SUPPORTING INFORMATION

Enzyme Architecture: Erection of Active Orotidine 5'-Monophosphate Decarboxylase by Substrate-Induced Conformational Changes

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EXPERIMENTAL

Materials. Orotidine 5'-monophosphate (OMP) was prepared using modifications of literature procedure for the enzymatic synthesis of uridine 5'-monophosphate (UMP).[1] Uridine (99%), 5-fluoroorotic acid hydrate (FO, 98%), 5-fluorouracil (FU, ≥ 99.0%), 3-(N-morpholino)propanesulfonic acid (MOPS, ≥ 99.5%), D-erythritol 4-phosphate lithium salt (DE4P, ≥ 95%), L-glycerol 3-phosphate lithium salt (LG3P, ≥ 95%), D,L-glycerol 3-phosphate sodium salt hydrate (DLG3P, ≥ 95%) and D-ribose 5-phosphate disodium salt hydrate (DR5P, ≥ 98%) were purchased from Sigma. Formic acid (88% solution), sodium hydroxide (1.0 N), hydrochloric acid (1.0 N) and Amicon® centrifugal filter units with a 10K molecular weight cutoff (MWCO) were purchased from Fisher. Ammonium acetate (HPLC grade) and sodium phosphite (dibasic, pentahydrate) were purchased from Fluka. The water content of sodium phosphite was reduced to $Na_2HPO_3 \cdot 0.4H_2O$ as previously described.[2] Wild type orotidine 5'-monophosphate decarboxylase from *Saccharomyces cerevisiae* (OMPDC) was overexpressed and purified by published procedures.[3-4] Water was purified using Milli-Q Academic purification

system. 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 OMP and standards (FU and uridine) were prepared by dissolving the solid in water and adding 1N HCl or 1N NaOH to adjust to the desired solution pH. The stock solution of FO was prepared by dissolving the free acid in water and adjusting the pH ≈ 7 by addition of 1 N NaOH. Stock solutions of OMP (15 mM) and FO (*ca* 50 mM) were stored at -20 ºC. Stock solutions of FU (20 mM) and uridine (20 mM) were stored at room temperature. The concentration of OMP in 0.1 M HCl was determined from the absorbance at 267 nm using a value of $\varepsilon = 9430\ M^{-1}\ cm^{-1}$.[5] The concentration of FO in 0.1 M HCl was determined from the absorbance at 285 nm using a value of $\varepsilon = 7070\ M^{-1}\ cm^{-1}$.[6] The concentration of FU in 0.1 M HCl was determined from the absorbance at 265 nm using a value of $\varepsilon = 7100\ M^{-1}\ cm^{-1}$.[6] The concentration of uridine in 0.1 M HCl was determined from the absorbance at 262 nm using a value of $\varepsilon = 10100\ M^{-1}\ cm^{-1}$.

MOPS buffers at pH 7.0 (45% free base) were prepared by addition of measured amounts of 1 N NaOH and solid NaCl to give the desired acid/base ratio and ionic strength. A stock solution of ammonium acetate was prepared by dissolving the salt in water and adjusting the pH to 4.2. Stock solutions of sodium phosphite (100 mM, 80% free base, I = 0.28), DE4P (100 mM, 90% free base, I = 0.29), LG3P (100 mM, 90% free base, I = 0.29) and DLG3P (100 mM, 90% free base, I = 0.29) at pH 7.0, were prepared by dissolving the salt in water and adjusting the pH to 7.0 with 1 N HCl or 1 N NaOH to give the desired acid/base ratio. Stock solutions (20–30

mg/mL) of wild type OMPDC were dialyzed exhaustively against 25 mM MOPS pH 7.0 at 4 °C. The concentration of enzyme in the stock solution was determined from its absorbance at 280 nm using a value of $\varepsilon = 29\ 900\ M^{-1}\ cm^{-1}$ that was calculated using the ProtParam tool available on the ExPASy server.[7-8]

Enzyme Assays. The activity of OMPDC was determined by monitoring the change in absorbance at 279 nm during the conversion of OMP to UMP ($\Delta\varepsilon = 2400\ M^{-1}\ cm^{-1}$) at 25 ºC in solutions that contain 10 mM MOPS (I = 0.105, NaCl), 44 μM OMP and ≈ 20–30 nM of OMPDC.[4, 9]

OMPDC-catalyzed Decarboxylation of 5-Fluoroorotate Monitored by HPLC. The decarboxylation of the phosphoribosyl truncated substrate FO in 10 mM MOPS, pH 7.0, 25 ºC and I = 0.15 (NaCl) in the absence and presence of phosphodianions catalyzed by wild type OMPDC was followed by monitoring the formation of FU by HPLC analyses. Reaction mixtures were prepared by mixing stock solutions of OMPDC in MOPS buffer and other reagents, with initiation by addition of the substrate FO in water. The unactivated reaction mixtures (500 μL) contained 25 mM MOPS (45% free base, pH 7.0), 5 or 10 mM FO and 690 μM OMPDC at I = 0.15 (NaCl). The reactions mixtures for the study of activation by phosphite dianion, LG3P, DLG3P and DR5P (500 μL) contained 25 mM MOPS buffer (45% free base, pH 7.0), 5 mM FO, 280–460 μM OMPDC at I = 0.15 (NaCl) and phosphite dianion, LG3P or DLG3P (5–40 mM). The reactions mixtures for activation by DE4P contained 25 mM MOPS buffer (45% free base, pH 7.0), 5 mM FO, 90 μM OMPDC and 2.5–20 mM DE4P. The unactivated OMPDC catalyzed decarboxylation of FO was followed for up to 17 days, during which time up to 0.01% of FO was converted to product FU. The phosphite dianion-, and G3P dianion-activated OMPDC-catalyzed reactions of FO were followed for up to 5 days during which time up to 3.5 % of FO

was converted to product FU. The DE4P dianion-activated FO reactions were followed for up to 10 hours, during which time up to 2.5 % of FO was converted to product FU.

At measured reaction times a 50 μL aliquot was withdrawn and the reaction was quenched by the addition of 150 μL of solution of formic acid (2.6 mM) that contained 20 μM uridine, which served as an internal standard for HPLC analyses. The enzyme was removed by ultrafiltration using an Amicon filter unit (10K MWCO) that had been prewashed 2 to 3 times with 400 μL of water. **Warning**: the failure to prewash the filtration device resulted in loss of reactant and/or erratic HPLC retention times. The resulting filtrate (90–125 μL) was analyzed by HPLC using a Waters Atlantis dC18 3 μm column (3.9 × 150 mm) with isocratic elution by 10 mM NH_4OAc pH 4.2 over 12 min, with a flow of 1 mL/min and peak detection at 265 nm. Under these conditions the unreacted FO eluted at 2.5 min, product FU eluted at 4.5 min and uridine eluted at 8.5 min. The observed HPLC peak area for FU was normalized using the observed peak area for uridine and the standard HPLC peak area for uridine determined by direct HPLC analysis of the formic acid/uridine quench solution. This procedure was necessary because there is a small variable dilution of the sample upon its passage through the prewashed filtration device. The concentration of the product FU in the reaction mixture at time t, $[FU]_t$ was then obtained from its normalized HPLC peak area by interpolation of a standard curve that was constructed using standard FU solution. The concentration of FU in the stock solutions used for this calibration was determined from the absorbance in 0.1 M HCl at 265 nm using $\varepsilon = 7100\ M^{-1}\ cm^{-1}$. These second-order rate constants from HPLC analyses are reproducible to better than ±10%.

Periodic standard assay of the OMPDC from these reaction mixtures, using OMP as substrate, showed that there was no significant decrease in the activity of OMPDC during these

reactions. The pH was determined at the end of each reaction, and in no case was a significant change observed in the starting pH. Observed *first-order* rate constants k_{obs} (s^{-1}) for the turnover of FO by OMPDC was determined from the linear-least squares fit of plots of reaction progress against time. The corresponding *second-order* rate constants, $(k_{cat}/K_m)_{obs}$ were calculated using the relationship $(k_{cat}/K_m)_{obs} = k_{obs}/[E]$.

RESULTS

Figures S1A and S1B show the effect of increasing concentrations of D,L-glycerol 3-phsophate and L-glycerol 3-phosphate, respectively, on $(k_{cat}/K_m)_{obs}$ for OMPDC-catalyzed decarboxylation of **FO**. The data from these Figures were fit to eq 1, derived for Scheme 3A, to give the kinetic parameters reported in Table 2 in the main text. The dashed-line in Figure S1A is the linear correlation of the $(k_{cat}/K_m)_{obs}$ data for ≤ 10 mM [DLG3P], the slope which gives the third-order rate constant $[(k_{cat}/K_m)_{Act}/K_d]$. The decarboxylation of 5 mM **FO** to form **FU** catalyzed by 390 μM OMPDC in the presence of 40 mM D-ribose 5'-phosphate (DR5P) was monitored for the reaction of 0.03% of **FO** over seven days, during which time OMPDC maintained full activity. The initial reaction velocity *v* was determined and used to obtain the approximate third-order rate constant $(k_{cat}/K_m)_{Act}/K_d = v/[E][EO][DR5P] \approx 3 \times 10^{-5}\ M^{-2}\ s^{-1}$ for DR5P-activated OMPDC-catalyzed decarboxylation of FO.

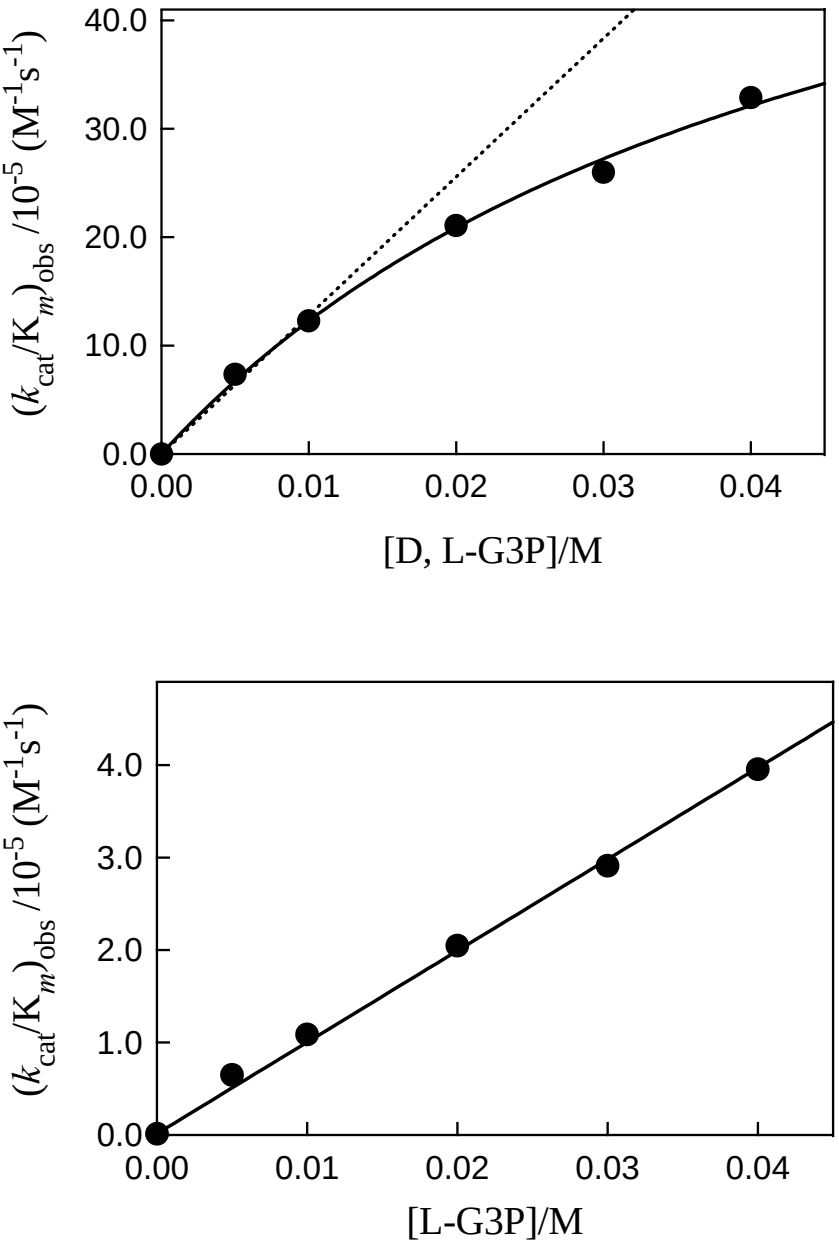

Figure S1. (A) The dependence of $(k_{cat}/K_m)_{obs}$ for OMPDC-catalyzed decarboxylation of **FO** on [D,LG3P]. (B) The dependence of $(k_{cat}/K_m)_{obs}$ for OMPDC-catalyzed decarboxylation of **FO** on [LG3P].

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