Published in final edited form as:

ACS Catal. 2018 November 2; 8(11): 10241–10253. doi:10.1021/acscatal.8b02554.

Experimental and Computational Studies Delineate the Role of Asparagine 177 in Hydride Transfer for *E. coli* Thymidylate Synthase

Ilya Gurevic^{1,#}, Zahidul Islam^{1,@,#}, Katarzyna widerek^{2,#}, Kai Trepka¹, Ananda K. Ghosh¹, Vicent Moliner^{2,*}, and Amnon Kohen^{1,*}

¹Department of Chemistry, College of Liberal Arts & Sciences, University of Iowa, Iowa City, Iowa 52242-1727, United States.

²Departament de Química Física i Analítica, Universitat Jaume I, 12071 Castelló, Spain

@present address: Institute for Quantitative Biosciences (QB3), University of California-Berkeley, Berkeley, 94720, United States.

Abstract

Thymidylate synthase (TSase), an enzyme responsible for the *de novo* biosynthesis of 2'-deoxythymidine 5'-monophosphate (thymidylate, dTMP) necessary for DNA synthesis, has been a drug target for decades. TSase is a highly conserved enzyme across species ranging from very primitive organisms to mammals. Among the many conserved active site residues, an asparagine (N177, using *Escherichia coli* residues numbering) appears to make direct hydrogen bonds with both the C4=O4 carbonyl of the 2'-deoxyuridine 5'-monophosphate (uridylate, dUMP) substrate and its pyrimidine ring's N3. Recent studies have reassessed the TSase catalytic mechanism, focusing on the degree of negative charge accumulation at the O4 carbonyl of the substrate during two critical H-transfers - a proton abstraction and a hydride transfer. To obtain insights into the role of this conserved N177 on the hydride transfer, we examined its aspartic acid (D) and serine (S) mutants - each of which is expected to alter hydrogen bonding and charge stabilization around the C4=O4 carbonyl of the 2'-deoxyuridine 5'-monophosphate (uridylate, dUMP) substrate. Steady-state kinetics, substrate binding order studies and temperature-dependency analysis of intrinsic KIEs for the hydride transfer step of the TSase catalytic cycle suggest the active site of N177D is not precisely organized for that step. A smaller disruption was observed for N177S, which could be rationalized by partial compensation by water molecules and rearrangement of

*corresponding authors; moliner@uji.es, amnon-kohen@uiowa.edu.

#these authors contributed equally

SUPPORTING INFORMATION

The Supporting Information is available free of charge on the ACS Publications website at DOI: details of the substrate binding order; scheme of the active site with definition of the QM sub-set of atoms; Circular dichroism spectra; representative Michaelis-Menten curves for N177S TSase; Observed and intrinsic KIEs for WT, N177S and N177D *E. coli* TSase; Commitment factors for hydride transfer; temperature dependence of the contribution to the total KIEs from Quasiclassical term computed at RM1/MM level of theory; temperature dependence of tunneling and recrossing transmission coefficients for tritium transfer computed at RM1/MM level of theory; Normalized autocorrelation functions of the forces acting on the reaction coordinate at the TSs for protium and tritium the four different temperatures.

Competing Financial Interests Statement

The authors declare no competing financial interests.

other residues toward preparation of the system for the hydride transfer under study. These experimental findings are qualitatively mirrored by QM/MM computational simulations, thereby shedding light on the sequence and synchronicity of steps in the TSase-catalyzed reaction. This information could potentially inform the design of mechanism-based drugs targeting this enzyme.

Graphical Abstract

Keywords

Thymidylate synthase; Steady-state kinetics; temperature-dependency KIEs; Kinetic Isotope Effects; QM/MM calculations; Free energy surfaces

INTRODUCTION

In order to perform the DNA synthesis that precedes cell division, 2'-deoxythymidine 5'-monophosphate (dTMP), or thymidylate, must be synthesized at adequate levels.¹ Most organisms, including *Escherichia coli* and *Homo sapiens*, rely on the enzyme thymidylate synthase (TSase) for *de novo* production of dTMP from 2'-deoxyuridine 5'-monophosphate (dUMP) and 5,10-methylene-5,6,7,8-tetrahydrofolate (MTHF), with the latter serving as both methylene and hydride donor.¹ Tumors rely on TSase to maintain the nucleotide pool that enables fast growth required for its proliferation. A number of clinically used cancer chemotherapeutics target TSase by mimicking either the nucleotide or folate substrate. Even better inhibitors with lower toxicity are being sought for chemotherapy, while the fine differences between the bacterial and human TSases are of interest for the development of antibiotics.

After the substrates bind, the long-accepted TSase mechanism – depicted in Scheme 1 – begins with the attack of the active-site cysteine (C146) thiolate on C6 of the pyrimidine ring to form an enolate, as seen in step 1.¹ In step 2, the nucleophilic C5 of this enolate attacks the iminium ion form of MTHF.¹ An array of side chains and ordered water molecules³ removes the proton at C5, reforming the enolate as step 3 and subsequently in step 4 eliminating tetrahydrofolate after protonation of its N5. This forms an exocyclic methylene intermediate, E.¹ Next, in step 5 a rate-limiting hydride transfer^{4–5} from C6 of tetrahydrofolate to the exocyclic C7 of this intermediate E takes place, generating

dihydrofolate and forming a new enolate.¹ Finally, in step 6, the enolate breaks down, rearomatizing the pyrimidine and eliminating the cysteine thiolate, thereby accounting for dTMP formation. This is followed by product release.¹ Importantly, all the enolate forms proposed – if present – are likely to be stabilized by H-bonding to N177.

More recently, revision to this mechanism has been proposed.^{6–11} Computational quantum mechanics / molecular mechanics (QM/MM) studies have centered on the nature of the proton abstraction and hydride transfer processes.^{6–7, 10, 12} Investigation of the proton transfer step by these *in silico* methods suggested that this event is concomitant with partial dissociation of the C6-S_{Cys} bond.^{6–7, 11–12} Later synthesis and kinetic characterization¹³ of the bridged bisubstrate intermediate with a C5=C6 double bond provided strong evidence supporting these QM/MM studies. The enolate is thus being bypassed in these steps.

The hydride transfer – which is the focus of the present study – was predicted to happen with scission of the C6-S_{Cys} bond, but with the degree of dissociation of the C5-H bond of tetrahydrofolate being higher than the degree of dissociation of the C6-S_{Cys} bond.^{6, 10, 12} This is referred to as a “concerted but asynchronous” process.^{6, 12} A secondary kinetic isotope effect (KIE) measurement⁹ with labelling on the C6 of dUMP suggested a concerted mechanism for this hydride transfer step but did not examine the synchronicity of dissociation of the C6-S_{Cys} bond and the hydride transfer. These suggested mechanistic modifications leading to the same exocyclic methylene hydride-accepting intermediate (E) are summarized in scheme 2. This work strives to clarify whether there is enolate character – and thus negative charge accumulation at O4 – in the transition state (TS) for the hydride transfer step. This question is summarized in Scheme 3. When it comes to inhibitor design, an enolate character of this TS would suggest installation of a polar functional group in place of the C4=O4 carbonyl (mechanism I in Scheme 3), while absence of enolate character would suggest using a less polar functionality (mechanism II in Scheme 3). The answer to the question of whether hydride transfer passes through an enolate-like TS or intermediate could guide the design of transition state analog (TSA) inhibitors of TSase. The crucial point is what charge character develops at O4 position along the reaction path. The drug design implications are independent of whether enolate formation or breakdown contributes to rate limitation.

Crystal structures of WT *E. coli* TSase (Figure 1a) imply that N177 forms six-membered cyclic hydrogen bonds with O4 and N3 of dUMP,¹⁴ presumably stabilizing O4 and any negative charge that it may accumulate during hydride transfer. This residue is strictly conserved across all organisms, and a crystal structure of the ternary complex of TSase-5-fluoro-dUMP-MTHF suggests that this residue repositions itself in response the puckering of the dearomatized pyrimidine.¹⁵ Inferences concerning the role of N177 could be possible by examining mutants that alter interactions with O4. N177D would serve as a potentially revealing mutant, as this residue takes up similar space but could be different in charge. It is expected to be anionic at pH 7.5, but active sites are known to shift pK_a values, sometimes very dramatically.¹⁶ This would change the nature of interactions near this carbonyl, and thus is a relevant consideration to be explored herein. In a prior study, a screening for growth of *E. coli* colonies harboring N177S TSase yielded slowly growing colonies.¹⁷ As serine is a polar, uncharged hydrogen-bond donor and is smaller in size than asparagine, study of the

N177S enzyme would also be worthwhile. The study of N177D and N177S at the molecular level could potentially shed light on the role of N177 in the TSase-catalyzed hydride transfer.

Schramm and co-workers used KIEs to predict the structure of the TS of human purine nucleoside phosphorylase and thereby refining TSA inhibitor structures.^{18–19} This demonstrated the general utility of KIEs for study of enzyme mechanisms, which in turn assist in inhibitor design. In this study, KIEs are utilized to provide evidence regarding the mechanism for TSase – in particular, the hydride transfer step; TSase has been subjected to such a line of inquiry.⁴ These further details on the mechanism of TSase could potentially allow for development of enhanced therapeutics. Another aspect that could play into future inhibitor development is the protonation state and tautomeric form of the hydride acceptor (**E**) in Scheme 2. These processes are difficult to assess experimentally and therefore are underexplored. Nonetheless, deprotonation of imide N3 of the pyrimidine has been proposed to promote chemical steps in the distinct flavin-20 dependent class of TSase,²⁰ and multiple tautomeric forms of the imide moiety of the uracil ring have been proposed to contribute to RNA-protein binding.²¹

In this work, the effects of neutral and supposedly anionic N177 mutations on structure, steady-state kinetics, substrate-binding order and hydride transfer kinetic isotope effects are examined. *QM/MM* calculations are brought to bear on these questions, and the outcomes corroborate experimental findings. Moreover, computational methods weigh in on the protonation state and the tautomeric forms of the exocyclic methylene hydride acceptor (**E**) in Scheme 2. The similarities and differences between the two mutants and the wild type enzyme emphasize the role of the chemical environment of the C4 carbonyl in TSase catalysis and provide further insight into the reaction coordinate of the hydride transfer process.

MATERIALS AND METHODS

Materials and instruments.

[5-³H]-dUMP, specific radioactivity ~14 Ci/mmol and [2-¹⁴C]- dUMP, specific radioactivity ~53 mCi/mmol were purchased from Moravsek Biochemicals. Unlabeled MTHF was from Merck. Radiolabeled MTHF derivatives were synthesized as described elsewhere.²⁴ Ultima Gold liquid scintillation cocktail was from PerkinElmer. liquid scintillation counting was performed on a Packard TRI-CARB 2900 TR instrument. Separations of reaction mixtures were conducted on reverse-phase Supelco Discovery C18 columns using Agilent Technologies 1100 HPLC systems. Initial velocities were measured on a Hewlett-Packard Model 8452A diode-array spectrophotometer connected to a water bath for temperature control. A Jasco CD J-815 instrument was used to record the circular dichroism spectra for the enzymes under study.

Protein expression and purification.

Mutagenic primers - forward and reverse - were designed and then ordered from Integrated DNA Technologies (IDT). These were then used in mutagenic PCR, followed by DpnI

digestion, purification, and transformation into *E. coli thyA*-BL21(DE3) cells. Sequencing by the Genomics Division of the University of Iowa Institute of Human Genetics confirmed that the desired mutation was present. The enzyme was expressed and purified according to the method described previously.²⁵

Circular dichroism spectroscopy.

Solutions at ~7.5 μ M concentration of apo enzyme were prepared for all three enzymes in the same buffer as was used in steady-state kinetic studies. A cuvette with a pathlength of 1 mm was used, and spectra were recorded from 260 nm to 190 nm at 20° C.

Steady-state kinetics.

Initial velocities for steady-state kinetics were measured by following the change in absorbance at 340 nm (A₃₄₀) and using $\Delta\epsilon_{340, \text{DHF} - \text{MTHF}} = 6,400 \text{ M}^{-1} \text{ cm}^{-1}$.²⁴ The reactions were performed in 100 mM tris pH 7.5 buffer, with 1 mM TCEP, 50 mM MgCl₂, and 7 mM HCHO. Typical enzyme concentrations were 1–10 μ M, and one substrate was varied, while the other was at saturating concentration of 1 mM. Non-linear fitting to the Michaelis-Menten equation was employed to extract steady state parameters; in contrast to measurements with the wild type TSase substrate inhibition by MTHF was not incorporated because its onset was well beyond a 1 mM MTHF concentration. All measurements were conducted in triplicate.

Kinetic isotope effect technique.

KIE measurements aim to arrive at ratios of rate constants of different isotopologues for a particular chemical step. For studies of hydride transfer catalyzed by TSase and its mutants (*R*)-[6-^XH] of MTHF was prepared, where X is 1, 2, or 3 for H, D, or T, respectively. Measured, or observed, KIEs incorporate the isotopically insensitive steps along the catalytic cycle thus an assessment of the intrinsic KIE on the hydride transfer per se require further analysis of the observed values. Here we used the Northrop method²⁶ to arrive at $KIE_{int}^{HT \text{ or } DT}$, which are the intrinsic KIEs comparing the rates of H to T or D to T, respectively. This method allows for the assessment of intrinsic KIEs by a mathematical approach that ‘cancels out’ the contribution of all the steps except the isotopically sensitive one under examination:

$$\frac{1/KIE_{obs}^{HT} - 1}{1/KIE_{obs}^{DT} - 1} = \frac{1/KIE_{int}^{HT} - 1}{1/(KIE_{int}^{HT})^{0.3} - 1}, \quad (1)$$

an equation with only one unknown, KIE_{int}^{HT} , because the KIE_{obs}^{HT} and KIE_{obs}^{DT} values are found out with the two experimental competitive one-pot observed KIE measurements.

The temperature dependence of the KIEs reflects the dynamic behavior of the reaction’s TS. Dividing Arrhenius equations for the H and T isotopologues yields

$$KIE_{int}^{HT} = \frac{k_H}{k_T} = \frac{A_H}{A_T} \exp \frac{\Delta E_{a,T-H}}{RT} \quad (2)$$

and allows us to generate a temperature-dependency plot of intrinsic KIE value vs. $1/T$, with the y-intercept expressing the isotope effect on the Arrhenius pre-exponential factor $\left(\frac{A_H}{A_T}\right)$

and the slope expressing the isotope effect on the activation energy,

$\Delta E_{a,T-H} = E_{a,T} - E_{a,H}$. To assess $\Delta E_{a,T-H}$, the intrinsic KIEs are fitted via non-linear regression to eqn 2. Intrinsic KIEs and their temperature dependence are a useful tool to probe how well-organized is the TS of the H-transfer in question. Temperature-independent KIEs reflect a precisely structured TS, while temperature-dependent KIEs indicate a loosely-held TS with low frequency sampling of the donor acceptor distance (DAD).²⁷ A phenomenological explanation called the activated tunneling model that implicates quantum mechanical behavior of hydrogen in these enzymatic systems has been developed to relate the temperature dependency profiles of intrinsic KIEs to the TS structure for the chemical step being examined.^{28–29} Most wild-type enzymes with their natural substrates have been experimentally seen to have a small or zero $\Delta E_{a,T-H}$,²⁷ reflecting a tight, high-frequency DAD sampling in the run-up to the TS.¹⁷ A large $\Delta E_{a,T-H}$, on the other hand, reflects a broad distribution of DAD with low sampling frequency, typical to mutants that disrupt the TS of the step under study.

Proton abstraction kinetic isotope effects.

The reaction mixtures had final concentrations equal to 100 mM tris(hydroxymethyl)aminomethane, 1 mM TCEP (antioxidant to keep cysteines, including the key residue performing the Michael addition, reduced), 50 mM MgCl₂ and 7 mM HCHO (formaldehyde, to stabilize MTHF). The process was very much in keeping with that previously reported for the WT and other mutants.^{3,30} A pre-mix of [5-³H]-dUMP and [2-¹⁴C]-dUMP was made with a ratio of ³H: ¹⁴C in the 5 and 9 range; higher radioactivity of tritium compensates for the lower average energy of β particles from ³H than ¹⁴C. The ratio chosen preserves the accuracy of LSC counting. A low [dUMP] was maintained at around 5–10 μ M, and was always the limiting reagent. The other substrate's concentration – that of MTHF, which is *not* radiolabeled in proton abstraction KIE studies – was different in each of the four experiments. Hydrochloric acid or sodium hydroxide was used to bring the pH to 7.5 while maintaining everything in a 25° C water bath, with a micro pH probe inserted into an eppendorf containing the reaction mixture of 500 μ L. Each t_0 was created by taking out ~30 – 40 μ L of the reaction mixture – which amounted to around 150 kdpn ³H and 20 kdpn ¹⁴C – and flash-freezing it in liquid nitrogen, followed by placement into the –80° C freezer until HPLC analysis. Enzyme was pre-diluted in 100 mM tris pH 7.5 to ten times higher than the final desired concentration; the latter was chosen so that the reaction progress would be well over 20% conversion within well under one hour. The volume of the enzyme solution added would be set to make about ~10% of the total volume of material. This addition of enzyme, with prompt thorough mixing by pipette, coincided with the

starting of a timer. For each time point, the process was pipetting the reaction mixture - always kept in the water bath - up and down and then withdrawing ~ 40 μ L of solution and ejecting it into a pre-chilled 0.6 mL eppendorf containing 10 μ L of 5-fluoro dUMP inhibitor to a final concentration of at least ten times the initial concentration of dUMP. The 0.6 mL eppendorff would be vortexed immediately and frozen in liquid nitrogen. Enzyme concentrations and aliquot withdrawal times corresponding to 20–80% fraction conversion (f), as assessed by ^{14}C radioactivity, were selected in all cases:

$$f = 100\% \cdot \frac{{}^{14}\text{C} - d\text{TMP}}{{}^{14}\text{C} - d\text{UMP} + {}^{14}\text{C} - d\text{TMP}}, \quad (3)$$

a value determined upon HPLC analysis. There would be typically eight to ten time points taken; after that, a large concentration of WT *E. coli* TSase would be added to push the reaction entirely to completion after 30–45 min of incubation. This was to guarantee that no starting materials remained in the infinity points used for R_{inf} determination. The dpm values for tritium in water - corrected for initial tritium in water at t_0 - divided by dpm values for ^{14}C in dTMP,

$$R = \frac{\text{corr. } {}^3\text{H in water}}{{}^{14}\text{C in dTMP}}, \quad (4)$$

at time t (R_t) and infinity (R_{inf}) were computed; there were at least four time points and two infinities per concentration of MTHF. The observed KIE was calculated from

$$\text{KIE}_{obs} = \frac{\ln(1-f)}{\ln\left(1-f\frac{R_t}{R_{inf}}\right)} \cdot {}^{31} \quad (5)$$

These observed KIEs determined in one-pot competitive measurements are the ratio of the catalytic efficiencies $\left(\frac{k_{cat}}{K_m}\right)$ for the light isotopologue of the substrate over the heavy isotopologue of the substrate.

Hydride transfer kinetic isotope effects:

There were two isotopic comparisons conducted, each in a one-pot competitive approach. MTHF was radiolabeled along with dUMP; MTHF was labeled with H/T or D/T at its C6_{fol} position. For fraction conversion determination, dUMP was remotely labeled with ^{14}C at C2 of the pyrimidine ring. The Northrop method was then applied to get the intrinsic KIEs, which are the actual rate constant ratios between isotopologues for the hydride transfer step alone. Buffer components included 100 mM tris pH 7.5, with 1 mM TCEP to avoid disulfide formation, 50 mM MgCl_2 , and 7 mM HCHO to keep MTHF stable. This was exactly the

same method used previously for the WT and other mutants. This first isotopic comparison was to get H vs. T observed KIEs and involved mixtures of $(R) - 6 - [^3H] - MTHF + (R) - 6 - [^1H] MTHF + 2 - [^{14}C]dUMP$, while the second isotopic comparison was to get D vs. T observed KIEs and involved mixtures of $(R) - 6 - [^3H] - MTHF + (R) - 6 - [^2H] MTHF + 2 - [^{14}C] - dUMP$. To ensure reproducibility for the counting of the lower-energy β particles of tritium, the ratio of $^3H: ^{14}C$ was between 4 and 9 in the original reaction mixtures. The total [MTHF] was 80–120 μM ; this was the limiting reagent in all hydride transfer work. On the other hand, the total [dUMP] - present in excess in all cases - was 100–150 μM . It was crucial to have MTHF as the limiting reagent here, and to verify this, a small amount of the final reaction mixture would be incubated with WT TSase until the reaction was over, and then HPLC coupled with radioactive flow detector use, or fraction collection and LSC counting, would confirm the desired relationship between reactants. The % excess of dUMP over MTHF was usually ~30 – 40%. The pH of each mixture was adjusted at the temperature in question (5° C, 15° C, 25° C, 35° C). Like in the proton abstraction situation, the reaction mixture would be in an eppendorf in a water bath set to the desired temperature, and a pH micro electrode probe, calibrated at the working temperature, would be used to gauge pH; adjustments would be achieved by addition of several μL of hydrochloric acid or sodium hydroxide. The final volume of the reaction mixture was 500 μL . A portion of the reaction mixture, normally 30–40 μL , with circa 150 kdpn 3H and 20 kdpn ^{14}C in it, would be withdrawn as a t_0 .

Enzyme was separately pre-diluted in 100 mM tris pH 7.5 to ten times higher than the final desired concentration; that final concentration was picked out so that the reaction progress would be well over 20% normalized conversion within the first hour. The volume of the enzyme solution added would be set to make about ~10% of the total volume of material. This addition of enzyme, with prompt thorough mixing by pipette, coincided with the starting of a timer. For each time point, the process was pipetting the reaction mixture – always kept in the water bath – up and down and then withdrawing ~40 μL of solution and ejecting it into a pre chilled 0.6 mL eppendorf containing 10 μL of 5-fluoro dUMP inhibitor to a final concentration of at least ten times the initial concentration of dUMP. The 0.6 mL eppendorff would be vortexed immediately and frozen in liquid nitrogen. Enzyme concentrations and aliquot withdrawal times were selected so as to obtain 20–80% normalized fraction conversion (f_{nml}). The final enzyme concentrations were typically 1–10 μM . The same eqn. 3 applicable for proton abstraction is applied to hydride transfer as well for determination of f ; the difference is that here, at the end of the reaction, we have not consumed all the dUMP, so we have an f_{inf} value, circa 70–80% based on a dUMP excess of 30–40% mentioned above, and $f_{nml} = 100\% \cdot \frac{f_{raw}}{f_{inf}}$. Approximately ten portions would be withdrawn over the course of the reaction (time points); then, to guarantee that the reaction proceeded to its endpoint, WT *E. coli* TSase was added, and the mixture was incubated an additional 30–45 min at 25° C to ensure that the reaction went to completion (these were the infinity points). Ratios of tritium in dTMP to ^{14}C in dTMP,

$$R = \frac{{}^3\text{H in } d\text{TMP}}{{}^{14}\text{C in } d\text{TMP}} \quad (6)$$

at time t (R_t) and infinity (R_{inf}) were computed, with at least four time points and three infinities per temperature. The observed KIEs, utilizing f_{nml} values, were calculated from eqn. 5.³¹ Thus, there were observed KIEs for two isotopic comparisons at each temperature. To assess the intrinsic KIEs (KIE_{int}) all pairings of H/T and D/TKIE_{obs} at each temperature were plugged into the Northrop equation (eqn. 1).²⁶ The KIE_{int}^{HT} values were obtained via numeric solution using a Mathematica program script developed by Roston et al.²⁹ A plot of KIE_{int}^{HT}/T was generated and its parameters were calculated from non-linear regression to the Arrhenius equation as described above. All the KIE_{int}^{HT} values were used for fittings, but only averages and standard deviations are displayed in graphs. Forward commitments to catalysis, C_f – defined as the rate constant for the isotopically sensitive forward step over the net rate constant for isotopically insensitive backward steps³¹ – were analyzed from

$$C_f = \frac{KIE_{int} - KIE_{obs}}{KIE_{obs} - 1}. \quad (7)$$

Computational QM/MM simulations:

The theoretical calculations, initiated with the structure of *Escherichia coli* TSase bound to 5-fluoro-dUMP (FdUMP) and CH₂H₄folate (PDB code 1TSN),¹⁴ were focused in the N177D mutant because this was the most dramatic mutation of the enzyme, as demonstrated within the experimental data shown below. The results of the reaction catalyzed by the wild-type are reported in ref.¹² After preparing the protein and neutralizing the system with counterions, the system was solvated using a box of water molecules of $100 \times 80 \times 80 \text{ \AA}^3$.

The whole system, protein, folate and dUMP solvated in a box of water molecules of $100 \times 80 \times 80 \text{ \AA}^3$ (see Supporting Information for details), was divided into a QM part and a MM part to perform the hybrid QM/MM simulations. The QM part contains 25 atoms of the folate, 21 atoms of the dUMP and the side chain of Cys146, which gives a total of 54 QM atoms. In order to satisfy the valence of the QM-MM frontier atoms, three hydrogen link atoms³² were inserted at the boundary between QM and MM regions. In the study of the tautomerization of the N177D mutant, the side chain of the Asp177 residue was also included in the QM region (7 atoms), and a new link atom was added (see Figure S1 in the Supporting Information). Only one active site of the full protein was treated quantum mechanically, leaving the second active site (ligands removed) in the MM region. The folate and dUMP, the rest of the enzyme, the water molecules and the sodium counterions were treated by molecular mechanics force fields (60826 atoms).

The QM sub-set of atoms were treated by the standard semiempirical method RM1,³³ in all the QM/MM energy optimizations and in the molecular dynamics (MD) trajectories, which has been recently shown to provide good performance in QM/MM studies of enzyme catalyzed hydride transfer reactions,³⁴ including TSase.¹² The force fields for the rest of the system, protein and water molecules, were OPLS-AA³⁵ and TIP3P³⁶, respectively, as implemented in the fDYNAMO library. It is important to point out that, as shown by Kaiyawet et al. in a computational study of this system,³⁷ apparently classical force fields can present some limitations for reproducing energies for distortion of the guanidinium side chain of the catalytic arginine residue correctly. Nevertheless, these can be minimized when comparing wild-type¹² and mutants, or when comparing the results at different temperatures. In fact, as described below, our results reproduce the same concerted molecular mechanism as the one obtained by Kaiyawet et al. In fact, as described below, our results reproduce the same concerted molecular mechanism as the one obtained by Kaiyawet et al. when including this residue in the QM region. After setting up and equilibration of the model, QM/MM potential energy surfaces, PESs, were generated for the study of the tautomer equilibrium in compound E, as well as for exploring the hydride transfer step of the TSase catalyzed reaction in the N177D mutant. Then, free energy surfaces, in terms of Potentials of Mean Force, PMFs, were performed using structures from the PESs as starting points of each window. Once the PMFs were computed, in order to get accurate results of rate constants and KIEs for the hydride transfer step in N177D, the Variational Transition State Theory (VTST)³⁸ has been applied, which amends TST limitations such as the recrossing trajectories and the quantum tunneling effects^{38–40}:

$$k(T) = \Gamma(T, \xi) \frac{k_B T}{h} e^{-\left(\frac{\Delta G_{act}^{QC}(T, \xi)}{RT}\right)} \quad (8)$$

where R is the ideal gas constant, T is the temperature, k_B is the Boltzmann constant, h is Planck's constant and ΔG_{act}^{QC} is the quasiclassical activation free energy calculated along the reaction coordinate ξ . In equation 1, $\Gamma(T, \xi)$ is the temperature-dependent transmission coefficient that contains the recrossing transmission coefficient, $\gamma(T, \xi)$, and the tunneling corrections, $\kappa(T)$, to the TST rate constant:

$$\Gamma(T, \xi) = \gamma(T, \xi) \kappa(T) \quad (9)$$

The recrossing transmission coefficient $\gamma(T, \xi)$ was computed by means of the Grote-Hynes theory as described in previous papers.⁴¹ The tunneling corrections, $\kappa(T)$, were computed by means of the VTST, where the zero-point energy for each mode of the quantum region atoms was obtained by evaluating an ensemble average over primary subsystems and making a quasiharmonic approximation. To perform these calculations, 10 TS structures were localized from the corresponding simulation windows. The reactant structures were localized by running intrinsic reaction coordinate (IRC) calculations. The Hessian matrix for

all of the stationary structures were computed. An average over all the structures was computed to get the final quantum mechanical corrections.

Computed Kinetic Isotope Effects.

KIEs were computed for isotopic substitutions of the hydride transfer from the TS and the reactant complex. As described in previous papers,^{12,34,42} KIEs were estimated based on the total partition functions computed as averages of all possible combinations from 10 optimized structures of TS and 10 optimized structures of reactant state at RM1/MM level. In addition, structures of TS and reactants were optimized at M06-2X hybrid density functional theory (DFT) functional⁴³ in combination with the MM force fields with an iterative micro-macro procedure,⁴⁴ to obtain KIEs at higher level. In all, the total KIE is expressed as:

$$KIE = KIE^{QC} \times \frac{\gamma_L}{\gamma_H} \times \frac{\kappa_L}{\kappa_H} = KIE^{QC} \times KIE^{\gamma} \times KIE^{\kappa} \quad (10)$$

The different terms appearing in equation 10 are the quasi-classical, KIE^{QC} , the recrossing, KIE^{γ} , and the quantum tunneling, KIE^{κ} , contributions to the total KIE.

RESULTS AND DISCUSSION

Circular Dichroism (CD) analysis.

To determine if the mutations at N177 causes major structural changes to the enzyme's secondary motifs, CD measurements were conducted for all three forms of the enzyme. All three enzymes exhibit similar circular dichroism spectra (Figure S2) with the typical α -helical signature of local minima at ~222nm nm and ~208 nm⁴⁵ present – which is as anticipated, since TSase is to a large extent α -helical. The similarities do not indicate any significant change in secondary structure between the three enzymes. Additionally, analysis of many crystal structures of other *E. coli* TSase mutants demonstrated that changes in the threedimensional structure of the protein backbone are rare, further supporting the possibility that the mutations did not significantly alter the enzyme's structure.

Effect of mutations on steady-state kinetic parameters.

A comparison of the mutants' Michaelis-Menten parameters in the presence of Mg^{2+} with those for WT is arrayed in Table 1, and representative plots are displayed in Figure S3(a-b). While substrate inhibition by MTHF has been reported for the WT TSase,^{1, 4} for the N177D and N177S mutants it is only observed at MTHF concentration above 1.5 mM. Therefore, analyses focus on lower concentrations where no substrate inhibition is observed. The data in Table 1 suggest that the reduction of the turnover number k_{cat} for N177D – by three orders of magnitude – as compared with wild-type is more dramatic than that for N177S – which is reduced by two orders of magnitude. On the other hand, both $K_{m,dUMP}$ and $K_{m,MTHF}$ are significantly larger for N177S than for N177D. These finding suggest that while N177D impairs the catalytic turnover number, it has a lesser effect on the Michaelis

constants of both reactants. Previously reported kinetic parameters obtained for the N177D mutant, which were obtained in different conditions,⁴⁶ are in qualitative alignment with our data. These findings accord well with previous reports that all substitutions – being they at N177 position of *E. coli* or at the corresponding N229 of *L. casei* TSase – reduce the turnover number, while the effect on the Michaelis constants of substrates varies depending on the side chain of the substitutions.^{46–47,23, 48}

The WT's N177 side chain seems to serve as a hydrogen bonding partner in six-membered ring fashion for N3 and O4 of the dUMP substrate.²² The mutants studied here likely alter stabilization of the C4=O4 carbonyl along the catalytic cycle (thus the reduction in k_{cat}) and also have some effect on the K_m for dUMP. Michaelis constants sometimes report in part on reactant binding. The changes in K_m values for N177S are more drastic. This is consistent with weakened hydrogen bonding interactions brought about by the shortened serine side chain. In regards to the catalytic efficiencies (k_{cat}/K_m), N177D and N177S have a similar impact on dUMP consumption, but N177D has a more substantial effect on MTHF consumption than N177S does.

Effect of mutations on substrate binding order.

It has been previously established that wild-type *E. coli* TSase exhibits an ordered substrate binding mechanism in which binding of dUMP is followed by MTHF.^{1, 3, 30, 50} Analysis of this pattern could imply conformational differences in binding, thereby foreshadowing alteration of the hydride transfer TS. One sensitive experiment to test the substrate binding order is the observed proton abstraction KIE for [5- H]-dUMP as a function of MTHF concentration. In a strictly ordered binding mechanism a high concentration of MTHF as the second-binding substrate leads to high forward commitment (C_f in eqn. 7) and no observed KIE ($KIE_{\text{obs}} = 1$).^{3, 30} In the random-binding scenario, however, the first substrate can dissociate from the ternary (EdUMPMTHF) complex regardless of the concentration of the MTHF, the C_f at high MTHF goes to a finite value and the KIE_{obs} also goes to a finite value (not unity). This is further discussed in the SI.

Figure 2 presents the proton abstraction H/T KIE_{obs} plotted against [MTHF]. An apparent plateau at $KIE_{\text{obs}} = 1$ appears to be reached for both WT³⁰ and N177S TSase. However, N177D appears to reach a plateau at $KIE_{\text{obs}} > 1$, suggesting that N177D exhibits less ordered substrate binding than WT and N177S TSases. The observed differences in binding mechanisms between N177D and N177S may relate to the altered enzyme-substrate hydrogen bonding networks seen in the crystal structure of the N177A mutant.

Intrinsic hydride transfer KIEs (KIE_{int}).

Figure 3a presents the Arrhenius plots of KIE_{int} vs. $1/T$ for the N177D, N177S and WT enzymes while the numeric values of the observed and intrinsic KIEs are presented with their experimental errors in Tables S1-S3 and discussed below. The parameters of the non-linear regression are the isotope effects on the Arrhenius pre-exponential factors and activation energies, and are summarized in Table 2.

The y-intercept in Figure 3a is the isotope effect on the pre-exponential Arrhenius factor A (A_H/A_T in Table 2). While the interpretation of that isotope effect is complex,⁵¹ it is more

similar to the WT in N177S than N177D, suggesting that mutation to S, while geometrically more dramatic, has a lesser effect on the TS of the hydride transfer step.⁵² More insight can be gleaned from analysis of the slope of the KIE_{int} , as reflected by $\Delta E_{a,T-H} = E_{a,T} - E_{a,H}$. As is typical for WT enzymes with their usual substrates, $\Delta E_{a,T-H}$ for the WT TSase is essentially zero.⁴⁹ However, for both mutants $\Delta E_{a,T-H}$ is significantly larger, and more so for N177D than N177S (Table 2). In light of the “Activated Tunneling” model described in Materials & Methods,^{27–29, 53} the larger $\Delta E_{a,T-H}$ in mutants indicates that in the WT enzyme N177 could be part of the reaction coordinate for the hydride transfer step. The trend in $\Delta E_{a,T-H}$ (N177D>N177S>N177) again indicates that the disruption to the DAD sampling at the TS of N177D TSase (while geometrically similar to that for N177) is far greater than that of N177S (where the side chain is truncated). It is worth noting that $\Delta E_{a,T-H} = 2.4 \pm 0.2$ for N177D is still somewhat smaller than the values obtained for other ecTSase mutants. As a comparison, for R166K – a mutant postulated to impair the polarization and motion of the cysteine that performs the Michael additions in the mechanism – $\Delta E_{a,T-H} = 3.6 \pm 0.1$ for hydride transfer.⁹ Thus, even though N177 may not be as integral to this reaction as R166, the N177 fulfills a major auxiliary role.

In addition to the Arrhenius analysis (leading to A_H/A_T and $\Delta E_{a,T-H}$), the same KIE_{int} data can also be analyzed by a model developed by Roston and co-workers.²⁹ Beginning

from the equation $KIE = \frac{k_{light}}{k_{heavy}} = \frac{\int_0^\infty P^{light}(DAD)e^{-E(DAD)/(k_B T)} dDAD}{\int_0^\infty P^{heavy}(DAD)e^{-E(DAD)/(k_B T)} dDAD}$, for temperature-

Independent results, both the transmission probability at each DAD and the Boltzmann factors can be expressed as a function of the force constant of DAD sampling and of the average DAD. Fitting to experimental data yielded a best-fit force constant and average DAD. For temperature-dependent results, Roston and co-workers proposed a bimodal distribution, expressed the transmission probability and Boltzmann factors as a function of the average DAD for the longer population and the Gibbs free energy difference between the populations. Least-squares fitting yielded average DAD values for the longer population and G between the two populations. This model predicts averages of DAD distributions with a unimodal distribution for $\Delta E_{a,T-H} < 1$ kcal/mol and a bimodal distribution for

$\Delta E_{a,T-H} > 1$ kcal/mol.²⁹ In the bimodal case, there would be a small share of the population in which the DAD is so short as to provide all isotopologues with more energy than the barrier height; for this population, there is no tunneling. The remainder of the active sites belong in the longer-DAD population, in which the product formation is essentially only through tunneling.²⁹ In agreement with the discussion of the Arrhenius analysis, Table 3 suggests that N177 mutations alter the DAD sampling for the hydride transfer step in WT TSase, and that the disruptive effect of D is greater than that of S.

The disruption of the optimal alignment of substrates, water molecules, and side chains in the active site at the TRS (or tunneling-ready state) in N177D and N177S indicated by inflated $\Delta E_{a,T-H}$ accords well with negative charge accumulation at carbonyl 4 of the

nucleotide at the TS of the hydride transfer. If N177D were negatively charged, then negative charge accumulation at the carbonyl oxygen could result in electrostatic repulsion and, consequently, longer DADs with a broader DAD sampling range (i.e., poorly organized TS). However, as discussed below, our computational prediction of the pK_a value of residue 177, indicates that N177D is neutral, yet has altered hydrogen bonding strengths and tautomeric equilibria, thereby predicting the same poorly organized TS but from different arguments. One interpretation consistent with the moderate $\Delta E_{a,T-H}$ for N177S as opposed to the higher $\Delta E_{a,T-H}$ for N177D is that the smaller side chain in the former allows active site residues (e.g., H147, E58, etc.) and active site water to rearrange and compensate (in part) for the H-bond perturbation. Apparently, such compensation is not possible with the N177D sidechain, which fills the same space as the N177. Indeed, in the crystal structure of the N177A mutant with dUMP and an analog of MTHF, a rearrangement of active-site water molecules and a striking repositioning of the imidazole ring of H147 is apparent (Figure 1b). Additional support to this hypothesis is also provided from the geometrical analysis of the equilibrated structures derived from the QM/MM MD simulations (see details in the Supporting Information). That rearrangement appears to form a hydrogen bond to O4 to substitute for the one lost because of the replacement of asparagine with alanine.²³ For the WT and two mutants studied here, the turnover number and $\Delta E_{a,T-H}$ appear to be correlated according to an Arrhenius relationship, with the points representing $\Delta E_{a,T-H}$ graphed against the logarithm of k_{cat} falling along a line (Figure 4). It thus appears there is a link between the hydride transfer TS and the overall enzyme performance.

N177D pK_a prediction.

Some experimental evidences suggest that D177 is deprotonated. First, the most straightforward way to explain a dramatic decrease in k_{cat} by three orders of magnitude for N177D relative to WT is by a drastic change in the characteristics of the active site. The simplest rationale is that the negatively charged carboxylate electrostatically repels the partial negative charge on the C5-C4=O4 enolate, slowing down the turnover. Also, the preference for dUMP to bind first is altered; this, too, suggests a significant change in the favorability of the contacts dUMP makes.

On the other hand, Hardy *et al.*⁴⁷ and Santi *et al.*⁴⁸ speculated that the N177D side chain carboxylic acid may be protonated at pH 7.4 in the mutant's resting state. Although the expected pK_a of aliphatic carboxylic acids is typically $\approx 4 - 5$, enzyme active sites offer a microenvironment in which pK_as can be shifted.¹⁶ The PROPKA ver. 2 package, which relies on X-ray crystal structural information as the basis for determining environmental modulation of pK_a values,⁵⁴ anticipates a substantially elevated pK_a of $\sim 9.0 - 9.5$ for the D177 side chain. Our computational work, based on the generation of the free energy surface (2D-PMF) for the tautomer equilibrium as shown in Figure 5, indeed implies that in the ground state for hydride transfer, compound E, the protonated and neutral D177 residue stabilizes the uracil ring of the nucleotide by hydrogen bond interactions with O4 and the protonated N3. This information could be consonant with the suggestion by Hardy *et al.* of the formation of an intermediate in the N177D system that "may be too stable." After hydride transfer, the resulting C5-O4 enolate could be intercepted by protonation of O4 by

the carboxylic acid of N177D into the enol before the cysteine thiolate is eliminated. This enol could be the intermediate suggested by Hardy *et al.* Moreover, if this protonation is also present in the apo enzyme, this could account for the reasonably low $K_{m,dUMP}$ for N177D. If D177 is protonated, this is a reasonable finding because protonated D177 can have a six-membered ring hydrogen bonding pattern with the N3 and O4 of the nucleotide as is the case in wild-type (see Figure 1 (a)). If D177 was negatively charged, then such a six-membered ring hydrogen bonding pattern cannot take place because D177 has no hydrogen bond to donate to O4 of the nucleotide. In summary, although arguments exist for both protonation states, prior proposals and the computational studies herein suggest that is more likely that D177 is protonated prior to hydride transfer. The computational study of the hydride transfer from this intermediate in N177D, confirming the predictions of its effect on the rate constant of the mutant, is presented below.

Tautomer analysis.

The tautomeric form of the enzyme-bound exocyclic methylene hydride acceptor could influence the nature of the TS for hydride transfer. However, it is challenging to experimentally assess the most prevalent tautomeric form for this hydride acceptor. The pKa of the proton at N3 of dUMP was experimentally determined to be $\sim 9.0 - 9.5$.⁵⁵ The hydride acceptor is likely to have this proton still present. This prediction is confirmed with the QM/MM free energy surface shown in Figure 5 that explores the equilibrium between N3(H) and O4(H) tautomers. As seen in the figure, just like WT, the N177D mutant strongly favors the N3(H) tautomer over the O4(H) (T1 is much more stable than T2). Interestingly, an eventual accumulation of negative charge in O4 during the reaction can be stabilized in this tautomer by the hydrogen bond interaction with D177.

Computational temperature dependency of KIEs:

The hydride transfer process and its KIEs have been previously examined for WT TSase.^{4, 6, 10, 12, 49, 56} Tunneling has been implicated as a major contributor in the KIEs of hydride transfer process,¹⁰ supporting the interpretation of experimental studies presented above. Although the use of an alternative crystal structure implied a moderate extent of stepwise character for the path from E to dTMP,⁵⁶ most analyses suggested a one-step process, but with a higher degree of hydride association with C7 of the incipient dTMP than the degree of C6-S_{Cys} dissociation.^{6, 10, 12, 56} Computationally determined KIEs at the M06-2X:RM1/MM level were temperature-independent for WT.¹² Here, the same methodology was used to examine the hydride transfer for N177D TSase. According to the tautomer analysis presented above, the T1 tautomer of the exocyclic methylene (with unprotonated O4 and protonated N3) was used as starting point for studying the hydride transfer step and KIEs at 5, 15, 25 and 35 °C in N177D. The computational results, plotted on Figure 3b, buttress the conclusion derived from our experimental kinetic studies that for N177D, hydride transfer is noticeably, significantly temperature-dependent, while it is essentially temperature-independent for WT. In order to analyze the origin of this temperature dependency for the N177D mutant, the total KIEs have been decomposed into the different contributions coming from the quasiclassical, recrossing and tunneling terms (see eq. 10). As derived from the results reported in Table 4, while the quasiclassical and recrossing terms are temperature-independent, the tunneling term appears to be significantly dependent

on the temperature, thus being responsible for the observed trend. This was not observed in the wild type enzyme (see Table 4), where the variations of the tunneling term was much less significant and, in any case, was compensated by the trends on the other two terms.

In an attempt to carry out a deeper analysis of the temperature variations of the KIE_K and KIE_γ terms, the recrossing and tunneling transmission coefficients for the protium transfer are listed in Table 5, where the average DADs measured from the structures of the TSs and the quasi-classical activation free energies are also provided (temperature dependence of transmission coefficients for the tritium transfer are deposited in Tables S5 and S6). Representative snapshots of the TS structures located on the wild type and on the N177D mutant are shown in Figure 6. The first conclusion that can be deduced from the information reported in Table 5 is that the free energy of activation on the N177D is significantly higher than the values computed for the wild type (see refs^{6, 12}). This result is in agreement with the experimental data reported in the present study, where the turnover k_{cat} decreases up to three orders of magnitude after the N177D mutation (see Table 1). It is important to point out that the experimental turnover rate constants do not correspond to the intrinsic hydride transfer step and, consequently, a direct comparison between theory and experiments is not possible in this regard (although the intrinsic KIE values can be compared directly). In addition, a note of caution must be introduced at this point since, despite the final values are corrected at DFT/MM level, the free energies of activation are computed based on original RM1/MM geometry sampling and, thus, a certain degree of error can be introduced in the calculations. Nevertheless, this effect, which is not expected to be dramatic, would affect calculations of the wild type and mutants at all temperatures making credible the comparison. In fact, the computationally predicted trends when comparing wild-type and mutants, are in good agreement with the experiments. The inspection of the structures shown in Figure 6 show no dramatic differences between the TSs of WT and N177D. In contrast, some differences appear when the preceding intermediates are compared, which can be responsible of an stabilization of the enzyme-bound exocyclic methylene hydride acceptor in a less reactive conformation. This is also in agreement with the experimental evidences and a suggestion by Hardy *et al.*^{46–47} The D177 residue strongly interacts with the proton of N3 and the O4 atoms of the uracil ring of the nucleotide in the reactants state of the hydride transfer step, rendering the DADs larger in the mutant and, consequently, higher energies are required to overcome the TS. As observed in Figure 6, these interactions are kept along the reaction coordinate, thus perturbing also the TS which is reflected in a slightly larger DAD in the mutant at 278 K. The DAD measured on the TS structures for the hydride transfer on the N177D TSase reported in Table 5 does not show any temperature dependence. The computed DAD values in the TSs, ranging from 2.74 to 2.77 Å, are slightly smaller than those deduced from the model of ref²⁹ (see Table 3). Nevertheless, the values of Table 5 and Figure 6 follow the same trend as in Table 3.

The values of the recrossing transmission coefficients, ranging between 0.477 and 0.555, do not evidence any temperature dependence and their magnitudes suggest low dynamic effects into the chemical reaction step with a contribution to the effective free energy barrier between 0.3–0.4 kcalmol⁻¹. Interestingly, these values are slightly lower than those previously computed for the WT, that were ranging between 0.53 and 0.60,¹² which can be

interpreted as the higher impact of the environment on the real reaction coordinate in the N177D mutant than in the WT, in agreement with the predictions previously proposed based on the “Activated Tunneling” model. On the contrary, the quantum tunneling effects are reduced with the temperature, which is in agreement with our previous study on the WT TSase¹² and with the small temperature dependence observed for the hydride transfer reaction catalyzed by *E. coli* dihydrofolate reductase reported by Truhlar and co-workers.⁵⁷ The smaller values of the tunneling transmission coefficients obtained for the N177D, by comparison with previous values obtained for the WT,¹² also show signs of a disruption of the active site that reduced the contribution of tunneling to the overall effective activation free energy.

In all, according to our computational results, the lower activity of the N177D mutant, by comparison with the WT enzyme, must be associated with an overstabilization of the reactant state that is trapped in a less reactive conformation, together with a slightly effect on the TS. It appears that the strong interaction with residue D177 provokes that the reactants state is stabilized at values of the reaction coordinate further away from the TS. As observed in Figure 6, the bond between C6 and the S atom of Cys146 is almost unperturbed in any of the TSs structures. This, together with the fact that the C6-S(Cys146) bond is broken in the products state located on the PMFs, confirms the asynchronous but concerted character of the hydride transfer and the C6-S(Cys146) bond scission, as suggested in our previous computational studies.^{6, 10, 12}

CONCLUSIONS

The studies herein dissect the role of the asparagine 177 (N177) residue in *E. coli* TSase. Because recent reassessment of the TSase mechanism seeks to determine whether an enolate form of the C4=O4 carbonyl of the nucleotide exists in the lead-up to the crucial hydride transfer step, mutational study of this residue was undertaken. Mutations to both aspartic acid (D) and serine (S) residues were performed, to potentially change charge and size of the residue. We found that steady-state parameters were disrupted very heavily in N177D TSase but somewhat less so in N177S TSase. Overall, the chemistry in *E. coli* TSase is impaired more in N177D, while Michaelis constants are higher, i.e. less favorable, in N177S. N177S appears to have ordered substrate binding as in WT, while N177D has partially randomized substrate binding preference.

Temperature dependency study of intrinsic KIEs – both experimentally and computationally – revealed that organization of active-site architecture in preparation for hydride transfer is impaired noticeably in N177D and more moderately in N177S ecTSase. The reduction in steady-state turnover (k_{cat}) corresponded to an increase in the isotope effect and the activation energies in the WT and the two mutants of N177 of the TSase. This shows that a slowdown of the turnover number can be traced back to a disruption in the preparation of the active site for hydride transfer.

These data could potentially be explained by repulsion between an anionic N177D carboxylate and partially negatively charged enolate O4. However, our computational efforts suggested that D177 is still protonated and overstabilizes an enzyme-bound hydride transfer

intermediate. As for N177S, our evidence suggests that it partially compensates for the mutation by adjust hydrogen-bonding and minimizing its disruption. It is thus likely that, despite the reevaluation of the TSase mechanism – there is partial enolate character in the C4=O4 carbonyl in the approach to hydride transfer and that N177 is relevant part of the reaction coordinate for that step.

The information reported in this study provides information for design of mechanism- based drugs targeting this crucial enzyme involved in severe human diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the USA National Institute of Health (Ref No. NIH R01 GM065368), the Spanish Ministerio de Economía y Competitividad and FEDER funds (project CTQ2015–66223-C2) and Universitat Jaume I (project UJIB2017–31). KS thanks the Spanish Ministerio de Economía y Competitividad for a Juan de la Cierva – Incorporación (ref. IJCI- 2016–27503) contract. Authors acknowledge computational resources from the Servei d'informàtica of Universitat Jaume I.

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Figure 1.

(a) WT *E. coli* TSase in a ternary complex with dUMP and folate analog CB3717 (Cys 146 is covalently attached to the pyrimidine ring; CB3717 is omitted for clarity). This view emphasizes hydrogen bonding around O4. PDB ID 1TRG.²² (b) N177A *E. coli* TSase in a ternary complex with dUMP and folate analog CB3717 (Cys 146 is covalently attached to the pyrimidine ring; CB3717 is omitted for clarity). The structure shows rearrangement of hydrogen bonding around O4 as compared with WT. PDB ID 1BQ1.²³

Figure 2. Proton abstraction KIE_{obs} vs. [MTHF] for WT (green),³⁰ N177S (purple), and N177D (red). (a) logarithmic scale of x-axis; (b) linear scale of x-axis. The curves, plotted solely to guide the eye, are from fitting to the ordered binding equation for N177S and WT and to the random binding equation for N177D.³

Figure 3.

Arrhenius plot of H/T intrinsic KIE for hydride transfer for WT (green),⁴⁹ N177S (purple), and N177D (red) derived from experimental measurements (a), and from QM/MM calculations (b). The values of the wild type on panel b (in green) are from ref. 12.

Figure 4.

An apparent correlation between steady-state turnover number (k_{cat}) and isotope effect on activation energies of the hydride transfer in WT,⁴⁹ N177S and N177D. The blue line of best fit shows that differences in hydride transfer activation energies are related directly to logarithms of the first-order rate constant k_{cat} . Rate constants of hydride transfer itself play into determining the overall k_{cat} .

Figure 5.

QM/MM free energy surface, computed as a 2D PMF at RM1/MM level at 298 K, for the tautomeric equilibrium between T1 and T2 of the N177D enzyme-bound exocyclic methylene hydride acceptor. The two reaction coordinates (in Å) correspond to the anti-symmetric combination of distances defining the position of the hydrogen (HD2) between the donor, O4, and the acceptor OD2 atom of D177, Y-axis, and the anti-symmetric combination of distances defining the position of the hydrogen (H3) between the donor O atom of D177 (OD1) and the N3 acceptor atom, X-axis. Energies of isoenergetic lines are in kJmol^{-1} .

Figure 6. Representative snapshots of the reactants states and TS for the hydride transfer on the wild type and N177D TSase obtained at 278 K. Distances are provided in Å.

Scheme 1. Traditional TSase mechanism proposed by Carreras and Santi.¹

Scheme 2.

Revised TSase mechanism leading to exocyclic methylene hydride acceptor **E**.

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Table 1.

Comparison of steady-state parameters for the three enzymes with Mg^{2+} at 25° C. The values of WT are taken from ref 49.

	WT	N177S	N177D
$k_{\text{cat}}, \text{s}^{-1}$	8.7 ± 0.2	0.082 ± 0.002	0.009 ± 0.001
$K_{\text{m,dUMP}}, \mu\text{M}$	2.4 ± 0.2	192 ± 18	13.2 ± 1.5
$k_{\text{cat}}/K_{\text{m,dUMP}}, \mu\text{M}^{-1}\text{s}^{-1}$	3.1 ± 0.3	$(4.3 \pm 0.4) \cdot 10^{-4}$	$(6.8 \pm 1.1) \cdot 10^{-4}$
$K_{\text{m,MTHF}}, \mu\text{M}$	15 ± 1	184 ± 16	118 ± 33
$k_{\text{cat}}/K_{\text{m,MTHF}}, \mu\text{M}^{-1}\text{s}^{-1}$	0.58 ± 0.04	$(4.5 \pm 0.5) \cdot 10^{-4}$	$(7.6 \pm 2.3) \cdot 10^{-5}$

Table 2.

Isotope effects on the Arrhenius parameters for the three enzymes under study as derived from regression to their KIE_{int} as presented in Figure 3. The values of WT are taken from ref. 49.

	WT	N177S	N177D
A_H/A_L	5.3 ± 1.0	1.4 ± 0.3	0.3 ± 0.1
$E_{a,H-T}$, kcal/mol	0.2 ± 0.1	1.5 ± 0.1	2.4 ± 0.2

Table 3.

Average DADs, in Å, as obtained from the Roston and co-workers model.²⁹

	WT	N177S	N177D
population	unimodal	bimodal	bimodal
average DAD	3.06	3.12	3.19

Table 4.

Total H/T primary KIEs ($\text{KIE}^{\text{total}}$) and their contributions from Quasiclassical (KIE^{QC}), Tunneling (KIE_{κ}) and recrossing (KIE_{γ}), as described in eq. 10, computed at M06-2X:RM1/MM level theory in four different temperatures for the hydride transfer step in WT (data from ref. ¹²) and N177D ecTSase. KIE^{exp} corresponds to the experimentally determined intrinsic H/T primary KIEs values.

T / K	KIE^{QC}	KIE_{κ}	KIE_{γ}	$\text{KIE}^{\text{total}}$	KIE^{exp}
wild type					
278	8.27	1.50 ± 0.23	0.69 ± 0.10	8.56 ± 0.37	7.54 ± 0.82
293	8.40	1.38 ± 0.20	0.62 ± 0.09	7.19 ± 0.29	7.58 ± 0.57
303	7.85	1.32 ± 0.19	0.79 ± 0.11	8.19 ± 0.26	7.22 ± 0.40
313	7.67	1.27 ± 0.18	0.70 ± 0.12	6.82 ± 0.25	7.39 ± 0.98
N177D					
278	11.45	2.48 ± 0.76	0.987 ± 0.005	28.01 ± 0.77	23.33 ± 2.90
288	9.96	2.19 ± 0.62	0.824 ± 0.007	17.96 ± 0.63	21.23 ± 3.00
298	8.53	1.97 ± 0.51	0.832 ± 0.010	13.96 ± 0.52	18.40 ± 2.96
308	6.99	1.80 ± 0.42	0.891 ± 0.0071	11.20 ± 0.42	14.86 ± 2.50

Table 5.

Temperature Dependence of the Tunneling transmission coefficient (κ) and the recrossing transmission coefficient (γ) computed for the transfer of protium in the N177D TSase, as described in eqs. 8 and 9. Quasi-classical activation free energies computed at M06-2X:RM1/MM level, ΔG_{act}^{QC} , are in kcalmol⁻¹, and averaged DAD at the TSs and RC are reported in Å.

T (K)	κ	γ	Γ	ΔG_{act}^{QC}	DAD_{N177D}^{TS}	DAD_{N177D}^{RC}
278	25.5 ± 10.3	0.555 ± 0.001	14.2 ± 5.7	35.9	2.77±0.06	4.48±0.10
288	18.6 ± 6.6	0.477 ±0.001	8.9 ± 3.1	36.6	2.76±0.07	4.58±0.09
298	14.1 ± 4.4	0.548 ±0.001	7.7 ± 2.4	35.6	2.74±0.06	4.54±0.12
308	11.2 ± 3.1	0.555 ±0.001	6.2 ± 1.7	36.1	2.75±0.07	4.49±0.10