

Bacterial Thymidylate Synthase Binds Two Molecules of Substrate and Cofactor without Cooperativity

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Supporting Information

ABSTRACT: Thymidylate synthase (TSase) is a clinically important enzyme because it catalyzes synthesis of the sole *de novo* source of deoxy-thymidylate. Without this enzyme, cells die a “thymineless death” since they are starved of a crucial DNA synthesis precursor. As a drug target, TSase is well studied in terms of its structure and reaction mechanism. An interesting mechanistic feature of dimeric TSase is that it is “half-the-sites reactive”, which is a form of negative cooperativity. Yet, the basis for this is not well-understood. Some experiments point to cooperativity at the binding steps of the reaction cycle as being responsible for the phenomenon, but the literature contains conflicting reports. Here we use ITC and NMR to resolve these inconsistencies. This first detailed thermodynamic dissection of multisite binding of dUMP to *E. coli* TSase shows the nucleotide binds to the free and singly bound forms of the enzyme with nearly equal affinity over a broad range of temperatures and in multiple buffers. While small but significant differences in ΔC_p° for the two binding events show that the active sites are not formally equivalent, there is little-to-no allostery at the level of $\Delta G_{\text{bind}}^\circ$. In addition NMR titration data reveal that there is minor intersubunit cooperativity in formation of a ternary complex with the mechanism based inhibitor, 5F-dUMP, and cofactor. Taken together, the data show that functional communication between subunits is minimal for both binding steps of the reaction coordinate.

Thymidylate synthase (TSase) catalyzes the synthesis of the sole source of dTMP in organisms ranging from viruses to humans.¹ The mechanism involves reductive methylation of the substrate, dUMP, using a cofactor, N₅,N₁₀-methylene-5,6,7,8-tetrahydrofolate (CH₂H₄fol), as both a methylene and hydride donor.² Given its key role in DNA synthesis and cell division, TSase is an attractive drug target for treating microbial infection and cancer. As such, it is highly scrutinized in terms of its structure and catalytic mechanism. TSase is a dimeric enzyme with two active sites, and one often cited feature is that there is allostery between the two sites, which are separated by ~30 Å. Among these reports are that TSase is a half-the-sites reactive enzyme,^{3,4} the enzyme binds to only a single molecule of substrate⁵ or binds it with negative cooperativity,⁶ and the enzyme binds to only a single molecule of cofactor^{7,8} or binds it with negative cooperativity.⁹ Contrary to these reports are the X-ray models of TSase, which for the case of the *E. coli* enzyme have

yet to capture singly bound forms. Rather, structures show symmetrical subunits with full occupancy of both active sites. These data, coupled with an NMR spectrum of substrate analog and cofactor-saturated TSase clearly showing binding to both subunits,¹⁰ are inconsistent with *extreme* negative cooperativity. However, the question of cooperativity remains open because there has yet to be a rigorous study of the binding events in this key enzyme. To settle this, we measured the thermodynamics of binding of substrate and cofactor to both sites of *E. coli* TSase. We employed isothermal titration calorimetry (ITC), which is exquisitely sensitive to strength, heat, and stoichiometry of binding, to provide the first detailed thermodynamic picture of the TSase–dUMP interaction. We show that *E. coli* TSase binds two molecules of dUMP and, unexpectedly, that both the free and singly bound forms have the same affinity for substrate. Further, our analysis highlights the challenges with analyzing multisite binding data in that very small errors in ITC cell concentration can lead to dramatically different pictures of cooperativity. Only by measuring titrations at multiple conditions and by including cell concentration as a fitted parameter were we able to obtain accurate binding parameters. For the case of cofactor binding, where heat of covalent-bond formation can complicate interpretation of ITC data, we used NMR spectroscopy to directly quantify populations of all states over the course of a titration with a substrate analog and cofactor. This is a powerful approach as it provides a rare^{11,12} opportunity to monitor microscopic states in a multibinding site system. The data show both sites are similar with respect to formation of the ternary complex, demonstrating that allostery is minimal for the two binding steps of the reaction cycle.

Given the general interest in the phenomenon of allostery and the question of dUMP binding in TSase, we set out to probe the binding thermodynamics of this dimeric system. Thermodynamics of substrate binding was measured by ITC at 25 °C (Figures 1 and S3). The data fit well to a single site model with a stoichiometry (*n*) of 1.8 (Figure 1A, Table S1), but based on reports of cooperativity in this⁵ and other forms⁶ of the enzyme, the data were also fit to a general model that can accommodate differences in affinities and heats between the two binding events. The general model fit to intrinsic $K_{D,1}$ of ~4 μM and $K_{D,2}$ of ~20 μM, for a ρ -value (ratio of K_{A2}/K_{A1}) of 0.22 (Figure 1A, Table S1). This suggests negative cooperativity, but a comparison of reduced χ^2 indicates the single model is a better fit to the data (Figure 1A, (χ^2 values for all models are compared in Table S1)).

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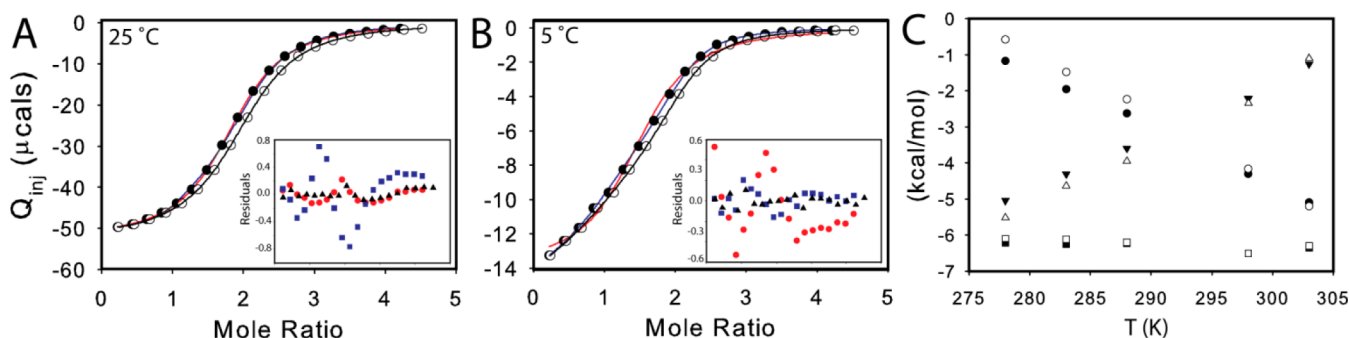


Figure 1. ITC measurement of dUMP binding to TSase. Conditions are 290 μM TSase in the cell and 6 mM dUMP in the syringe, both in 25 mM NaPO₄, 1 mM EDTA, and 2 mM TCEP, pH 7.5. (A, B) Fits are shown for dUMP titrations using models for one-site binding (red line against closed circles), general two-site binding (blue line against closed circles), and modified general binding (black lines against open circles); insets show residuals for one-site binding (red circles), general two-site binding (blue squares), and modified general binding (black triangles). For both 25 °C (A) and 5 °C (B), original data points are shown as filled circles, and data points corrected for cell concentration are shown as open circles. (C) The modified general model was used to fit ITC data at multiple temperatures. The thermodynamic parameters, ΔH° (circles), $-T\Delta S^\circ$ (triangles), and ΔG° (squares), for binding to free (filled) and singly bound (open) TSase are shown as functions of temperature. The slope of ΔH° versus T yields $\Delta C_p^\circ = -157$ and -183 cal/mol-K for binding to free and singly bound TSase, respectively. Errors in parameters were determined from Monte Carlo simulations, and the error bars are smaller than the points. Values for fitted parameters from the modified general model are shown in Table S2.

Even though the single sites model gives the superior fit, both models make assumptions that might not be accurate for this system. The main assumption with the single sites model is that each of the n sites has the same K and ΔH° , and it could be the case that $K_1 \neq K_2$ and/or $\Delta H^\circ_1 \neq \Delta H^\circ_2$. The potential pitfall of the general model is that all of the calculated and fitted parameters are dependent on a fixed n ($n = 2$ in the case of TSase). The fact that the best fitting single site model gave a non-integral stoichiometry of 1.8 raised the possibility that errors in ITC cell concentration, which are not taken into account by the general multisite model within the Origin ITC package, could lead to erroneous fits.

To overcome these issues, the data were fit using a general binding model that included cell concentration as a fitted parameter (see Supporting Information (SI)). Treating protein concentration as an adjustable parameter is reasonable given the possibility that the active fraction of TSase is <100% and potential discrepancy between actual and theoretical extinction coefficients. Further, fitting protein concentration within the general model was employed previously with other multibinding site systems.^{13,14} This is a rigorous fitting approach because the only assumption is the total number of binding sites, which is justified here by the X-ray model of the *E. coli* TSase-dUMP complex in which both sites have full occupancy.¹⁵ Fits to this modified general model (Figure 1A) give $\rho \approx 1$, a lower reduced χ^2 than either the single or unmodified general models (Table S1), and a fitted protein concentration 10% lower than that measured by UV spectroscopy. To ensure that the improved χ^2 associated with the modified general model is not simply the result of overfitting, we doubled the ratio of observables to fitted parameters by performing global fits to paired titrations with either two cell or syringe concentrations. This approach was shown previously to break degeneracies and increase robustness of fitted ITC parameters.¹⁶ Global fits to the paired titrations described above yield $\rho \approx 1$ (Figure S3, Table S1) in support of using the modified general model and the conclusion that binding affinities are similar. This analysis underscores the importance of accounting for inaccuracies in ITC cell concentration as errors of even 10% here can lead to a misinterpretation of up to 4-fold negative cooperativity when binding sites are truly identical (Table S1).

Because the heat capacity change upon binding is a sensitive probe of changes in structure and dynamics upon binding,¹⁷ we looked at dUMP binding at additional temperatures. The data fit poorly to the single site model at some temperatures other than 25 °C (Figures 1B and S4, Table S1), indicating that either cooperativity is temperature dependent or that ΔH°_1 and ΔH°_2 are not equivalent at all temperatures. The data were then fit to the modified general model which, for cases where $\Delta H^\circ_1 \neq \Delta H^\circ_2$, fits significantly better than either the single or unmodified general models (Figures 1B and S4, Table S1). Data for all five temperatures showed both active sites have nearly equivalent binding affinity (Figure 1C, Tables S1 and S2), and all data sets required a similar correction to enzyme concentration, which is expected if the enzyme originates from the same preparation. In contrast to binding affinities, ΔH° for the two binding events diverges as a function of temperature (Figure 1C and Table S2). The temperature trends highlight the difficulty with fitting all of the data to the single sites model because both sites are not described by the same set of ΔH° parameters (Table S2). The slope of ΔH° versus T yields a ΔC_p° of -157 ± 1 cal/mol-K for site 1 and -183 ± 2 cal/mol-K for site 2 (Figure 1C). Overall, the apo and singly bound forms of TSase bind dUMP with similar affinities, but the sites are not equivalent based on small but significant differences in ΔC_p° .

To determine if the differences in ΔH° were the result of proton exchange with the solution upon dUMP binding, titrations were conducted in a series of four buffers with different heats of buffer ionization, ΔH_b° . The chosen buffers were phosphate, HEPES, TES, and Tris in order of increasing ΔH_b° .¹⁸ The measured ΔH° from ITC is linked to the heat of ionization of the buffer (ΔH_b°) and is related to the number of protons exchanged during binding.¹⁹ The data were fit to the general binding model with adjustable protein concentration and fit with $\rho \approx 1$ for all buffers (Figures 2 and S5, Table S3), a convergence which further supports this fitting model. Binding is 2- to 3-fold weaker in phosphate than in the other buffers (Table S3). The difference in binding affinity may be attributable to preferential interaction with phosphate, as the ion alone is shown to bind TSase at the same site as the phosphate moiety of dUMP.¹⁵ Thus, phosphate may be a better competitor for dUMP binding than the other buffers used here. The slopes of ΔH° versus ΔH_b° (Figure 2D) indicate that <0.1 mol of H⁺ is taken up by the

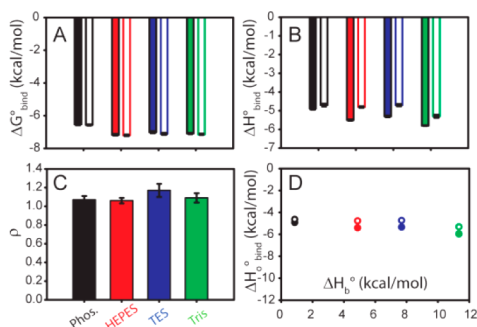


Figure 2. Minimal proton linkage accompanies dUMP binding to TSase. Modified general model was used to fit ITC experiments in multiple buffers with different enthalpies of ionization (ΔH_b°). Buffer color key in (C) applies to all panels. In panels A, B, and D, sites 1 (2) are represented by filled (open) bars/symbols. Linear fits to data in panel D give number of protons (n) linked to binding. Site 1 (\bullet) fits to n of -0.09 ± 0.02 protons and ΔH_1° of -4.8 ± 0.17 kcal/mol. Site 2 (\circ) fits to n of -0.06 ± 0.03 protons and ΔH_2° of -4.5 ± 0.19 kcal/mol. Example fits are given in Figure S5, with values in Table S3.

protein upon dUMP binding and that H^+ linkage does not play a large role in dUMP binding.

It is possible that cooperative effects alternatively reside at the cofactor binding step. Substrate binding results in only modest conformational changes in TSase.²⁰ Addition of cofactor or cofactor analog causes more dramatic rearrangements in the local binding site²⁰ that could influence the neighboring subunit. This, coupled with reports of cofactor analog binding cooperativity in human⁹ and bacterial TSase,^{7,8} prompted us to further investigate cofactor binding. We chose to monitor binding of the substrate analog, 5F-dUMP, and the biological cofactor, CH_2H_4Fol , by NMR titration. In the resulting ternary complex, C146 makes a covalent bond to 5F-dUMP, which is covalently attached via a methylene bridge to the cofactor;²¹ the covalent attachments make it such that the two small molecules can be treated as a single “di-ligand”. Importantly, the di-ligand is considered a mechanism-based inhibitor, as these covalent bonds are formed during the normal reaction cycle.² Therefore, the complex is an excellent model for cooperativity in ternary complex formation during catalysis. Further, structures of this complex are isosteric with other complexes involving dUMP and cofactor analogs.^{22,23} Lastly, the stability of the complex yields high-quality NMR spectra with resonances that are in slow-exchange on the NMR time scale, facilitating the quantification of all species (see below).

Titration of TSase with the di-ligand were monitored by TROSY- 1H - ^{15}N HSQC spectra. A subset of residues at the dimer interface yielded two resonances corresponding to the singly bound state that are distinct from symmetrical free and doubly bound resonances (Figure 3A,B). This allowed us to directly measure the fraction of free, singly bound, and doubly bound microstates.¹² Four amides give well-resolved peaks for all three states; intensities from these residues were fit to the two-site binding polynomial (see SI). At the limit of stoichiometric binding, which is observed in this case, we cannot determine the absolute binding affinities. However, differences in the relative binding affinities between the two sites are readily apparent (Figure S6). In the case of di-ligand binding to TSase, the ρ values range from 0.55 to 0.90 for fits of single residue data (Figure S7 and Table 1). A global fit of all four residues yielded a ρ of 0.65 ± 0.075 (Figure 3C,D and Table 1), which indicates a

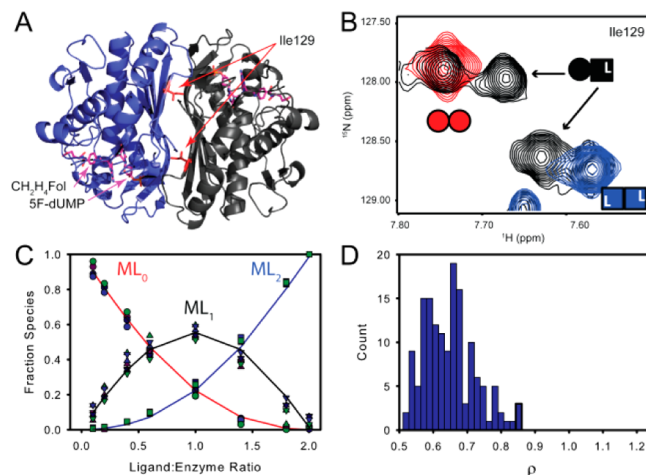


Figure 3. Both TS active sites have similar affinity for the 5F-dUMP- CH_2H_4Fol “di-ligand”. (A, B) In NMR spectra, at intermediate titration points (black, (B)), resonances from a subset of residues near the dimer interface (e.g., Ile 129 in (A)) have chemical shifts from the singly bound state that are different from the free (red, (B)) and doubly bound states (blue, (B)). For these residues there are four resonances total at intermediate titration points, as the singly bound state produces two peaks: one from the free subunit and one from the bound subunit (B). (C) Global fit of peak intensities from the four resonances having all three states resolved in NMR spectra. Circles and squares represent free and doubly bound data, respectively. Upward triangles are from the free subunit of singly bound species, and downward triangles are from the bound subunit. (D) Histogram of ρ (K_2/K_1 ratio) from fits of 150 Monte Carlo simulated data sets.

Table 1. Relative binding constants for TSase 5F-dUMP- CH_2H_4Fol di-ligand binding from NMR titration

residue	ρ^a
Gln33	0.88 ± 0.067
Ile129	0.90 ± 0.12
Asn134	0.57 ± 0.080
unassigned Trp indole	0.77 ± 0.14
global fit	0.65 ± 0.075

^aRatio of intrinsic association constants (K_2/K_1).

slight degree of negative cooperativity with a *maximum* magnitude of <2-fold.

The data presented herein unequivocally show that substrate binds to the free and singly bound forms of *E. coli* TSase with similar affinity. This finding contrasts with a previous fluorescence study showing only one molecule of dUMP bound per TSase dimer.⁵ It is unlikely that solution conditions account for the discrepancy, as we observe equivalent binding affinity at multiple temperatures and in all buffers tested. It is more probable that the fluorescence results were confounded by complex interactions between the seven tryptophan probes per TSase subunit. These issues are circumvented by the direct link between binding and heat of reaction measured by ITC. It is noteworthy that while dUMP binding affinities are the same in both the free and singly bound enzyme, ΔH°_1 and ΔH°_2 are not equivalent at some temperatures and in some buffers. This phenomenon, in which binding is similar at the level of ΔG° but different based on ΔH° and $T\Delta S^\circ$, was termed “silent allosteric coupling”.²⁴ The recent linkage between binding and redistribution of side-chain dynamics^{25–28} and the connection

between side-chain dynamics and conformational entropy^{26,29,30} suggests this type of coupling is widespread.

We also show by NMR that the ternary complex is formed with nearly equal probability in both subunits, which disagrees with an ITC study showing roughly one molecule binds to dimeric *E. coli* TSase.⁷ It is difficult to compare our data with the published ITC study because the fitting models are not described in detail and our work provides a dramatic example of how choice of fitting model can affect interpretation of ITC in multibinding site systems. However, our data are in agreement with crystal structures^{15,21,23,31} and NMR spectra¹⁰ that show two molecules of cofactor are bound. Interestingly, binding the first di-ligand does elicit chemical shift changes in the empty subunit (Figure 3B), leading us to conclude that the effects of binding are communicated across the dimer interface. We are currently investigating this coupling. Lastly, we should emphasize that our work does not necessarily reflect on reports that eukaryotic TSase is a cooperative enzyme because, unlike the enzyme studied here, symmetry is broken in forms from higher organisms by an active site appendage that adopts multiple conformations in the absence of ligands.^{32,33}

■ ASSOCIATED CONTENT

📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b10128.

Detailed experimental procedures including ITC and NMR titration fitting methods, additional ITC curves, NMR spectra, and local fits to NMR titrations (PDF)

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Notes

The authors declare no competing financial interest.

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