Supporting Information for

Inhibition of *E. coli* CTP synthetase by NADH and other nicotinamides, and their mutual interactions with CTP and GTP

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List of Supporting Figures

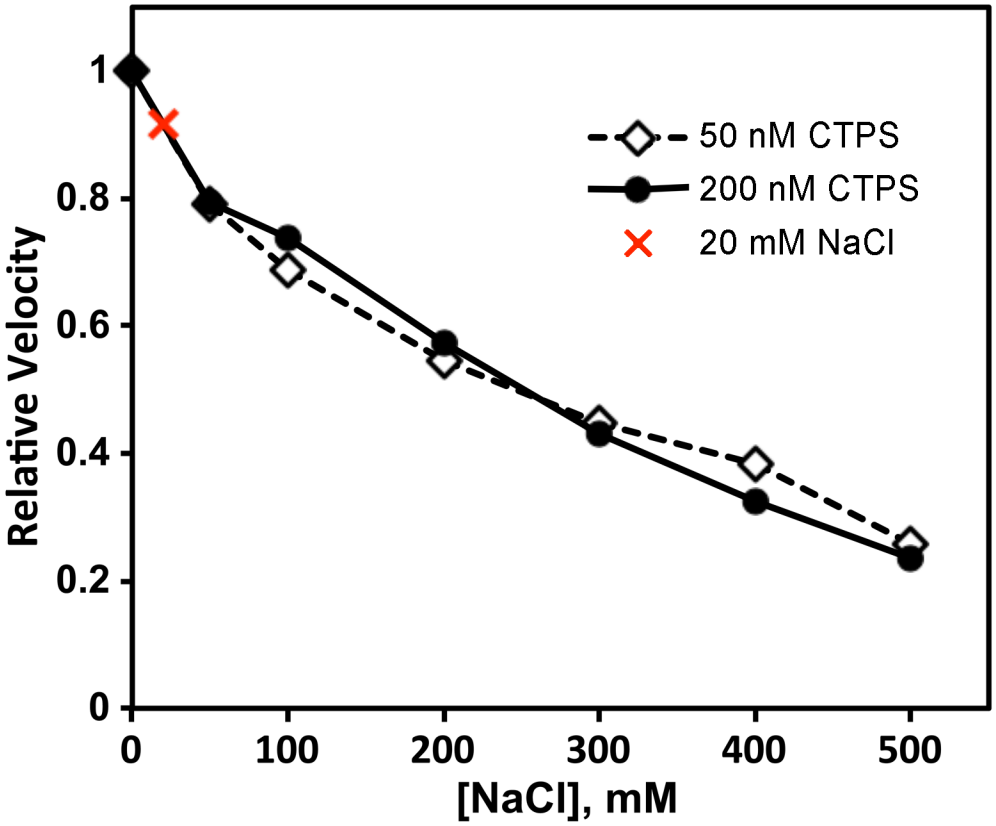


Figure S1. "At 8 uM final EcCTP concentration, the NaCl contribution from enzyme stocks did not exceed 20 mM, which has a negligible effect on rate (data not shown)."

Standard assay conditions were utilized at 50 nM (white diamonds) and 200 nM (black circles) *Ec*CTPS. For preincubation, 0, 50, 100, 200, 300, 400 and 500 mM NaCl (final concentration in assay) was added to the A tubes (See **Materials and Methods**). At 20 mM NaCl, activity was reduced ~8%.

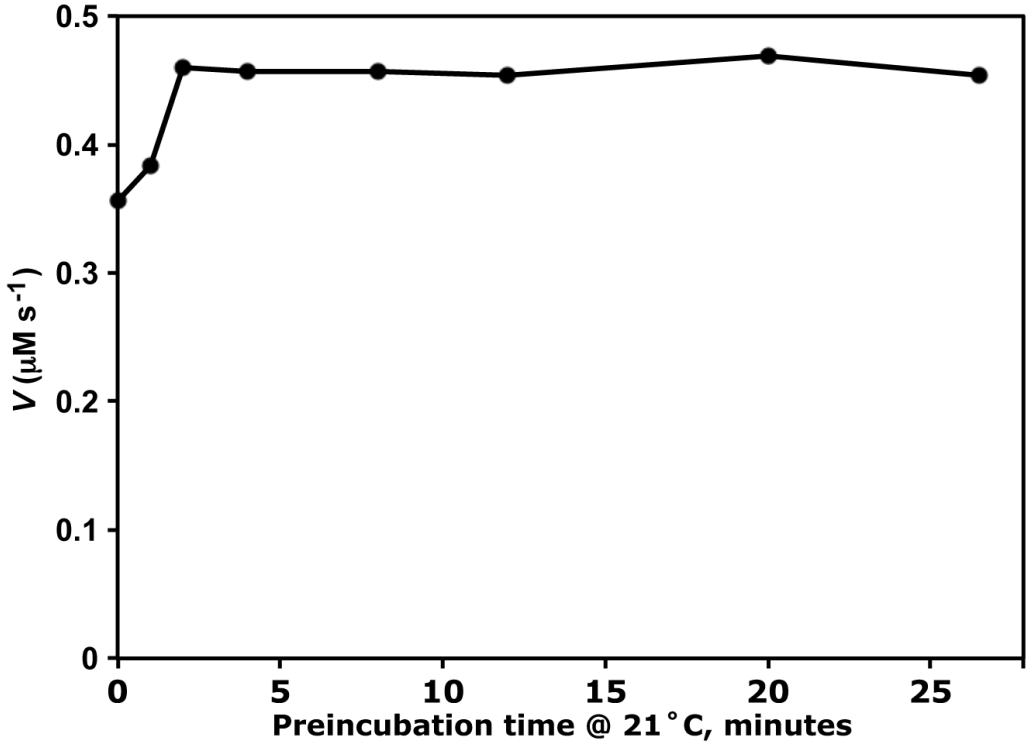


Figure S2. "The annealing procedure, which is aimed at maximizing dimer reassembly, improved assay reproducibility and increased the specific activity by 20-40%" Representative experiment showing the effect of 21˚C preincubation for 0, 1, 2, 4, 8, 12, 20 and 27 minutes at 1 μM CTPS in reaction buffer (60 mM Na-HEPES, 10 mM $MgCl_2$, pH 8.0), followed by mixing with A tubes containing reaction buffer, 150 uM UTP, 1 mM ATP, 0.2 mM GTP (final concentrations) and further incubation at 37˚C. After 90 seconds, 10 mM glutamine was added and the change in A_{291} was measured. In this experiment, activity increased 28%. Increases ranged 20-40%. The experiment also demonstrates that EcCTPS retains its activity for at least 25-30 minutes at 21˚C.

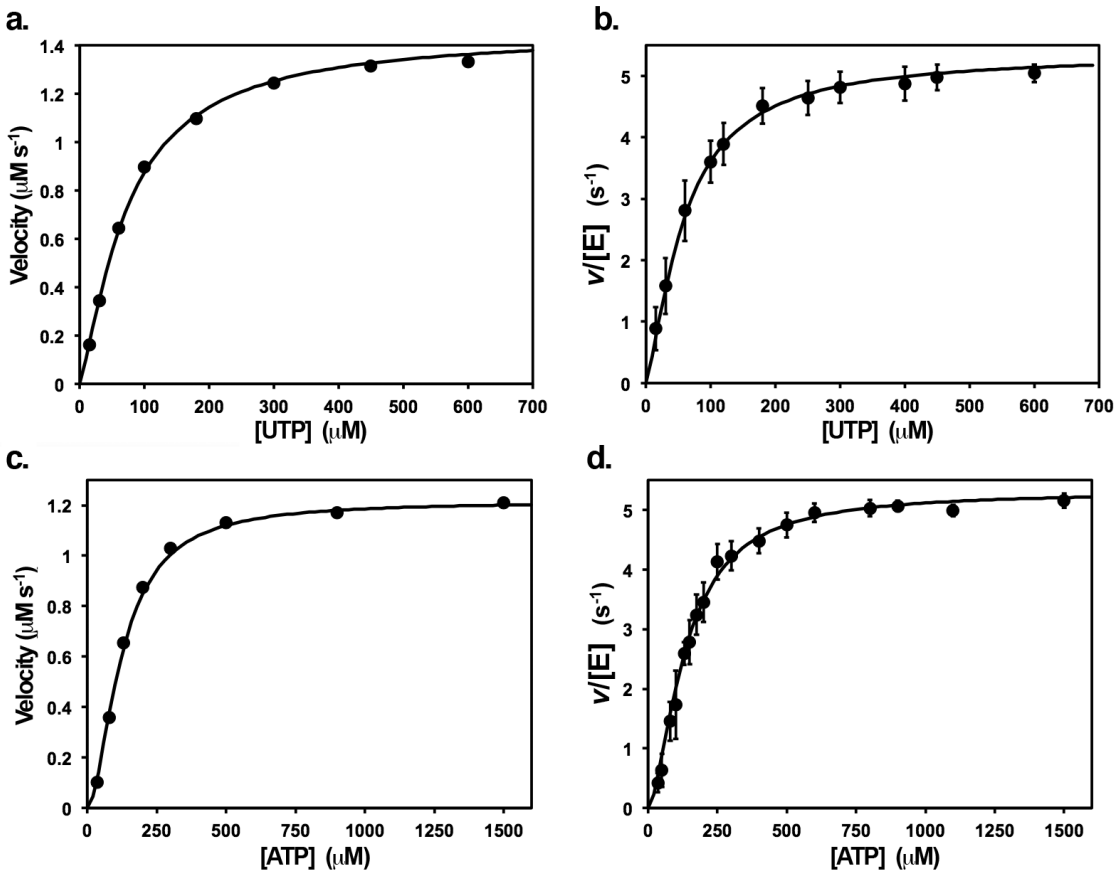


Figure S3. Representative kinetic data for UTP and ATP concentration dependence of *Ec*CTPS velocity and apparent k_{cat}.

Kinetic data were acquired as described in **Materials and Methods**. **(a)** Sample single experiment for UTP dependence at 15, 30, 60 100, 180, 300, 450, and 600 μM UTP, 200 nM *Ec*CTPS, 1.5 mM ATP, 200 μM GTP in 60 mM Na-HEPES, 10 mM $MgCl_2$, 0.5 mM Na-EGTA, pH 8.0. Additional Na^+ ion contributions from nucleotides range from 8 mM (0 μM UTP) to 11 mM (600 μM UTP). The fit curve was calculated using the Hill equation with the following parameters: $S_{0.5}$ = 72 μM, V_{max}= 1.45 μM s^{-1} and n_H = 1.3. **(b)** Merged data from 21 separate experiments titrating 0-600 μM UTP as described in (a) and **Materials and Methods**. Data from different enzyme concentrations were used since the $S_{0.5}$ and n_H values did not differ

significantly from 15-2000 nM enzyme, although apparent k_{cat} values ranged 2.4 – 7.2 s^{-1}. V/[E] values were normalized to the mean for all of the experiments (5.4 ± 1.4 s^{-1}) and then averaged. Each data point represents the mean of normalized data for 5 to 21 individual measurements. The fit line was calculated from averaged parameters for 23 experiments $S_{0.5}$ = 59 μM, V/[E]= 5.35 μM s^{-1} and n_H = 1.4 (R^2 = 0.999, Table 1). The Solver non-linear regression solution for the averaged data was $S_{0.5}$ = 54 μM, V/[E]= 5.27 μM s^{-1} and n_H = 1.33. **(c)** Sample data for the velocity dependence on ATP concentration (35, 80, 130, 200, 300, 500, 900, and 1500 μM ATP, 600 μM UTP, and as in (a)). Additional Na^+ ion contributions varied from 4-11 mM. The fit curve was calculated using the Hill equation: $S_{0.5}$ = 125 μM, V_{max}= 1.22 μM s^{-1} and n_H = 1.8. **(d)** Merged data from 18 separate experiments titrating 0-1500 μM ATP, and [*Ec*CTPS] ranging 50-400 nM, with apparent k_{cat} values ranging 3.7 – 6.6 s^{-1}. V/[E] values for each point were normalized to the mean for all of the experiments (5.3 ± 0.9 s^{-1}) and then averaged (n=3-18). The fit line was calculated from averaged parameters for 18 experiments $S_{0.5}$ = 130 μM, V/[E]= 5.30 μM s^{-1} and n_H = 1.7 (R^2=0.995, Table 1). The Solver non-linear regression solution for the averaged data was $S_{0.5}$ = 134, V/[E]= 5.16 μM s^{-1} and n_H = 1.8.

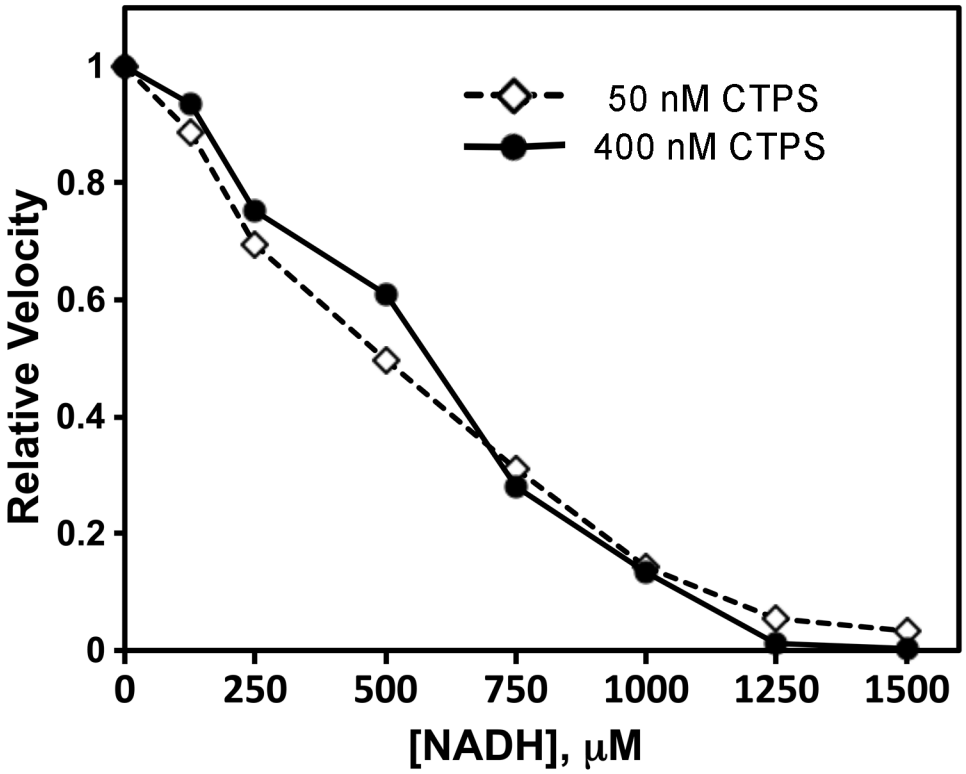
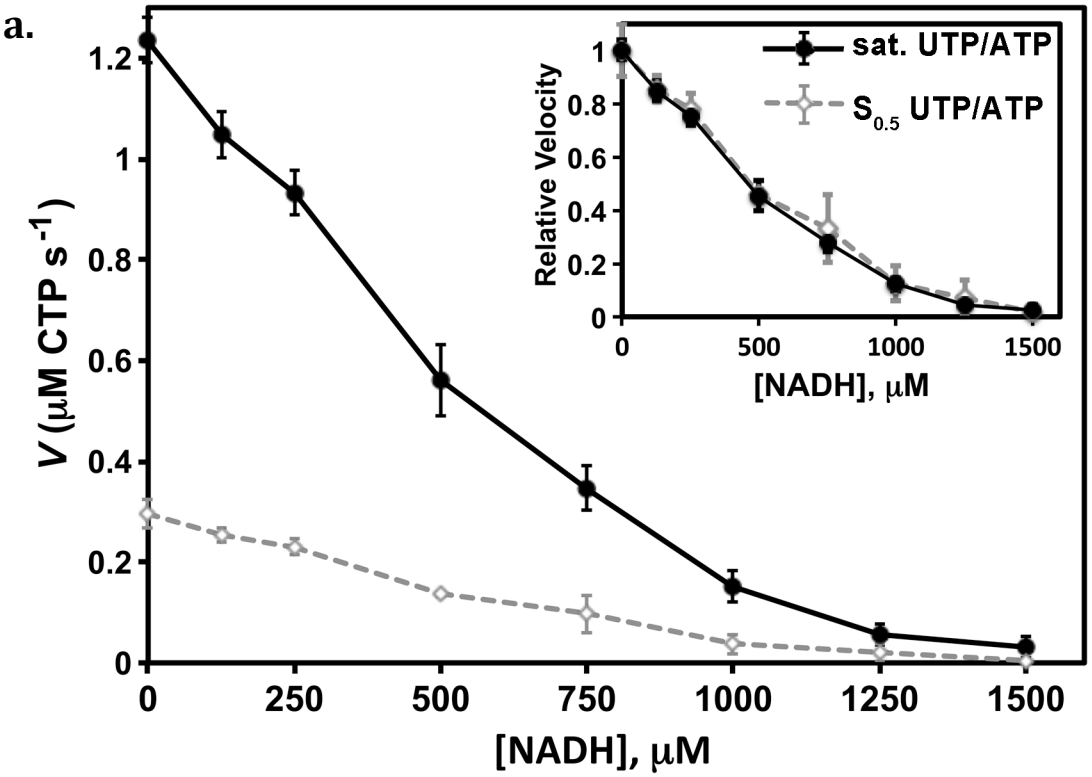


Figure S4. "(NADH) Inhibition potency was independent of enzyme concentration from 50-400 nM."

In a representative experiment, *Ec*CTPS, 50 and 400 nM (final), was assayed under standard conditons in the presence of 0, 100, 250, 500, 750, 1000, 1250 and 1500 μM NADH. Both experiments yielded similar titration curves after normalizing V_o = 1 (V_o values were 0.33 μM s^{-1}(50 nM) and 2.58 μM s^{-1} (200 nM)).


a.
V (μM CTP s-1)
[NADH], μM
sat. UTP/ATP
S0.5 UTP/ATP
Relative Velocity
[NADH], μM

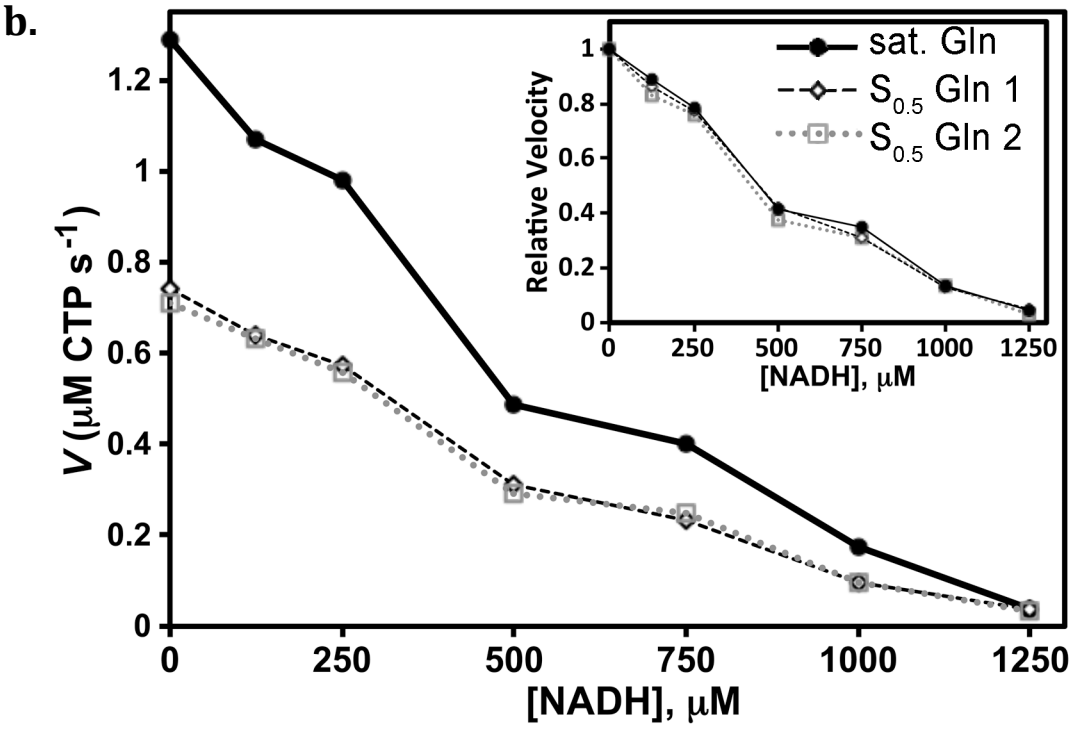

b.
V (μM CTP s-1)
[NADH], μM
sat. Gln
S0.5 Gln 1
S0.5 Gln 2
Relative Velocity
[NADH], μM

Figure S5. "Conversely, when titrated in the presence of ATP, UTP or glutamine substrates at their $S_{0.5}$ values, the NADH dose-response curves were identical to those with saturating substrates (data not shown)."

*Ec*CTPS activity was assessed in the presence of 0-1250 μM NADH, and $S_{0.5}$ or saturating (10x $S_{0.5}$) concentrations of **(a)** both ATP and UTP or **(b)** glutamine. Insets show data normalized to V_o. For (a), the mean values and standard deviations of three experiments are shown. For (b), a representative experiment with one set determined with saturating glutamine and two at $S_{0.5}$ glutamine. For $S_{0.5}$ values, see Table 1. V_o values are (a) 0.30 μM s^{-1}($S_{0.5}$ ATP and UTP) and 1.24 μM s^{-1} (saturating ATP and UTP); and (b) 0.74 μM s^{-1} and 0.71 μM s^{-1} ($S_{0.5}$ glutamine) and 1.29 μM s^{-1} (saturating glutamine).

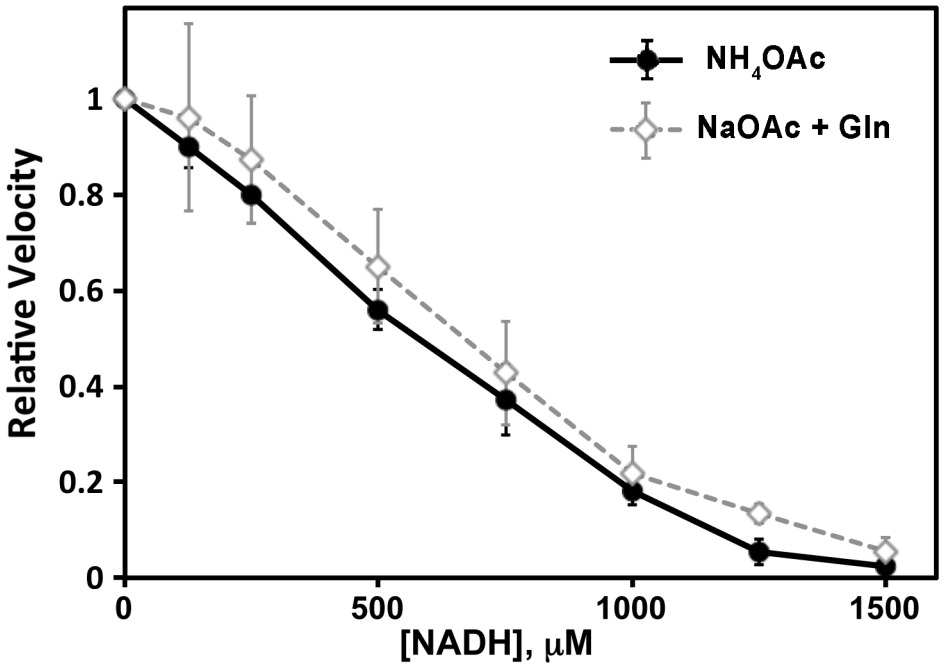


Figure S6. "Like guanosine, NADH inhibited ammonia-dependent synthesis**…**"

*Ec*CTPS activity was assayed using ammonia (black filled circles) or glutamine (grey open diamonds) as a nitrogen source. Reactions were carried out under standard conditions as described in **Materials and Methods**, except that C tubes contained either 1 M ammonium acetate or 100 mM glutamine in 1 M sodium acetate (pH 8.0, 100 mM final acetate). The data were averaged after normalizing V_o values of each experiment to one (n=3). The apparent IC50 values are nearly identical. The differences in ionic strength and composition slightly increased the IC50 values (580±60 mM for NH_4OAc, and 660±130 mM for NaOAc+glutamine) and reduced the apparent k_{cat} values (5.3±0.1 s^{-1} for NH_4OAc, and 3.7±0.3 s^{-1} for NaOAc+glutamine), compared to standard conditions without acetate.

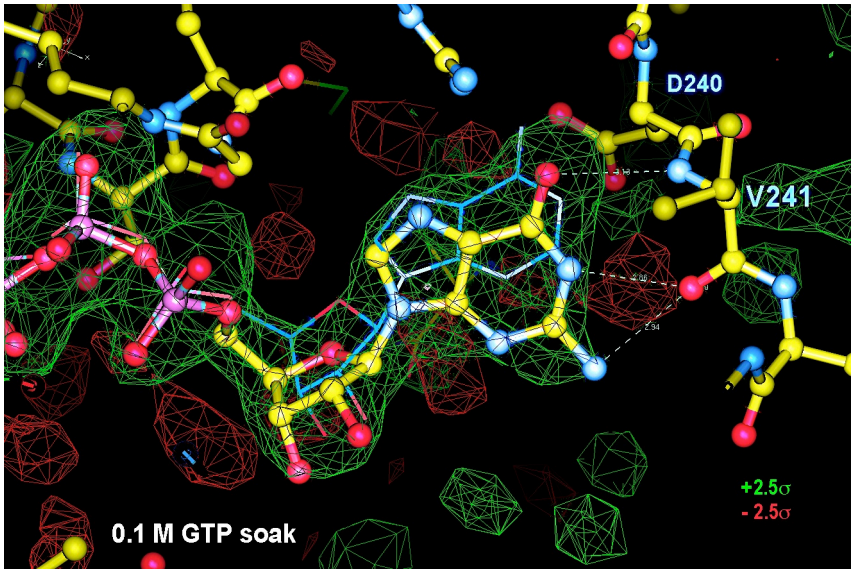


Figure S7. "Soaking *Ec*CTPS crystals in 100 mM GTP loads GTP into the ATP site (James Endrizzi and E.P.B., *unpublished data*). "

Crystals of recombinant His6-tagged EcCTPS, grown from ammonium sulfate as previously described for native CTPS (Endrizzi *et al.*, (2005)), were soaked with 0.1 M GTP for 1 hour at 4˚C before cryprotection with 25% MPD. Data were collected at SSRL Beamline 1-5 (100-2.4-Å resolution, 99.9/3.6 completeness/multiplicity (2.54-2.4 Å; 99.9/3.6), Rmerge=0.088 (2.54-2.4 Å; 0.42)). Refmac 5 maximum likelihood refinement starting with ligand/water-free PDB model 1S1M (R/Rfree = 21.6/25.1) (Endrizzi *et al.*, (2005)), yielded the above difference map (visualized using COOT). The GTP position was derived with 30 cycles of further refinement with GTP (R/Rfree = 20.9/24.2%). Shown for comparison is the ADP pose from PDB model 2AD5 after superposition of residues 1-260. Note the change in H-bonding pattern due to the shift in purine ring positions.

Although GTP binds the ATP site in the crystal, we view this as an artifact since there is no kinetic evidence for competition between ATP and GTP in ATP titration experiments.

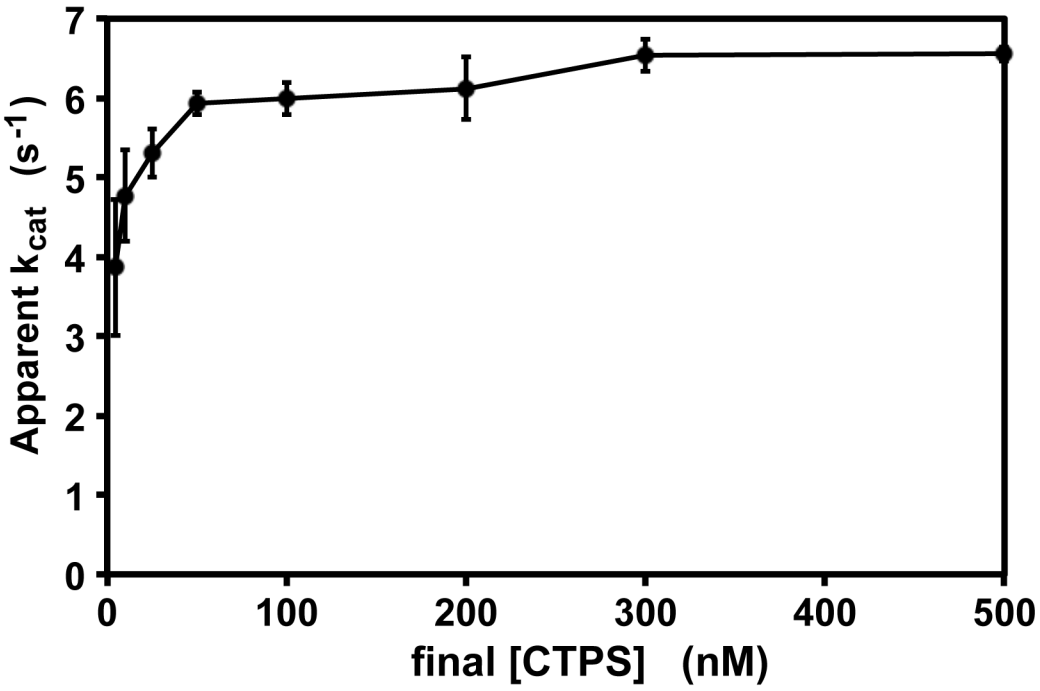


Figure S8. Figure 2 Legend: "Under these conditions, k_{cat} increases approximately 5-9% going from 100-500 nM final *Ec*CTPS (data not shown)."

Assay was performed as described in the Figure 2 legend.