Supporting Information

Mg^{2+} binds to the surface of thymidylate synthase and affects hydride transfer at the interior active site

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Supplementary Figures

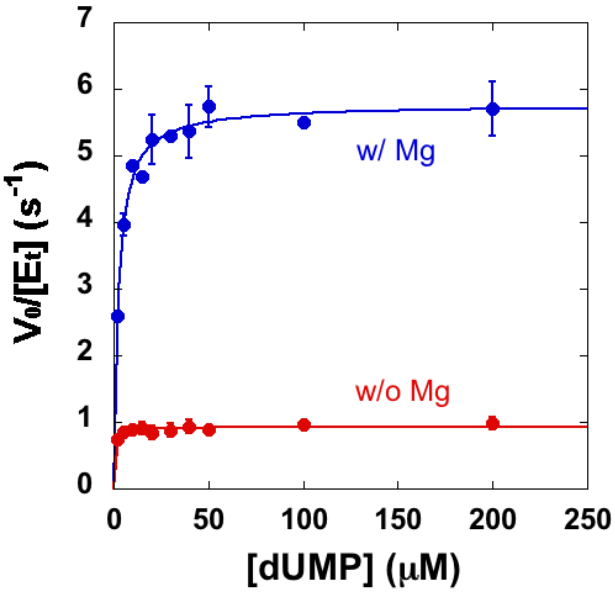

Figure S1. The initial velocity of ecTSase-catalyzed reaction *vs*. concentration of dUMP, in the absence (red) and presence (blue) of 50 mM $MgCl_2$.

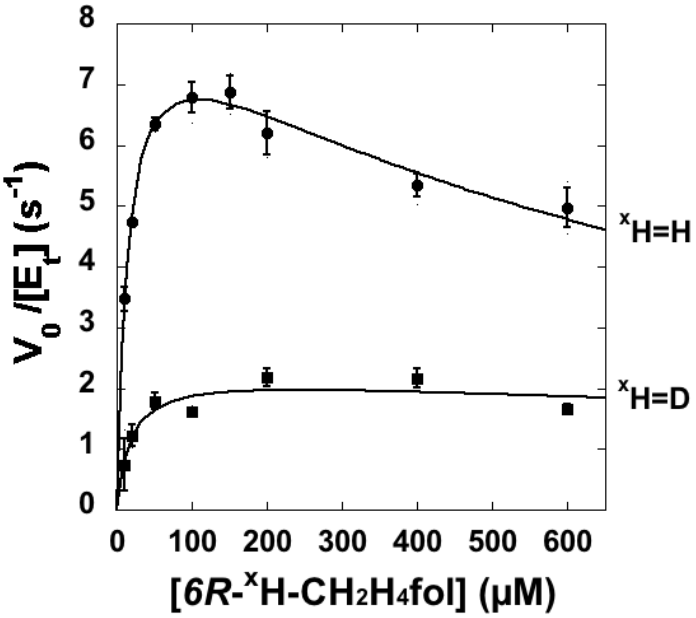

Figure S2. The initial velocity of TSase-catalyzed reaction *vs*. concentration of *6R*-^{x}H-CH_2H_4fol (^{x}H = H or D), in the presence of 50 mM $MgCl_2$ at 25 ˚C.

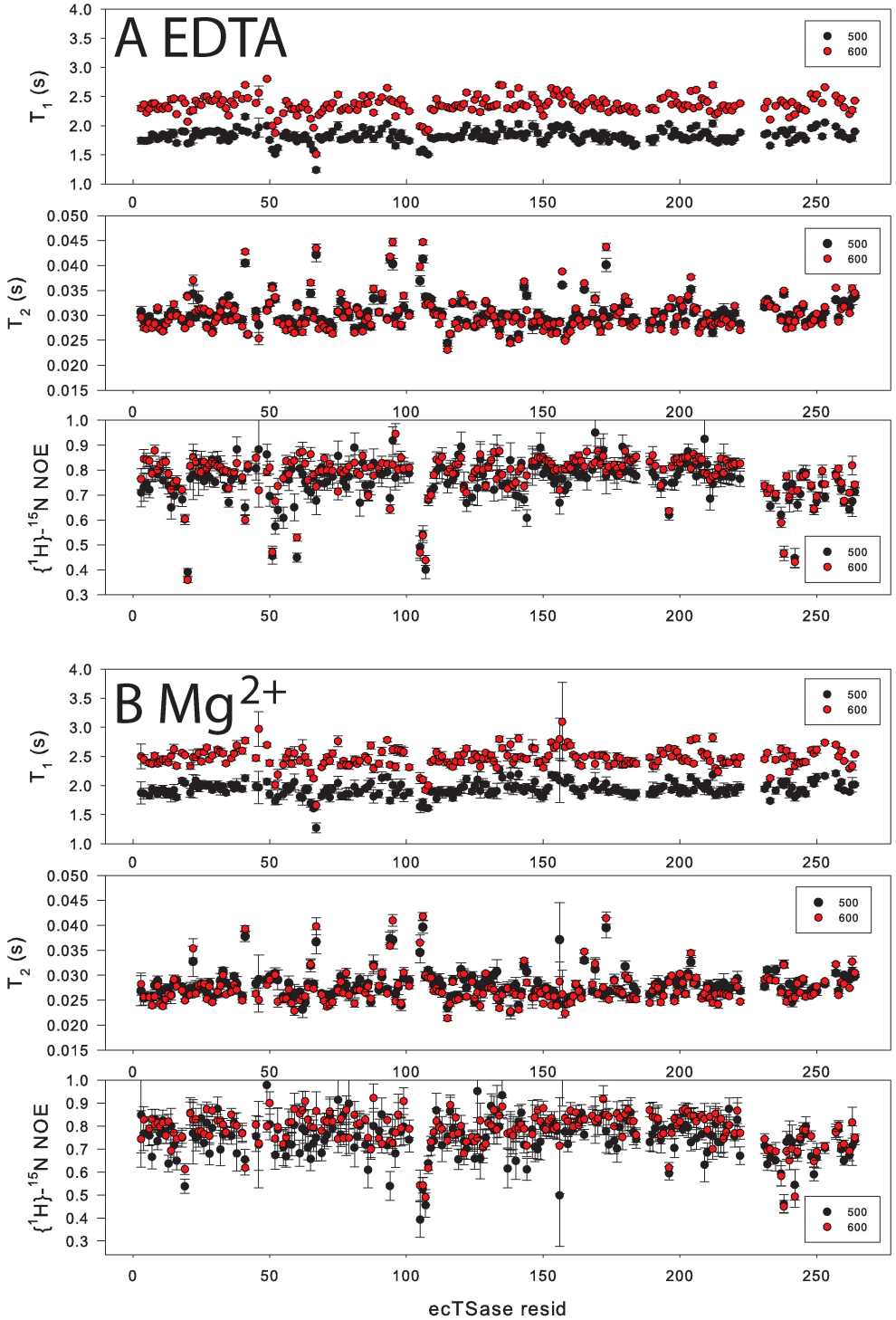

Figure S3. T_1, T_2, and $\{^1H\}$-^{15}N NOE for ecTSase-FdUMP-CH_2H_4Fol (A) without and (B) with $MgCl_2$.[1] Data at 500 and 600 MHz are in black and red, respectively.

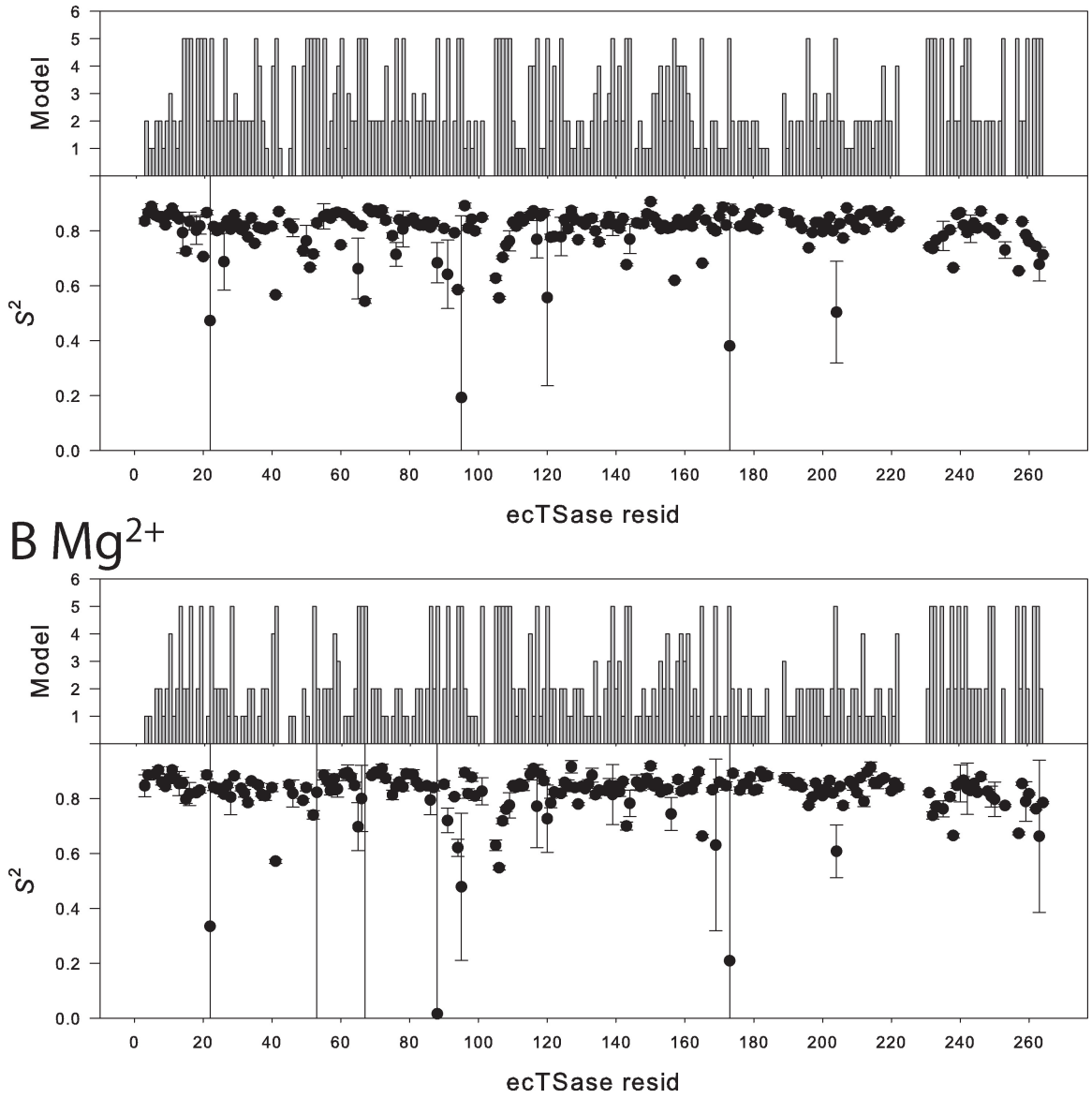

Figure S4. Model-free ^{15}N-^{1}H order parameters for the ecTSase-FdUMP-CH_2H_4fol complex (A) without and (B) with $MgCl_2$. The top plot in each panel shows the model that best fits the raw relaxation data.[1] Models are: 1) S^2 only, 2) S^2, t_e, 3) S^2, R_{ex}, 4) S^2, t_e, R_{ex}, 5) S^2_f, S^2, $t_e = t_s$.[2]

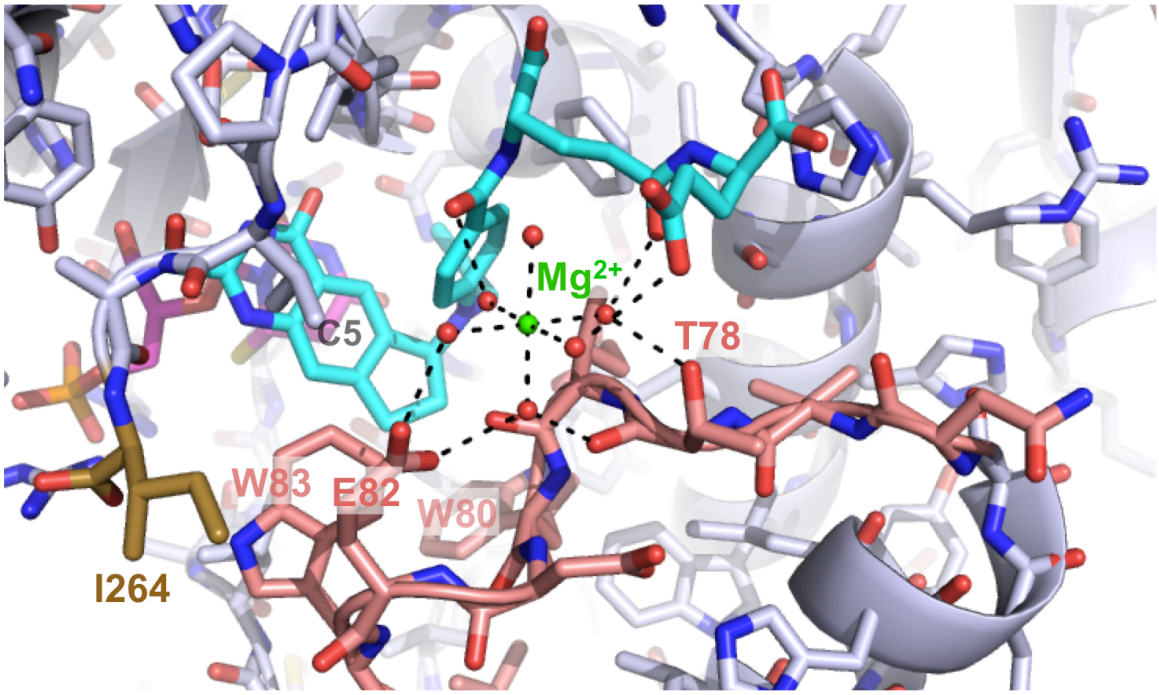

Figure S5. Mg^{2+} (green) binds to the surface of the ternary complex of ecTSase with dUMP and a di-Glu antifolate inhibitor BGC945 (cyan).[3] All other molecules are colored in the same way as Figure 5 in the main text. Mg^{2+} mediates an H-bond network between the first Glu of the di-Glu moiety of the inhibitor and the backbone carboxylates and side chains (*e.g.* T78 and E82) of the loop, which resembles the interactions in the ecTSase-dUMP-CB3717 complex (Figure 5).

Supplementary Tables

Table S1. Steady state kinetic parameters of ecTSase in the absence (w/o, red) and presence (w/, blue) of 50 mM $MgCl_2$.

w/ Mg^{2+} w/o Mg^{2+}	5 °C 5 °C	15 °C 20 °C	25 °C 30 °C	35 °C 40 °C
k_{cat} (s^{-1})	5.1 ± 0.3 0.73 ± 0.06	6.5 ± 0.2 1.02 ± 0.03	8.7 ± 0.2 1.32 ± 0.02	10.3 ± 0.3 1.62 ± 0.06
$K_M^{CH_4H_4fol}$ (μM)	8 ± 1 24 ± 5	11 ± 1 20 ± 2	15 ± 1 14 ± 1	19 ± 2 24 ± 3
$K_I^{CH_4H_4fol}$ (mM)	0.6 ± 0.1 0.43 ± 0.05	0.52 ± 0.05 0.53 ± 0.02	0.76 ± 0.07 1.09 ± 0.07	1.5 ± 0.2 1.5 ± 0.3

Table S2. The observed KIE on k_{cat}/K_M, the intrinsic KIE on the hydride transfer, and C_f in the presence of 50 mM $MgCl_2$.

Temper-ature ˚C	Observed KIE $^{T}(k_{cat}/K_M)_L$		Intrinsic KIE on the hydride transfer [a]			C_f
	H/T	D/T	H/T	H/D	D/T	
5	2.59 ± 0.02	1.47 ± 0.02	7.5 ± 0.8	4.0 ± 0.3	1.83 ± 0.06	3.1 ± 0.5
15	3.51 ± 0.02	1.60 ± 0.02	7.6 ± 0.6	4.1 ± 0.2	1.83 ± 0.04	1.6 ± 0.2
25	4.14 ± 0.02	1.65 ± 0.01	7.2 ± 0.4	3.9 ± 0.2	1.81 ± 0.03	1.0 ± 0.1
35	3.91 ± 0.05	1.63 ± 0.03	7.4 ± 0.9	4.0 ± 0.4	1.82 ± 0.07	1.2 ± 0.3

[a] The intrinsic H/T KIE is numerically solved from Eq 8 in the main text, and the intrinsic H/D and D/T KIEs are calculated from the intrinsic H/T KIE using the Swain-Schaad relationship.[4]

Table S3 Data collection and refinement statistics from ref 3.

PDB ID	4ISK		
Data collection		**Refinement**	
X-ray source	ALS 8.3.1	Resolution (Å)	29.81-1.75
X-ray wavelength (Å)	1.115869	R_{work}/R_{free} (%)	19.4/24.8
Space group	P21	No. reflections	200717
Cell dimensions		Completeness for range (%)	97.8
a, b, c (Å)	95.9,85.5,134.3		
α, β, γ (°)	(90, 109.4, 90)		
Resolution (Å)	30-1.75	r.m.s. deviations	
No. of unique reflections	204820	Bond length (Å)	0.013
Rmerge (%)	7.0	Bond angles (°)	1.57
I/σI	8.79	Ramachandran Plot (%)	
Completeness (%)	99.8	Favored regions	98.0
Redundancy	2.98	Allowed regions	1.60

References

(1) Sapienza, P. J., Lee, A. L. *Biomol. NMR Assigm.* **2013**, Submitted.

(2) Mandel, A. M., Akke, M., Palmer, A. G., 3rd *J. Mol. Biol.* **1995**, *246*, 144-163.

(3) Tochowicz, A., Dalziel, S., Eidam, O., O'Connell 3rd, J. D., Griner, S., Finer-Moore, J. S., Stroud, R. M. *J Med. Chem.* **2013**, Submitted.

(4) Swain, C. G., Stivers, E. C., Reuwer, J. F., Schaad, L. J. *J. Am. Chem. Soc.* **1958**, *80*, 5885-5893.