Gua]} \text{[X]}$ × 10⁻³ | $k_{cat}/K_{i_GA}\text{[Gua]} \text{[X]}$ × 10⁻³ |
|--------|-----------|------------------|------------------|
| HPi⁻² | 10        | 3.4 ± 0.12       | 340 ± 10         |
|        | 15        | 5.3 ± 0.19       | 52.0 ± 0.2        |
|        | 20        | 6.7 ± 0.15       | 10.2 ± 0.2        |
|        | 25        | 8.4 ± 0.10       | 10.2 ± 0.2        |
|        | 30        | 10.0 ± 0.13      | 10.2 ± 0.2        |
| FPi⁻² | 10        | 28.2 ± 0.6       | 2850 ± 50        |
|        | 15        | 42.6 ± 0.7       | 4250 ± 50        |
|        | 20        | 54.8 ± 1.4       | 5450 ± 50        |
|        | 25        | 71.9 ± 1.5       | 7150 ± 50        |
|        | 30        | 85.5 ± 1.3       | 8550 ± 50        |
| SO₄²⁻ | 10        | 0.257 ± 0.004    | 27 ± 1           |
|        | 15        | 0.350 ± 0.002    | 350 ± 2           |
|        | 20        | 0.494 ± 0.004    | 494 ± 2           |
|        | 25        | 0.681 ± 0.007    | 681 ± 2           |
|        | 30        | 0.884 ± 0.013    | 884 ± 2           |

*For reactions at pH 7.5, 25 °C, saturating [NADH] = 0.2 mM and I = 0.12 (NaCl). The advantage obtained from connecting the pieces GA and Gua⁺ by interaction with 1.0 M phosphate dianion ($\Delta G_{\text{Pi}}^{\ddagger} = 0.12$ (NaCl)).*
from earlier work are summarized in Table 2. We use the connection energy (ΔG^‡)_{GA-X} = 3.3 kcal/mol for activation by HPO_4^{2-} in our analyses, because phosphite dianion is the best steric and electronic dianion analogue for the phosphodianion of the whole substrate.

(ΔG^‡)_{GA-X} = -RT ln \left( \frac{[k_{cat}/K_m]_{GA}}{[k_{cat}/K_m]_{GA-X}} \right) = 3.3 \text{ kcal/mol (2)}

(ΔG^‡)_{X + GA} = RT ln \left( \frac{[k_{cat}/K_m]_{DHAP}}{[k_{cat}/K_m]_{HPi}} \right) = 7.5 \text{ kcal/mol (3)}

(ΔG^‡)_{GA} = -RT ln \left( \frac{[k_{cat}/K_m]_{GA}}{[k_{cat}/K_m]_{GA-HPi}} \right) = 10.8 \text{ kcal/mol (1)}

Energetics for the Self-Assembly of Substrate and Enzyme Pieces. The R269A mutant of hGPDH shows no detectable activity toward catalysis of reduction of the truncated substrate GA by NADH (k_{cat}/K_m \leq 0.003 \text{ M}^{-1} \text{s}^{-1}) or for reduction of GA in the presence of 30 mM HP, or Gua^+. However, R269A hGPDH is activated for catalysis of reduction of GA by the combined binding of HP, or Gua^+. Activation is also observed by FPO_4^{2-} or SO_4^{2-} in combination with Gua^+ (Figure 3).

Table 1 reports k_{cat}/K_m for R269A hGPDH for the fourth order rate constant for activation of R269A hGPDH for catalysis of reduction of GA by the combined action of X^2= HP, or Gua^+ (Scheme 7). This represents a 340-fold rate enhancement, at a standard state of 1.0 M substrate and activator, compared with R269A mutant-enzyme catalyzed reaction of DHAP (k_{cat}/K_m)_{WT} = 4.6 \times 10^5 \text{ M}^{-1} \text{s}^{-1} for wildtype hGPDH-catalyzed reduction of DHAP shows that the connection of the enzyme and substrate pieces to give the whole enzyme and substrate results in a 13 500-fold rate enhancement, which corresponds to a connection energy of (ΔG^‡)_{X+GA} = 5.6 \text{ kcal/mol (eq 7). Table 2 summarizes the values of k_{cat}/K_m for activation of hGPDH-catalyzed reduction of DHAP by interaction with the R269 side chain, (ΔG^‡)_{R269} (eq 4), or (b) the stabilization of the transition state for hGPDH-catalyzed reduction of DHAP by interaction with 1.0 M Gua^+, (ΔG^‡)_{Gua} (eq 5). The advantage obtained from connecting the enzyme pieces R269A hGPDH and Gua^+ is calculated as (ΔG^‡)_{E+Gua} = (ΔG^‡)_{R269} + (ΔG^‡)_{Gua} (eq 6). The relationships from eq 4-6 are illustrated by Scheme 6.

(ΔG^‡)_{R269} = -RT ln \left( \frac{[k_{cat}/K_m]_{R269A}}{[k_{cat}/K_m]_{WT}} \right) = -9.1 \text{ kcal/mol (4)}

(ΔG^‡)_{Gua} = -RT ln \left( \frac{[k_{cat}/K_m]_{Gua}}{[k_{cat}/K_m]_{R269A}} \right) = -6.7 \text{ kcal/mol (5)}

(ΔG^‡)_{E+Gua} = RT ln \left( \frac{[k_{cat}/K_m]_{WT}}{[k_{cat}/K_m]_{GA}} \right) = 2.4 \text{ kcal/mol (6)}
Scheme 7

Table 2. Kinetic Parameters and Derived Gibbs Free Energy Terms for Reactions of the Substrate and Enzyme Pieces Catalyzed by TIM, OMPDC and hGPDH

of hGPDH-catalyzed reactions of GA by the combined action of dianions HPO$_4^{2-}$, FPO$_4^{2-}$ or SO$_4^{2-}$ and Gua$^*$.  

$$
\Delta G_f^{R} = RT \ln \left( \frac{k_{cat}/K_m}{k_{cat}/K_{GA}K_{Gua}} \right)_{WT} = 5.6 \text{ kcal/mol}
$$

TIM and GPDH both recover a part of the activity of the whole phosphorylated substrate when catalyzing the reaction of truncated substrate and phosphate dianion pieces, and a part of the reactivity of the whole enzyme when the mutant (R269A hGPDH or K12G TIM) is combined with the truncated cation piece (Table 2). However, only R269A hGPDH binds both substrate (GA + HP) and enzyme (Gua$^+$) pieces to form a robust active enzyme. By contrast, no activity is observed for K12G TIM in the presence of the corresponding substrate and enzyme (GA + HP + EtNH$_3^+$) pieces. This difference in the tendency of the respective pieces for hGPDH and TIM to assemble into an active catalyst is reflected by the large difference between the sum of the connection energies for the hGPDH substrate and enzyme pieces ($\left( \Delta G_f^{R}_{GA+X} + \Delta G_f^{R}\right)_{GA+Gua}$) = 3.3 + 2.4 = 5.7 kcal/mol, which favors the reaction of the pieces, and the larger sum for TIM ($\left( \Delta G_f^{R}_{GA+X} + \Delta G_f^{R}\right)_{EN(RH)N} = 6.7 + 4.4 = 11.1$ kcal/mol (Table 2). The intermediate value of ($\Delta G_f^{R}_{GA+X} + \Delta G_f^{R}\right)_{E(GA)+Gua}$ = $4.1 + 3.0$ = 7.1 kcal/mol determined for reactions catalyzed by wildtype and R235A mutant OMPDC predicts that if activation of R235A mutant OMPDC catalyzed decarboxylation of 1-[(β-D-erythrofuranosyl)orotic acid (EO) by the combined action of HP$^+$ and Gua$^*$ is observed, then this activation will be weaker than for hGPDH.

Capturing the Reactivity of the Whole in the Two Pieces. The substrate phosphorydian and exogenous phosphate dianion each strongly activate TIM for catalysis of the isomerization reaction; and, the effect of connecting the dianion to the carbon acid substrate is mainly to reduce the change in entropy associated with the binding of two or three ligands, compared to a single ligand.

(1) The total connection energy for the reaction of the collection of enzyme and substrate pieces, ($\Delta G_f^{R}_{HP+ER+Gua}$ = 5.6 kcal/mol (eq 7), is essentially equal to the sum of the connection energies ($\Delta G_f^{R}_{GA+HP}$).
= 3.3 kcal/mol for the wildtype hGPDH-catalyzed reactions of the substrate pieces GA + HP, and (ΔG‡)cat = 2.4 kcal/mol for the reaction of whole substrate catalyzed by the R269A + Gua" enzyme pieces (Table 2). This observation of similar catalytic advantages from connection of the pieces at the E_{R269A}Gua"-GA-HP, quaternary complex (Figure 4D) and at the correspond-

Figure 4. (A) Representations of the X-ray crystal structure (PDB entry 1WPQ) of the nonproductive ternary Michaelis complex between wildtype hGPDH, DHAP and NAD+. (B–D) Representations, generated in silico from Figure 4A by deletion of the relevant covalent linkage(s) and maintaining a fixed position for the remaining atoms, of the following hypothetical Michaelis complexes: (B) wildtype hGPDH, GA and HP, (C) R269A hGPDH, DHAP and Gua"; (D) R269A hGPDH, GA, HP, and Gua".

Figure 5 shows the linear logarithmic correlation, with slope of 1.0, between the fourth order rate constants kcat/Kcat/Km for activation of R269A hGPDH-catalyzed reduction of GA by the combined action of diions X2- and Gua" and the third order rate constants kcat/Kcat/Km for activation of wildtype hGPDH-catalyzed reduction of GA by the same diions.

The Range of Connection Energies. Table 2 shows a variety of connection energies (ΔG‡)cat/(E) for reactions of substrate pieces catalyzed by TIM, OMPDC, and hGPDH. The connection energy reflects, first of all, the entropic advantage to the binding and reaction of a single whole ligand, compared to essentially the same ligand that has been cut into pieces (Figures 4A and 4B). However, it is difficult or impossible to rationalize variations in connection energies from Table 2 by consideration of entropic effects alone. For example, TIM and hGPDH catalyze reactions of the same GA + HP pieces and similar GAP and DHAP substrates. These enzymes might therefore be expected to show similar differences in the entropic price to the efficient binding and reaction of these substrates and pieces. By contrast, there is a large difference between the values of (ΔG‡)cat = 6.7 and 3.3 kcal/mol determined, respectively, for TIM and hGPDH.

The explanation for the small connection energies observed for hGPDH is of particular interest, because this allows for efficient self-assembly of the enzyme and substrate pieces into a reactive complex. We propose that the large connection energy (ΔG‡)cat = 6.7 kcal/mol for TIM reflects mainly the entropic advantage to the reaction of triosephosphates, and that the whole substrate and pieces show essentially the same binding interactions with the catalyst. By contrast we propose that the smaller connection energy (ΔG‡)cat = 3.3 kcal/mol for hGPDH includes a similar large (≈ 7 kcal/mol) entropic contribution, that is offset by the intrinsically tighter binding interactions to the pieces compared with whole substrate.

There is a larger total contribution of phosphodianion binding energy to catalysis by TIM (13.0 kcal/mol, Table 2) compared with hGPDH (10.8 kcal/mol). This reflects the limited total substrate binding energy available to be utilized for catalysis of the reactions of triosephosphate substrates for TIM, and the imperative that TIM make the best possible use of these binding interactions. By comparison, there is a weaker imperative to optimize the utilization of the binding interactions of the phosphodianion of DHAP in catalysis by hGPDH, because the binding energy from the large NADH cofactor is alone sufficient to obtain nearly the entire rate acceleration. This is the case for reactions catalyzed by alcohol
We propose that hIgPDH fails to optimize the binding interactions for the whole substrate DHAP, and that there is a small, but significant, preference for binding the substrate in pieces. This may reflect the independent, unhindered, movement of the GA + HP, pieces to tightly bound conformations not accessible to the whole substrate DHAP, where such motion may be restricted by the covalent connection. Such preferential binding of the pieces would result in a reduction in the connection energy (ΔGfi)GA+HPi.

Table 2 shows systematic variations in the connection energy (ΔGfi)E+cation for the reactions of enzyme pieces that reflect, mainly, variations in the stabilization of the complex to the cationic piece by interactions with the protein catalyst. For example, the 1.9 kcal/mol decrease in the connection energy (ΔGfi)E+RNH3 for rescue of K12G TIM from (ΔGfi)E+HPh3 = 6.3 kcal/mol (R = H) to (ΔGfi)E+RNH3 = 4.4 kcal/mol (R = Et, Table 2) reflects increasing stabilization of the bound ammonium cation by interactions at a hydrophobic protein cleft. The value of (ΔGfi)E+RNH3 = 6.3 kcal/mol for the minimal activator NH4+ was proposed to represent the approximate entropic advantage of covalent connection of enzyme pieces. This is similar to (ΔGfi)E+GAi = 6.7 kcal/mol, which was estimated above as the approximate entropic advantage for connection of the substrate pieces for TIM. We likewise propose that the small connection energy of (ΔGfi)E+RNH3 = 2.4 kcal/mol for R269A hIgPDH is due to stabilization of the complex between the cation and mutant enzyme by interactions with the amide side chain of Gln295 and the cofactor pyrophosphate (Figure 4).

The binding interactions to the alkyl groups of RNH3+ to TIM, which we propose are expressed as decreases in (ΔGfi)E+RNH3, are not sufficiently strong to give detectable saturation of K12G TIM by 80 mM RNH3+. Similarly, there is no detectable saturation of hIgPDH by 80 mM Gua+. These results emphasize the difference between the readily measurable intrinsic binding energy associated with formation of complexes of these activating cations to the enzyme-bound transition state and the observed ligand binding energy, which is too small to measure in these experiments.

Importance of Enzyme—Substrate Ion-Pairs. The upper limit for the contribution of the substrate phosphodianion and the R269 guanidine side chain to the enzymatic rate acceleration is <19.9 kcal/mol, the sum of the contributions of the phosphodianion (10.8 kcal/mol, Scheme 4) and the side chain cation (9.1 kcal/mol) to the rate acceleration. This includes a direct interaction between these ions, which is counted twice in the sum of the contributions, and estimated as the 2.8 kcal/mol effect of the R269A mutation on the stability of the Michaelis complex to DHAP. The total stabilization of the transition state for hIgPDH-catalyzed hydride transfer by interactions with these ionic groups is therefore ≤ (19.9 – 2.8) ≤ 17.1 kcal/mol.

We speculate that 17 kcal/mol is larger than the true contribution of interactions between the enzyme cation and the substrate phosphodianion to transition state stabilization, because of cooperativity between development of these interactions. The magnitude of these cooperative stabilizing interactions, which are eliminated by truncation of either the substrate phosphodianion or the enzyme cation, may be estimated by comparing the sum of the effects of separate truncation of the phosphodianion and cation ((10.8 + 9.1 – 2.8) = 17.1 kcal/mol) with the total effect of the two truncations on kcat/Km = 4.6 × 10^6 M^-1 s^-1 for the reaction catalyzed by wildtype hIgPDH. A rate constant of 1.2 × 10^-6 M^-1 s^-1 is predicted for a total 17 kcal/mol destabilization of the transition state for wildtype hIgPDH-catalyzed reduction of DHAP. By comparison, our protocol fail to detect R269A hIgPDH-catalyzed reduction of GA and set an upper limit of kcat/Km ≤ 0.003 M^-1 s^-1 for this reaction. This sets limits of 17 kcal/mol ≥ ΔΔG‡ ≥ 12.4 kcal/mol for the stabilization of the transition state for hIgPDH-catalyzed reduction of DHAP by interactions that involve the transition state, the protein catalyt, the enzyme cationic side chain and the substrate phosphodianion. These limits correspond to a rate acceleration of between 10^2- and 10^{12}-fold.

Cooperativity in the expression of the electrostatic interactions of these ionic groups will cause the transition state stabilization determined by suming the effects of mutation of the enzyme [R269A mutation] and substrate [truncation of phosphodianion] to exceed the true stabilization obtained from these interactions. For example, if closure of the enzyme flexible loop (Figure 1B) over the substrate phosphodianion results in a change in the environment of the enzyme active site that enhances electrostatic stabilization of the transition state, then truncation of the phosphodianion will both weaken the driving force for enzyme-activating loop closure and weaken the transition state stabilization from ion pairing interactions to the cationic side chain of R269. These ion pairing interactions are also lost at R269A mutant of hIgPDH, so that this part of this side chain stabilization of the transition state will be included twice when summing the effects of the separate truncation of the phosphodianion and the cationic side chain.

Other Enzymes. Other enzymes that catalyze reactions of phosphodianion substrates have been examined to determine whether the large 10^2- and 10^{12}-fold contribution of this binding determine to the stabilization of the transition state for catalysis by GPDH is an exception, the rule, or if the contribution to catalysis varies systematically from enzyme to enzyme. The present results are not exceptional, but the data is too limited to establish rules.

Triosephosphate Isomerase. The total stabilization of the transition state for TIM-catalyzed isomerization by interactions with the substrate phosphodianion and K12 side chain is [13.0 + 8.0 – 2.3] = 18.7 kcal/mol, where the first two terms give the sum of the effects of separate truncation of these groups (Table 2), and the third is the estimated 2.3 kcal/mol effect of the K12G mutation on the stability of the Michaelis complex to DHAP, which is counted twice in the first sum. This sets an upper limit of 18.7 kcal/mol ≤ ΔΔG‡ for transition state stabilization that is greater than the upper limit of 17 kcal/mol ≤ ΔΔG‡ for hIgPDH.

Orotidine 5’-Monophosphate Decarboxylase. The placement of K12 and R269 at the active sites of TIM and hIgPDH, respectively, is substantially different from that of R235 at OMPDC. All three side chains interact with the substrate phosphodianion, but only the side-chain of OMPDC interacts exclusively with the dianion, because its 10 Å separation from the orotate ring precludes direct interaction with the ring at the decarboxylation transition state. Consequently, the K12G and R269A mutations result in significant decreases in the respective values of kcat/Km for catalysis of reaction of the truncated substrate GA_{10},12 but the R235A mutation has no significant effect on kcat/Km for decarboxylation of the truncated substrate 1-(β-d-erythrofuranosyl)orotic acid (EO) by wildtype OMPDC. This absolute requirement for the presence of...
the substrate phosphodianion to observe stabilization of the
decarboxylation transition state by interactions with the cationic
side chain of R269 and the phosphodianion of substrate DHAP. The large (12.4–17.1) kcal/mol stabilization obtained from these interactions provides a telling
element of the power of electrostatic interactions in promoting
catalytic process.18 We propose the following imperatives for evolution
of this type of enzyme architecture.20

(1) GPDH, TIM and OMPDC exist in an open form that
allows substrate access to the active site, and then undergo a change in enzyme conformation to a caged complex that provides for optimal protein ligand interactions.19

(2) These conformational changes, which convert the open
enzymes to their closed form, are sufficiently fast to support turnover numbers of as large as \( k_{\text{cat}} = 8000 \text{ s}^{-1} \) for TIM-catalyzed isomerization of GAP.15 Such rapid reorganization of enzymes to their catalytic conformation is possible for floppy proteins with the "TIM barrel fold," that contain unstructured loops. By comparison, the caged complex between substrate diaminopimelate (DAP) and DAP-epimerase is formed by an "oyster shell-like" clamping that involves movement of relatively rigid fixed protein domains.23 The smaller \( k_{\text{cat}} \approx (70–80) \text{ s}^{-1} \) for DAP epimerase14 compared with 8000 s\(^{-1}\) TIM15 is consistent with an intrinsically slower motion of these rigid domains compared with the unstructured protein loops at a TIM barrel.20

(3) The closed form of GPDH is relatively rigid and
presumably provides for optimal positioning of protein side
chains next to the enzyme-bound ligand.14 The entropic cost of this conformational change, which restricts the motion of the flexible peptide backbone and catalytic side chains attached to this backbone, is unclear. This cost is recovered as an enhancement of stabilizing electrostatic interactions at the preorganized closed form of GPDH.10b,22

Our treatment emphasizes the large transition state stabilization obtained from electrostatic interactions at the rigid closed conformation of GPDH,17 and the similarity in the total stabilizing electrostatic interactions of the protein catalyst with either the bound whole substrate or bound substrate pieces (Figure 5).10b,14b We suggest that the covalent connection, which reduces the entropic cost of binding the whole substrate compared to the substrate pieces, plays little or no role in positioning the substrate at the enzyme active site.

Conformational changes of GPDH and other enzymes must be sufficiently fast to support catalytic turnover. Proposals for a more profound influence of the dynamics of the enzyme conformational change on the enzymatic rate acceleration add one more layer of complexity to our understanding of enzyme
catalysis. These proposals have staying power, in part because it is difficult to rigorously demonstrate the absence of a contribution of dynamics to catalysis. Furthermore, almost anything done to a protein changes its dynamics, so that an explanation for any experimental result is immediately at hand.14a,26

We therefore emphasize that the results reported in
this paper are consistent with a model that considers the
conformational change that connects the open and closed forms of GPDH, independent of the protein dynamics along the low free-energy pathway that connects these structures.10a,19b,25

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Notes

The authors declare no competing financial interest.

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