Supplementary Data for:

Asymmetric Allosteric Signaling in Aspartate Transcarbamoylase

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Supplementary Methods

ATCase Kinetic Assays

The activity of ATCase was measured at 25 °C by the colorimetric method (1). First, the concentration of HCE-Gly52$_R$ ATCase was determined by the BioRad version of the Bradford dye binding assay using wild-type holoenzyme as a standard (2). For assays at pH 8.3, the enzyme was diluted to a concentration of 0.06 μg ml^{-1} in 100 mM Tris-acetate buffer at pH 8.3 and for assays at pH 7.0, the enzyme was diluted to a concentration of 0.09 μg ml^{-1} in a tripart buffer consisting of 40 mM Bis Tris, 40 mM Tris, 40 mM CAPS at pH 7.0. Sample tubes were prepared by adding 500 μl of enzyme solution, varying concentrations of aspartate from 0–40 mM, and water to a final volume of 900 μl. The reaction was initiated by the addition of 100 μl of 48 mM carbamoyl phosphate solution every 10 seconds, vortexing the tube and placing it in a 25 °C water bath. After ten minutes, each tube was quenched by the addition of 1mL color mix and incubated in a black box at room temperature overnight (16 hours). Standard tubes were also prepared containing known concentrations of carbamoyl aspartate. The following day all tubes were positioned under a fluorescent lamp in a 45 °C water bath for 24 minutes then placed on ice. The absorbance at 466 nm for each tube was measured immediately. Aspartate saturation curves (**Supplementary Figure 2**) were performed in duplicate.

ATP activation curves, and CTP inhibition curves (**Supplementary Figure 3**) were performed in duplicate after the aspartate saturation curves. The concentration of Asp used for the assays depended upon the $[Asp]_{0.5}$ determined from the aspartate saturation curves. For the CTP and ATP saturation curves, a concentration of 1/5 the $[Asp]_{0.5}$ was used. Assays were performed in 50 mM Tris acetate buffer, pH 8.3, or in a tripart buffer at pH 7.0 containing 20

mM Bis Tris, 20 mM Tris, and 20 mM Caps buffer, in the presence of a saturating concentration of carbamoyl phosphate (4.8 mM).

Data analysis of the steady-state kinetics was carried out as described previously (3). Fitting of the experimental data to theoretical equations was accomplished by non-linear regression. The data was analyzed using an extension of the Hill equation that included a term for substrate inhibition (4).

HCE-Gly52$_R$ ATCase Steady-State Fluorescence Measurements

All steady-state measurements were performed using a JASCO spectrofluorometer FP-6300 in a quartz SUPRASIL fluorescence cell (Hellma) with a slit width of 5 nm at 25 °C. All measurements at pH 8.3 were performed in 50 mM tris-acetate buffer utilizing 50 μg ml^{-1} HCE-Gly52$_R$ ATCase or HCE-Gly52$_R$ r$_2$, and all measurements at pH 7.0 were performed in 20 mM Bis-Tris, 20 mM Tris, 20 mM CAPS buffer utilizing 100 μg ml^{-1} HCE-Gly52$_R$ ATCase or HCE-Gly52$_R$ r$_2$.

First, an excitation spectrum from 300–500 nm was acquired to determine the maximum excitation wavelength. Using an excitation wavelength of 360 nm at pH 8.3 or 330 nm at pH 7.0, a fluorescence spectrum from 400–600nm was acquired in the absence of nucleotides with maximum emission at 455 nm. Microliter amounts of the nucleotides ATP, CTP, UTP, or GTP were added to the fluorescence cell up to a concentration of 15 mM. At each concentration of nucleotide, a fluorescence spectrum from 400–600 nm was acquired. These tests were repeated using the same volume of buffer in place of the nucleotides to adjust for dilution. The tests were also repeated using free amino acid HCE-Gly and nucleotides in the absence of the HCE-Gly52$_R$ ATCase.

The maximum fluorescence intensity at each concentration was determined. The data was normalized to the first point in the absence of any nucleotides. The data was fit by non-linear least squares to an equation for binding at two classes of sites (Supplementary Table 1 & 2).

Supplemental Results

Supplementary Table 1. ATP, CTP, UTP, and GTP binding to HCE-Gly52$_R$ ATCase measured by fluorescence[a]

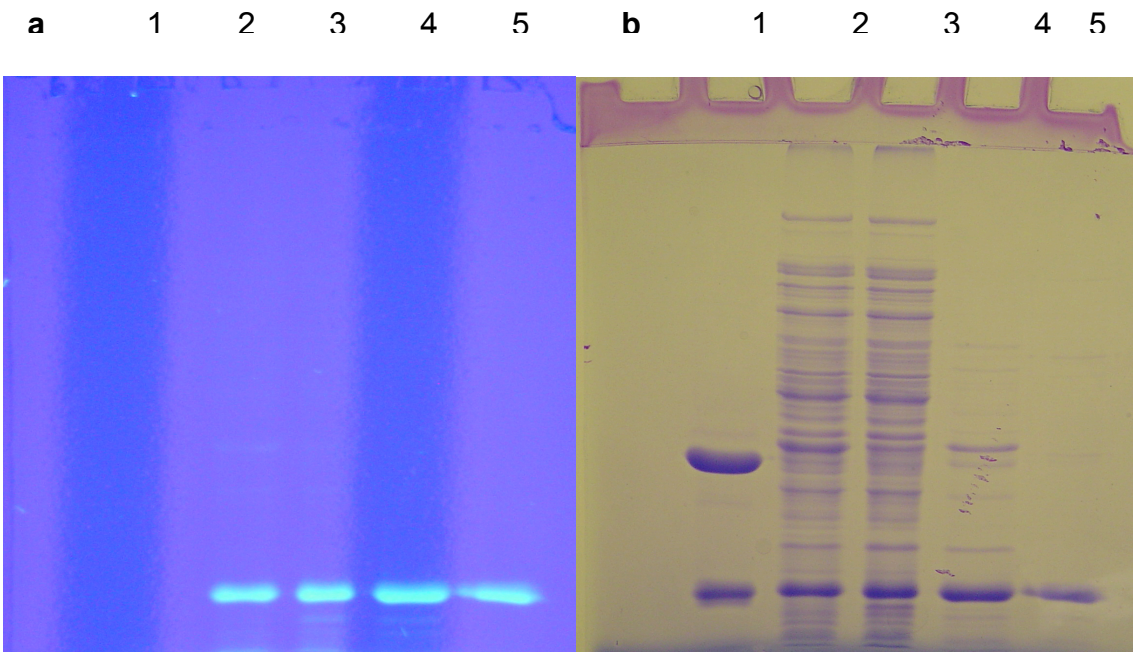
NTP	Conditions	K_1 (pH 8.3)	K_2 (pH 8.3)	K_1 (pH 7.0)	K_2 (pH 7.0)
		M^{-1}	M^{-1}	M^{-1}	M^{-1}
CTP	-	5.67 (±0.8) x10^4	1.43 (±0.3) x10^3	5.06 (±0.4) x10^5	7.40 (±0.9) x10^2
	2mM UTP	1.83 (±0.8) x10^4	8.16 (±0.5) x10^2	-	-
ATP	-	8.47 (±0.7) x10^3	3.10 (±0.4) x10^2	2.97 (±1.0) x10^4	5.67 (±1.4) x10^2
UTP	-	4.70 (±0.8) x10^3	4.39 (±0.7) x10^2	6.08 (±1.5) x10^1	-
	2mM CTP	2.73 (±2.0) x10^3	3.24 (±1.2) x10^2	-	-
GTP	-	1.16 (±0.5) x10^4	9.30 (±4.7) x10^2	1.43 (±0.3) x10^4	5.79 (±1.2) x10^1

[a] The binding constants reported for HCE-Gly52$_R$ enzyme were determined from fluorescence changes in the presence of ATP, CTP, UTP, and GTP. All measurements were performed at 25 °C in 50 mM Tris-acetate buffer, pH 8.3 or 20 mM Bis-Tris, 20 mM Tris, 20 mM CAPS buffer, pH 7.0. The values reported are the average deviation of two determinations.

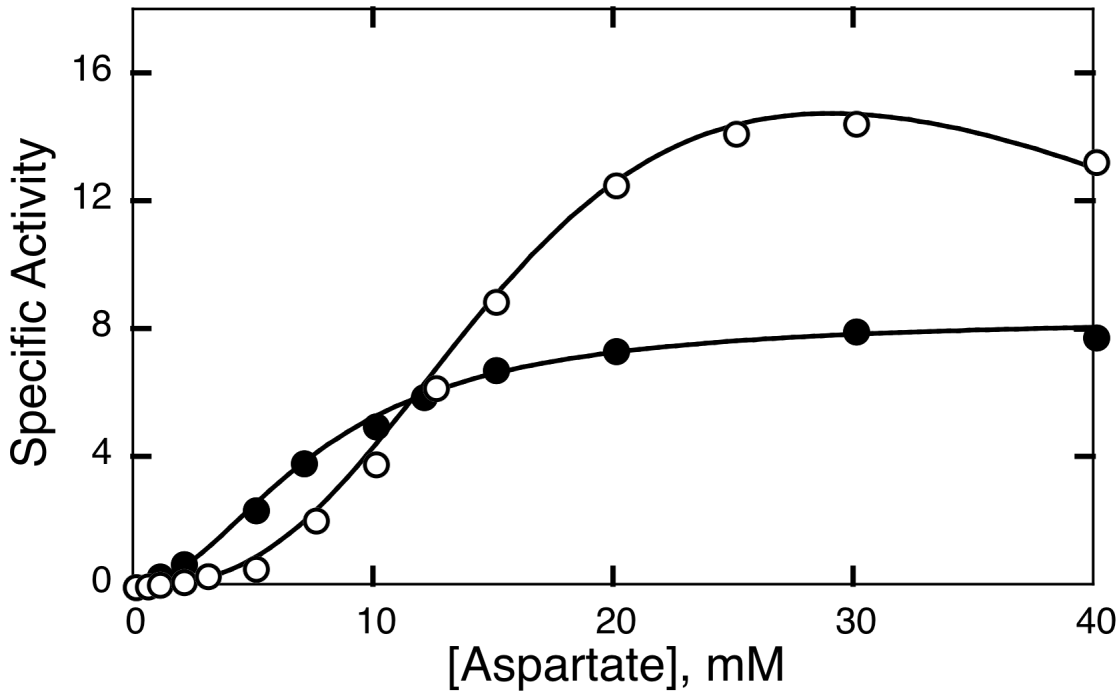
Supplementary Table 2. ATP, CTP, UTP, and GTP binding to HCE-Gly52$_R$ regulatory dimers measured by fluorescence[a]

NTP	K_1	K_2
	M^{-1}	M^{-1}
CTP	2.41 (±0.3) x10^4	4.13 (±1.4) x10^1
ATP	1.05 (±0.2) x10^4	1.08 (±0.3) x10^2
UTP	5.88 (±0.9) x10^3	3.21 (±0.6) x10^2
GTP	4.91 (±0.4) x10^3	-

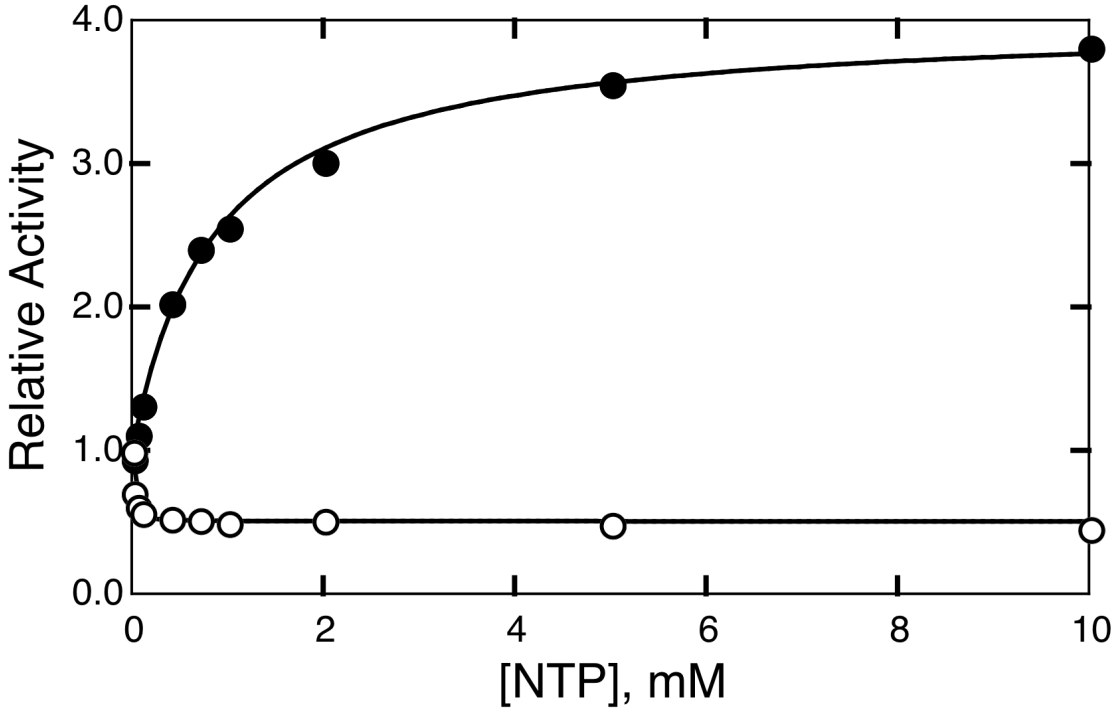
[a] The binding constants reported for HCE-Gly52$_R$ enzyme was determined from fluorescence changes in the presence of ATP, CTP, UTP, and GTP. All measurements were performed at 25 °C in 50 mM Tris-acetate buffer, pH 8.3. The values reported are the average deviation of two determinations.



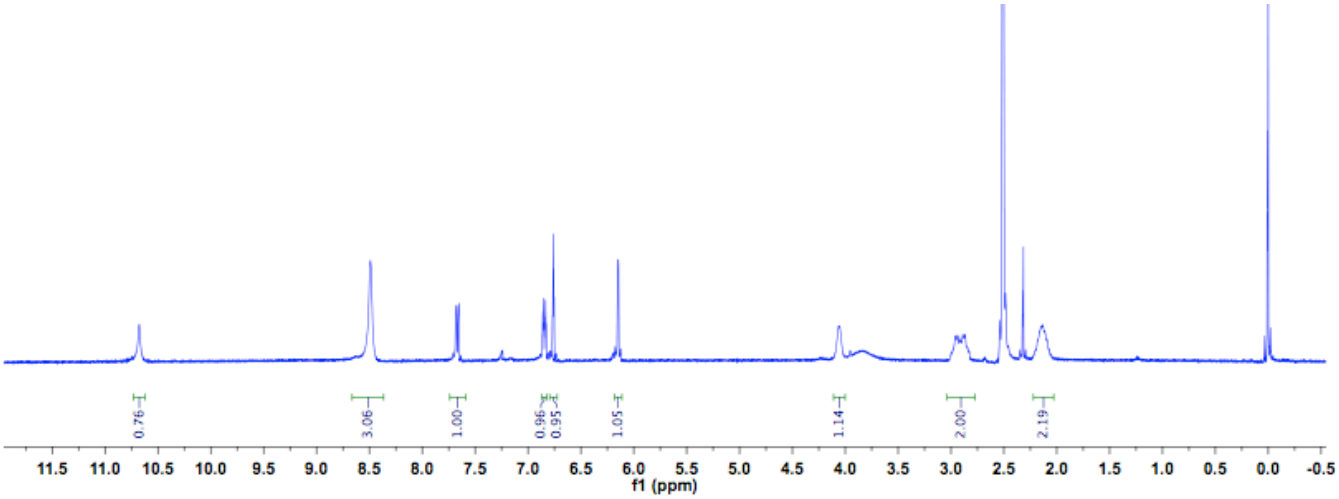
Supplementary Figure 1: Purification of HCE-Gly52$_R$ regulatory dimers. SDS PAGE visualized by fluorescence (**a**) or coomassie blue stain (**b**) *Lanes 1*, wild-type ATCase; *Lanes 2*, total soluble protein from cell lysate; *Lanes 3*, HCE-Gly52$_R$ r_2 sample after ammonium sulfate precipitation; *Lanes 4*, HCE-Gly52$_R$ r_2 after ion exchange chromatography employing Quaternary Fast Flow (GE Healthcare) and elution with 0.5M NaCl; *Lanes 4*, HCE-Gly52$_R$ r_2 after column purification employing Phenyl Sepharose (GE Healthcare).



Supplementary Figure 2. Aspartate saturation curves of HCE-Gly52$_R$ ATCase at pH 8.3 (open circles) and pH 7.0 (closed circles). The assays were performed at 25 °C at a saturating concentrations of carbamoyl phosphate (4.8 mM) in 50 mM Tris-acetate buffer, pH 8.3 or 20 mM Bis-Tris, 20 mM Tris, 20 mM CAPS buffer, pH 7.0. Specific activity is measured in units of mmoles CA formed•hr^{-1}•mg^{-1}.



Supplementary Figure 3. Alteration in the activity of HCE-Gly52$_R$ ATCase upon the binding of CTP (open circles) and ATP (closed circles). ATCase activity was measured at 25 °C by the colorimetric method. Assays were performed in 50 mM Tris-acetate buffer at pH 8.3 in the presence of a saturating concentration of carbamoyl phosphate (4.8 mM).



Supplementary Figure 4. ^{1}H NMR spectra of purified L-(7-hydroxycoumarin-4-yl) ethylglycine in dmso-d_6. ^{1}H NMR (400 MHz, dmso-d_6): δ 2.06-2.20 (m, 2H), 2.82-2.99 (m, 2H), 4.03-4.08 (m, 1H), 6.15 (s, 1H), 6.75-6.77 (m, 1H), 6.84 (d, J = 8.4 Hz, 1H), 7.67 (d, J = 8.8 Hz, 1H), 8.5 (s, 3H)

References

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