SUPPORTING INFORMATION

Enzyme Architecture: Optimization of Transition State Stabilization from a Cation-Phosphodianion Pair

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EXPERIMENTAL

Materials. Water was from a Milli-Q Academic purification system. Q-Sepharose was purchased from GE Healthcare. Dowex 50WX4-200R (H\(^+\) form), nicotinamide adenine dinucleotide reduced (NADH, disodium salt), dihydroxyacetone phosphate hemimagnesium salt, 2-(N-morpholino)ethanesulfonic acid sodium salt (MES, ≥99.5%), triethanolamine hydrochloride (≥99.5%) and D,L-dithiothreitol (DTT) were purchased from Sigma-Aldrich. Protease inhibitor tablets (Complete®) and bovine serum albumin, fraction V (BSA) were purchased from Roche. Guanidinium hydrochloride (electrophoresis grade, min. 99%), sodium hydroxide (1.0 N) and hydrochloric acid (1.0 N) were purchased from Fisher Scientific. All other chemicals were reagent grade or better and were used without further purification.

Preparation of Solutions. Solution pH was determined at 25 °C using an Orion Model 720A pH equipped with a Radiometer pHC4006-9 combination electrode that was standardized at pH 4.00, 7.00 and 10.00 at 25 °C. Stock solutions of NADH were prepared by dissolving the disodium form of the coenzyme in water, and stored at 4°C. The concentration of NADH in these solutions was determined spectrophotometrically at 340 nm using ε = 6220 M\(^{-1}\)cm\(^{-1}\). Stock solutions of DHAP were prepared by hydrolysis of the hemimagnesium form of DHAP by
treatment with Dowex 50WX4-200R (H\(^+\) form) in water at 25 °C for 5-10 minutes. Solutions of the free acid form of DHAP (pH ~2.0) were adjusted to pH 7.5 by adding 1 M NaOH, and then stored at -20 °C. The concentration of DHAP was determined, as the concentration of NADH oxidized in a reaction catalyzed by wild-type human liver L-glycerol 3-phosphate dehydrogenase (hsGPDH), from the change in absorbance at 340 nm,

Stock solutions (10 – 20 mg/mL) of wildtype hsGPDH and the R269A mutant enzyme were dialyzed exhaustively against 20 mM triethanolamine at 4 °C at pH 7.5. The concentration of wildtype and R269A mutant hsGPDH was determined from the absorbance at 280 nm, using the extinction coefficient of 18450 M\(^{-1}\)cm\(^{-1}\) calculated for a subunit molecular weight of 37 500 Da using the ProtParam tool available on the Expasy server.\(^1,2\) Stock solutions of guanidine hydrochloride were prepared by dissolving the salt in water and adjusting the pH to 7.5.

**Mutagenesis of hsGPDH.** The plasmid pDNR-dual donor vector containing the gene for wild-type hsGPDH was purchased from the Harvard plasmid repository. The insert gene was subcloned into a bacterial expression vector pET-15b from Novagen, as described in earlier work.\(^3\) Site-directed mutagenesis to introduce the R269A mutation was carried out using the Quickchange II kit from Stratagene. The primers used to introduce the R269A mutation differ from the sequence for the wildtype gene as follows:

Wildtype: 5’-CCTGCTATGGAGGGC\underline{G}GAACC\underline{G}GAAGTGGCTGAGGCC-3’

R269A: 5’-CCTGCTATGGAGGG\underline{C}GAACC\underline{G}GAAGTGGCTGAGGCC-3’

The R269A mutant was prepared from 30 ng of the plasmid pET-15b, purified from \(dam^+\) XL1-Blue cells, which contained the gene for wildtype hsGPDH. This was added to 5 µL 10X \(Pfu\) Ultra Buffer, 125 ng each of the forward and reverse mutagenesis primers, 5 µL of 2 mM dNTP mixture, 2.5 units of \(Pfu\) Ultra HF DNA polymerase and water to give a final volume of 50 µL.
The DNA was amplified by PCR using the following parameters: 30 sec at 95 °C followed by 19 cycles of 30 sec 95 °C, 1 min at 55 °C and 6 min at 68 °C. Next, 20 units of the \textit{Dpn}I restriction enzyme were added and the reaction mixture was incubated at 37 °C for 1 hour to degrade the methylated template DNA. One µL of the reaction mixture was electroporated into XL1-Blue cells and a single colony was used for mutant plasmid purification. DNA sequencing at the Roswell Park Cancer Institute DNA Sequencing Facility verified the presence of the mutant gene in the plasmid.

**Expression and Purification of R269A Mutant \textit{hs}GPDH.** Wildtype \textit{hs}GPDH was expressed and purified by published procedures.\textsuperscript{3} The plasmid coding for R269A mutant \textit{hs}GPDH was transformed into \textit{Escherichia coli} BL21(DE3) strain. These cells were then grown overnight in 200-300 mL of LB medium that contained 100 µg/mL ampicillin at 37° C. This culture was diluted into 4 L of LB medium (100 µg/mL ampicillin), and grown at 37° C to \textit{OD}_{600} = 0.6, at which point 0.6 mM isopropyl-1-thio-D-galactoside was added to the culture to induce protein expression. After 12 hours, the cells were harvested and stored in 30 mL of 25 mM MES buffer that contains 0.5 M NaCl at pH 6.7. The cell pellets were suspended in 25 mM MES at pH 6.7 in the presence of protease inhibitors (Complete®) and lysed using a French press. The lysate was diluted to 60 mL with the same buffer and centrifuged at 12 500 rpm for 60 minutes. The lysate supernatant was treated with 2% polyethyleneimine to precipitate the nucleic acids, and then centrifuged at 12 500 rpm for another 60 minutes. Solid ammonium sulfate was added to the resulting supernatant to give a 40% saturated solution, which was then centrifuged at 14 000 rpm for 20 minutes. The supernatant was mixed with solid ammonium sulfate to give a 50% saturated solution. After 20 minutes the mixture was centrifuged at 14 000 rpm for 20 minutes and the
solid was dissolved in 25 mL of 25 mM MES buffer at pH 6.7. The protein solution was dialyzed overnight against 25 mM MES buffer pH 6.7 at 4°C.

The dialysate was loaded onto a Q-Sepharose ion-exchange column previously equilibrated with 25 mM MES at pH 6.7 that contains 30 mM NaCl. The column was eluted with a 1.0 L linear gradient of 30 - 120 mM NaCl in the same buffer and 10 mL fractions were collected. The protein concentration and the apparent specific activity of *hsGPDH* (units/mg) at [DHAP] = 0.50 mM were determined to give the protein elution profile shown in Figure S1A. Fractions with apparent specific activity of \( \leq 0.002 \) units/mg were pooled, concentrated and dialyzed overnight against 25 mM MES buffer at pH 6.7 at 4°C. The dialysate was reloaded onto a Q-Sepharose column, the column was eluted with a 1.0 L linear gradient of 30 - 90 mM NaCl in the same buffer and fractions of 10 mL fractions were collected. The protein concentration and the apparent specific activity of *hsGPDH* (units/mg) at [DHAP] = 0.50 mM were determined to give the protein elution profile shown in Figure S1B. Fractions that eluted with a constant apparent specific activity of \( 1 \times 10^{-3} \) units/mg and for which A ≥ 1.0 were pooled, concentrated and stored at -80°C in 20% glycerol, 25mM MES buffer of pH 6.7, and 100 mM NaCl. The R269A mutant *hsGPDH* obtained from this column was judged to be homogeneous by gel electrophoresis. The final yield of R269A mutant *hsGPDH* obtained from the second Q-Sepharose column was 80 mg.
Figure S1. Elution profiles for purification of R269A *hs*GPDH by chromatography over Q-Sepharose, equilibrated with 25 mM MES at pH 6.7 that contains 30 mM NaCl. The eluant was monitored for protein at $A_{280}$ and for *hs*GPDH activity at [DHAP] = 0.5 mM. A: First chromatographic purification of an ammonium sulfate precipitate, eluting with a 1.0 L linear gradient of 30 - 120 mM NaCl in 25 mM MES at pH 6.7 and collecting 10 mL fractions. B. Second chromatographic purification of material obtained from the first column (Figure S1A). Elution was with a 1.0 L linear gradient of 30 - 90 mM NaCl in 25 mM MES at pH 6.7 and 10 mL fractions were collected.
Kinetic Parameters for Reduction of DHAP by NADH Catalyzed by R269A Mutant

*hsGPDH in the Absence and Presence of the Guanidinium Cation (Gua⁺).* Wildtype *hsGPDH* was assayed by monitoring the oxidation of NADH by DHAP, as described in previous work. Stock solutions of R269A mutant *hsGPDH* were prepared in 100 mM triethanolamine buffer (pH 7.5) that contains 10mM DTT and 0.1 mg/mL BSA. The standard assay mixtures (1 mL) for R269A mutant *hsGPDH*-catalyzed reduction of DHAP by NADH contained 100 mM triethanolamine (pH 7.5), 0.1 mg/mL BSA, 50 µM DTT, 0.025 – 15 mM DHAP, 10 µM R269A mutant *hsGPDH*, and either 0.20 or 0.10 mM NADH at *I* of 0.12 (NaCl). Figure S2 shows the increase in \(v/|E|\) with increasing [DHAP] for the R269A mutant *hsGPDH*-catalyzed reactions. The observation of a single fit for data obtained at 0.20 mM NADH (closed red circles) or 0.10 mM NADH (open triangles), shows that NADH is effectively saturating at 0.10 mM. The kinetic parameters \(k_{cat}\) and \(K_m\) for the R269A mutant *hsGPDH*-catalyzed reaction were determined from the fit of kinetic data from Figure S2 to the Michaelis-Menten equation. The value of \(K_m\) for the reactive carbonyl form of DHAP was calculated from the observed \(K_m\) and a value of \(f_{car} = 0.55\) for the fraction of DHAP present in the free carbonyl form.

The standard assay mixture for the R269A mutant *hsGPDH*-catalyzed reduction of DHAP by NADH in the presence of guanidinium cation contained 20 mM triethanolamine (pH 7.5), 0.1 mg/mL BSA, 50 µM DTT, 200 µM NADH, 0.050 – 1.2 mM DHAP, 2 - 80 mM guanidine hydrochloride and *ca* 0.12 – 1.2 µM R269A mutant *hsGPDH* at an *I* = 0.12 (NaCl).
Figure S2. Dependence of $v/[E]$ for reduction of DHAP by NADH catalyzed by R269A mutant hsGPDH on the concentration of DHAP for reactions at 25 °C, pH 7.5 (100 mM triethanolamine) and at $I = 0.12$ (NaCl). The closed red circles show data for reactions in the presence of 0.2 mM NADH and the open triangles show data for reactions in the presence of 0.1 mM NADH.
RESULT.

Figure S3 shows the protein surface of a complex between a substrate analog [(S)-1-\(\beta\)-phosphonofluoromethylene-1-deoxy- D-glucopyranose] and a mimic of phosphorylated \(\beta\)-phosphoglucomutase. Note the similar positioning of the capping loop (Ala114 – Lys117) shaded red and the surface side chain of Arg49, shaded black, to the capping loops and cationic side chains for OMPDC (Figure 1A) and \(hs\)GPDH (Figure 1B).

Figure S3. Representation, from an X-ray crystal structure, of the surface of \(\beta\)-phosphoglucomutase in a complex with (S)-1-\(\beta\)-phosphonofluoromethylene-1-deoxy- D-glucopyranose and magnesium trifluoride. The capping loop (Ala114 – Lys117) is shaded red and surface side chain of Arg49 is shaded black. (PDB entry 4C4S).
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