SUPPORTING INFORMATION

Enzymes

TP-synthase and 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate kinase (ThiD) were over-expressed and purified as previously documented[1,2] except that all buffers included 2 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP). Upon purification the proteins were desalted into storage buffer consisting of 50 mM tris(hydroxymethyl)aminomethane (tris buffer, pH 7.6), 100 mM NaCl, 2 mM TCEP, and 20% glycerol using a 10 DG column purchased from Bio-Rad (Hercules, CA). The protein was flash frozen and stored at -80^{o}C until use. TCEP was included in the buffers to slow oxidation during long term storage and is a replacement for DTT. Control experiments showed that TCEP made no difference in terms of reaction kinetics.

Chemicals and Substrates

4-methyl-5-(β-hydroxyethyl)thiazole phosphate (Thz-P), 4-amino-5-(hydroxymethyl)-2-methylpyrimidine (HMP), 4-amino-5-(hydroxymethyl)-2-(trifluoromethyl)pyrimidine (CF_3-HMP), and 4-amino-5-(hydroxymethyl)-2-methoxypyrimidine (CH_3O-HMP), were synthesized as previously described[3]. The pyrophosphorylation of HMP and HMP analogs was carried out enzymatically under the following conditions in a total reaction volume of 15 mL: 50 mM tris buffer pH 7.5, 2 mM TCEP, 10 mM adenosine 5′-triphosphate (ATP), 5 mM $MgCl_2$, 2.5 mM HMP or HMP analog, and 1 mg/mL ThiD. The reaction was allowed to incubate at room temperate overnight at which time the protein was removed by ultrafiltration using an Amicon ultra-15 centrifugal filter unit with a 10-kDa cutoff membrane (Millipore Corporation, Billerica, MA). The filtrate was diluted to 50 mL with ddH_2O and loaded onto a Mono Q HR 10/10 column equilibrated with 20 mM ammonium acetate pH 6.5 at a flow rate of 4 mL/min. The product was eluted with a linear gradient of 0.02 – 1 M ammonium acetate at a flow rate of 4 mL/min over a period of 2 hours. The fractions containing product were pooled and lyophilized to constant weight by consecutive rounds of resuspension in ddH_2O and lyophilization. The final stock concentration was determined by ^{1}H-NMR (300 MHz, Varian) using glycine as an internal standard. Thz-P was synthesized in two steps from Thz as previously described[4]. TCEP was obtained from Soltec Ventures (Beverly, MA). All buffers, salts and other chemicals were purchased from Sigma-Aldrich.

Active site titration

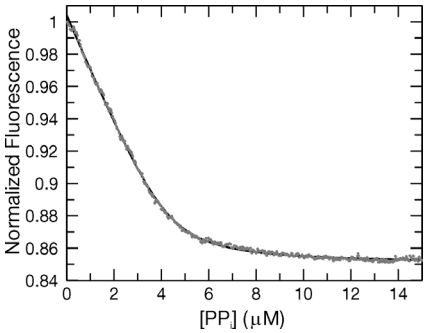

Figure S1. Shown above is the change in intrinsic protein fluorescence as a concentrated solution of PP_i was titrated into a solution containing 5 μM TP-synthase and 15 μM thiamin-P. A value of 5 μM TP-synthase was derived from the sum of the burst

amplitude and the Y-intercept as mentioned in the main text. A final volume of 10 μL's of PP_i solution was added to 310 μL's of the TP-synthase/thiamin-P binary complex and the data were corrected for the small dilution. The data were best fit by nonlinear regression analysis to the following quadratic equation:

$$F = F_o + \Delta F \cdot \frac{E_o + S_o + K_d - \sqrt{(E_o + S_o + K_d)^2 - 4 \cdot E_o \cdot S_o}}{2 \cdot E_o}$$

An *available* active site concentration (E_o) of 4.34 ± 0.01 μM and a K_d (dissociation constant) of 210 ± 3 nM for PP_i binding was obtained. There was an overall change in fluorescence of 15.4% (ΔF = 0.1544 ± 0.0002). The data were normalized by dividing each point by the fluorescence value of the sample prior to the addition of PP_i, which results in a starting fluorescence (F_o) equal to 1.0041 ± 0.0002. The burst phase amplitude predicts a slightly larger amount of active sites than is observed and is due to the fact that some TP-synthase (15-20%) co-purifies with TP and PP_i[5].

Chemical Quench-Flow and Titration Equipment

The chemical quench-flow (model RQF-3) and stopped-flow (model SF-2004) were manufactured by the KinTek Corporation (Austin, TX). The titration was performed using a titration module designed to work in conjunction with the SF-2004 and was also manufactured by KinTek.

Assay Conditions

All of the reported experiments were done in a buffer system which was composed of 50 mM Tris-Cl pH 7.6 @ RT, 100 mM NaCl, 2 mM TCEP, and 8 mM $MgCl_2$. Chemical quench-flow experiments were performed in the double mixing mode. The first mixing event combined TP-synthase and substrates (HMP-PP or MeO-HMP-PP and Thz-P) (~15 μLs each) and was programmed to allow the reaction to age for an appropriate amount of time before quenching with 85 μLs of 0.25 M NaOH and 125 μg/mL $K_3Fe(CN)_6$ in water (as the second mixing event). $K_3Fe(CN)_6$ oxidizes TP and its derivatives to a fluorescent compound and this reaction was allowed to occur for 30 seconds in the quench-flow. The oxidation reaction was then neutralized with 85 μLs of 0.25 M HCl. The sample was diluted to 500 μLs with ddH_2O and the fluorescence was measured using a Cary Eclipse fluorescence spectrophotometer (Varian Inc., Palo Alto, CA) exciting at 386 nm and observing emission at 490 nm, with a slit width of 5 nm. The CF_3 derivative of thiochrome was not sufficiently fluorescent under aqueous conditions; therefore, the procedure was modified accordingly. The reaction was not fast enough to require a quench-flow apparatus; therefore the mixing was done manually on the bench top. For each time point 45 μLs of reaction mixture was quenched and product was oxidized by the addition of 12.75 μLs of 2.5 M NaOH and 1.25 mg/mL $K_3Fe(CN)_6$ and allowed to react for 1 min at which time 12.75 μLs of 2.5 M HCl was added. The samples were diluted to 500 μL with isopropanol, mixed thoroughly and centrifuged at 13,000 RPM for 5 minutes in a tabletop centrifuge to remove the precipitated salts. The fluorescence was measured by exciting at 340 nm and observing emission at 423 nm, with a slit width of 5 nm. For the reaction using CF_3-HMP-PP, the amount of product formation was taken to be 80 μM based upon the complete consumption of the limiting substrate (Thz-P = 80 μM).

As mentioned in the main text no significant difference in the rate or amplitude of the burst phase using HMP-PP was observed when the concentration of substrate was varied (under pseudo first order conditions). Concentrations ranged from 10-500 μM for HMP-PP and 20 to 500 μM for Thz-P. The substrate concentration was maintained in at least a 5 fold excess of enzyme. Furthermore, single turnover experiments performed with as little as 5 μM TP-synthase and 2 μM Thz-P showed no significant difference in rate compared to the reported burst experiment.

Data Analysis

Linear and nonlinear regression analysis was performed in the program GraFit 5 (Erithacus Software Ltd., Horley, Surrey, UK) by the least squares method. The equations used are described and explicitly written out in the main text or figures. Fluorescence measurements for chemical quench data was converted to absolute concentration based upon linear standard curves.

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