INTRODUCTION

The potential of the folic acid biosynthesis pathway as a target for the development of antibiotics and chemotherapeutic drugs has been recognized for many years and validated by the clinical use. One function of folic acid metabolism is the support of DNA synthesis and repair through the generation of nucleic acid building blocks such as thymidine triphosphate (dTTP). This process involves the last step of *de novo* synthesis of a precursor of DNA, 2’-deoxythymidine-5’-monophosphate (dTMP), using (6R)-N⁵,N¹⁰-methylene-5,6,7,8-tetrahydrofolate (CH₂H₄folate) as a cofactor and 2’-deoxyuridine-5’-monophosphate (dUMP) as the substrate with subsequent phosphorylation to dTTP. The reductive methylation of dUMP to dTMP is catalyzed by Thymidylate synthase (TSase, EC 2.1.1.45), an important enzyme in the folate biosynthetic pathway.¹⁻³

Owing to its essential role in cellular metabolism, TSase has served as an antibiotic and chemotherapeutic drugs target for several decades (e.g. tomudex and 5-fluorouracil). The use of high concentration of chemotherapeutic drug to inhibit highly expressive tumor cells, damage the healthy tissues resulting toxicity in patients. This urges the development of more specific drugs that would specifically act against TSase in tumor cells without affecting normal cells. Therefore knowing the chemical mechanism of TSase in detail would help in the development of more cancer specific inhibitors. Although a large collection of X-ray crystallographic structures of TSase from several organisms were resolved and its complex mechanism has comprehensively studied, many aspects of its catalysis still remain unresolved.²

There had been a debate on the catalytic mechanism of the TSase. Scheme 1 illustrates the two main variations proposed for the chemical mechanism of TSase. In the traditional mechanism, the initial step is the nucleophilic attack of the conserved Cys146 to give 5,6-
dihydropyrimidine intermediate. The E58 assisted iminium ion formed on CH$_2$H$_4$folate (A) is nucleophilically attacked by the C5 enolate formed on dUMP forming a ternary complex (C). The abstraction of proton from C5 of dUMP results forming enol (step 4) again on C5 of dUMP and followed by the release of (6S)-H$_4$folate generating exocyclic methylene intermediate (E). Finally, a hydride transfer from (6S)-H$_4$folate to exocyclic methylene intermediate via 1,3S$_N$2 mechanism forms the product dTMP. It is still not clear whether step 6 proceeds via another enolate intermediate or 1,3S$_N$2 substitution reaction step as illustrated in scheme 2. In the alternate mechanism, the steps 2 and 3 are concerted reactions and the proton abstraction step involves E2 elimination of the thiolate C146. Moreover, the dissociation of CH$_2$H$_4$folate occurs via 1,3S$_N$2 substitution reaction (step 5'). $^3$

Figure 1 presents the crystal structure of the active site of wild type (WT) *Escherichia coli* (*ec*) TSase (PDB entry 2KCE). Histidine at 147 position is 3.6 Å away from the O4 of the dUMP. This histidine controls a network of H-bonds that acts as the general acid/base in the protonation/deprotonation of the C4 carbonyl during the catalytic cycle.$^2$ Previous results have shown that several mutants of H147 with nearly identical $K_m$s with WT TSase from either *ec* or *Lactobacillus casei* (*lc*) species but with significant reduction of $k_{cat}$.$^4,5$ More interestingly, TSase from *Bacillus subtilis* (*bs*) has a valine at the position 147 and even more active than the both species above.$^4$ Due to the high conservation of amino acids in TSases, the way by which *bs* TSase compensates for the H to V conversion and its effect on the nature of the H-transfer, in contrast to the barely active H147V *ec*TSase, would address the differences between the two proposed elimination steps (Scheme 1, step 4) and the role of this residue in the tuning of the reaction coordinate toward tunneling. The focus of this work is to examine the effect of H147V of *ec* TSase on two distinct chemical steps namely, the proton abstraction from C5 of dUMP
dissociating the ternary complex (step 4) and the hydride transfer step (step 6). Our recent QM/MM studies suggested a mechanism for the proton transfer (step 4\textsuperscript{	extprime}) that does not involve protonation of the C4 carbonyl and predict little effect of the H147 mutation on step 4\textsuperscript{	extprime} and the traditional mechanism on the other hand, predicts that H147 mutants should have a substantial effect on step 4 due to their limited ability to stabilize the proposed enol.\textsuperscript{6} Thus, the examination of the effect of H147V mutation on step 4 will assist in distinguishing between the two proposed mechanisms (Scheme 1).

The investigation on the effect of H147V mutation on the hydride transfer due to altered H-bond network would shed light upon the chemistry of the catalyzed reaction and also to resolve the ambiguous mechanism of whether step 6 proceeds via another enolate intermediate or 1,3\textit{S}\textsubscript{N}\textsuperscript{2} substitution reaction step (scheme 2).

**MATERIALS AND METHODS**

*Materials*

[2-\textsuperscript{14}C] dUMP (specific radioactivity 53 Ci/mol) and [5-\textsuperscript{3}H] dUMP (specific radioactivity 13.6 Ci/mmol) was from Moravek Biochemicals. [\textsuperscript{3}H]NaBH\textsubscript{4} (specific radioactivity 15 Ci/mmol) was from American Radiolabeled Chemicals. [\textsuperscript{2}H]NaBH\textsubscript{4} (> 99.5% D) was from Cambridge Isotopes. Dihydrfolate (H\textsubscript{2}-folate) was synthesized by following the procedure of Blakely.\textsuperscript{7} Unlabeled CH\textsubscript{2}H\textsubscript{4}folate was a generous gift from Merck and Cie (Switzerland). [2-\textsuperscript{3}H]iPrOH and [2-\textsuperscript{2}H]iPrOH were prepared by reduction of acetone with [\textsuperscript{3}H]NaBH\textsubscript{4} and [\textsuperscript{2}H]NaBH\textsubscript{4}, respectively as described in elsewhere.\textsuperscript{8} The WT ecTSase enzyme was produced and purified following a published procedure.\textsuperscript{9} Site-directed mutagenesis of ec TSase thyA gene was done by using the Quick-Change site-directed mutagenesis kit from Stratagene. The mutant protein was
purified as above for WT. Ultima Gold liquid scintillation cocktails were from Packard Bioscience. Liquid scintillation vials were from Research Products International Corp. All other materials were purchased from Sigma. The initial velocity experiments were performed using a Hewlett-Packard Model 8452A diode-array spectrophotometer equipped with a temperature-controlled cuvette assembly. All the purifications and analytical separations were performed using an Agilent Technologies model 1100 HPLC system. The radioactive samples were analyzed using a flow scintillation analyzer (Model RT505 from Packard, now Perkin Elmer Biosciences) or a Liquid Scintillation Counter (LSC).

**Synthesis of [6R-\(^3\)H]CH\(_2\)H\(_4\)folate for KIE (Kinetic Isotope Effect) Experiments on Hydride Transfer**

The [6R-\(^3\)H]CH\(_2\)H\(_4\)folate was synthesized by the published procedure.\(^{10}\) Briefly, the synthesis is a one-pot preparation that utilize two enzymes and a single chemical reaction: The reduction of NADP\(^+\) by [2-\(^3\)H]iPrOH to produce [4R-\(^3\)H]NADPH was catalyzed by alcohol dehydrogenase from *thermoanarobium brockii* (tbADH) and the reduction of H\(_2\)folate by [4R-\(^3\)H]NADPH to produce [6S-\(^3\)H]H\(_2\)folate was catalyzed by DHFR. [6S-\(^3\)H]H\(_2\)folate was trapped by adding formaldehyde, forming [6R-\(^3\)H]CH\(_2\)H\(_4\)folate. Strict anaerobic conditions were maintained by glucose/glucose oxidase *in situ* oxygen scavenging system. The synthesized [6R-\(^3\)H]CH\(_2\)H\(_4\)folate was purified by reverse phase HPLC (RP HPLC), lyophilized, and stored at -80 °C prior to use.

**Steady-State Kinetics**

The optical method which measures the increase of absorbance at 340 nm upon conversion of CH\(_2\)H\(_4\)folate to H\(_2\)folate (\(\Delta \varepsilon_{340\text{nm}} = 6.4 \text{ mM}^{-1}\text{cm}^{-1}\)), was used here to study the initial velocities for the mutant.\(^{11}\) The temperature dependence of initial rates of CH\(_2\)H\(_4\)folate were measured with
200 µM dUMP within a range of 5-35 °C. The initial rate of dUMP was measured with 50 µM CH₂H₄folate at 25 °C. These experiments were conducted in TCEP at least in triplicates.

**Competitive KIEs on the Hydride Transfer (step 6 in scheme 1)**

The competitive method for H/T and D/T was used to measure the KIE on the hydride transfer step in the temperature range of 5-35 °C, according to the procedure published for the Y209W enzyme. All experiments were carried out in Tris HCl (pH=7.5, adjusted at each temperature), 2 mM TCEP, 1 mM EDTA, 5 mM HCHO, 1.5 Mdpm [6R-³H]CH₂H₄folate (x=3 for H/T and x=2 for D/T experiments respectively), 0.5 Mdpm [2-¹⁴C]dUMP, CH₂H₄folate and dUMP to enable 20% molar excess of [2-¹⁴C]dUMP as published. The reaction mixture was pre incubated at each temperature of the study and the reaction was started adding H147V. Five time points of 100 µl aliquots were removed and quenched with 30 µM F-dUMP. Three infinity time points (t∞) were obtained by adding concentrated WT ecTSase. Two t₀ time points were obtained as controls for the experiment. All quenched samples were analyzed by HPLC and LSC as described in pervious publications. The competitive observed and intrinsic KIEs on the second order rate constant (V/K) were calculated as described elsewhere.

**Competitive KIEs on the Proton Transfer (step 4 in scheme 1)**

The competitive method for ¹° H/T was used to measure the KIE on the proton transfer step at 25 °C, according to the procedure published for the WT enzyme. Briefly, All experiments were carried out in Tris HCl (pH=7.5), 25 mM DTT, 1 mM EDTA, 5 mM HCHO, 50 mM MgCl₂, 3 µM CH₂H₄folate, 0.3 Mdpm [2-¹⁴C]dUMP, and 0.9 Mdpm [5-³H]dUMP. The reaction mixture was pre incubated at 25 °C and the reaction was started adding H147V. Five time points of 100 µl aliquots were removed and quenched with 30 µM F-dUMP. Three infinity time points (t∞) were obtained by adding concentrated H147V. Two t₀ time points were obtained
as controls for the experiment. The competitive observed KIEs on the second order rate constant were calculated as described elsewhere:

RESULTS AND DISCUSSION

Steady State Kinetics

The initial velocity studies were carried out for a temperature range 5-35 °C. We employed TCEP, a non-thiol reducing agent to avoid any 5-(2-hydroxyethyl)thiomethyl-dUMP (HETM-dUMP) structures formation ensuring more precise kinetic measurements of H147V. Similarly to the WT and Y209W ecTSase, the rates measured with dUMP with varying substrate concentrations, were fitted to the Michelis-Mentan hyperbolic equation, whereas the rates with CH$_2$H$_4$folate showed substrate inhibition (Figure 2A) and the data at each temperature were fit to the non-linear Michaelis-Menten equation published elsewhere. Table 1 compares the kinetic parameters of WT and H147V TSase with β-ME or with TCEP at 25 °C. There was an approximately ~ 15 fold decrease in $k_{cat}$ for H147V. Similar data for the mutant has reported.

For H147V mutant the $K_m$ for dUMP was unchanged and the $K_m$ for CH$_2$H$_4$folate was increased by ~2 fold. This data clearly indicate that H147 is not significant in binding but contributes to the catalysis process.

Mutational Effects on the Thermodynamic Parameters of the TSase Reaction

The steady state kinetic data at each temperature were analyzed with the least squares nonlinear regression available in KaleidaGraph (Version 4.03). This analysis provided the steady state rate constants of the reaction at different temperatures, which were fit to the Eyring equation to evaluate the activation parameters (Figure 2.B, Table 2) the entropy, enthalpy, and free energy of activation ($\Delta S^\ddagger$, $\Delta H^\ddagger$ and $\Delta G^\ddagger$ respectively) published elsewhere. This mutation
shows ~ 1.5 increase in the enthalpy of activation and compared with the WT enzyme, the H147V mutation has a significant effect \( \Delta G^\ddagger \) at the temperatures under this study (5-35 °C).

**Competitive KIEs on the Proton Transfer Step**

An inverse KIE was observed for the second order rate constant \((V/K)\) for competitive KIE experiment conducted at 25 °C. This could be explained by the observation of high \(^3\)H/\(^{14}\)C ratios in the product dTMP. The observation of high ratio of \(^3\)H/\(^{14}\)C in the product dTMP can be attributed to the slow formation of \([2-{^{14}\text{C}}]dTMP\) compared to release of tritium from \([2-{^{14}\text{C}},5-{^3\text{H}}]dUMP\). Similar results were observed for mutants of E58, involved in the charge stabilizing water-mediated hydrogen bond network with H147V above the pyrimidine ring of dUMP and the nucleophile, thiolate of C146. Focusing on WT TSase, the experiment releasing one tritium per molecule of dTMP formed owing to an ideal situation.

The observation of rapid release of tritium could be explained with an exchange reaction with the solvent protons. According to the traditional reaction mechanism (Scheme 1), the abstraction of proton at C5 of dUMP forms the intermediate D. Rather than converting from intermediate D to E, re-protonation of intermediate D with water will reverse the reaction in the direction of dUMP formation. This further suggests the rate-determining step of the H147V TSase catalyzed reaction is subsequent to formation of the covalent intermediate C. This is well accordant with the results of competitive KIEs on the \((V/K)\) for the hydride transfer reaction discussed below.

**Competitive KIEs on the Hydride Transfer Step**

Since Hydride transfer step is the only irreversible step in scheme 1, H, D, or T labeled at \(R\text{-C6}\) position allow to expose the KIE on this step as all the other steps are isotopically insensitive.
Previous studies in our lab reported H/T and D/T KIEs on V/K of 6.91±0.05 and 1.78±0.02 respectively with a Swain-Schaad exponent of 3.35±0.07 suggesting hydride transfer is rate limiting at 20 °C for WT TSase. This was intensely reinforced by the measured H/D KIE on kat by spencer and co-workers. We measured the competitive V/K KIE measurements on H147V mutant at various temperatures (15-35 °C). The observed H/T and D/T KIEs did not follow the semi-classical swain schaad exponent (SSE), i.e. $\frac{\ln(k_H/k_T)}{\ln(k_D/k_T)} \approx 3.34$ at any temperature, suggesting the hydride transfer step is not rate limiting at the temperatures we studied.

We noticed smaller observed KIEs than intrinsic KIEs. This observation can be rationalized by the kinetic complexity, in which the “masking” of intrinsic KIEs by isotopically insensitive steps in the enzymatic reaction cascade. The inflated commitment of the observed KIE on V/K of H147V suggests that hydride transfer step is not rate limiting and a preceding step becomes rate limiting. These data corroborates with the inverse KIE reported for proton abstraction step and indicating a reaction step between proton abstraction and hydride transfer reactions become rate limiting for the H147V mutant.

The intrinsic KIEs were fit to the Arrhenius equation published elsewhere. Table 3 summarizes the H/T isotope effects on the Arrhenius parameters of both the WT and H147V catalyzed hydride transfers. The KIEs on the Arrhenius preexponential factor was inverse ($A_H/A_T < 1$). Traditionally this could be explained by using the tunneling correction to transition state theory. $A_H/A_T$ values below unity corresponds to more extensive tunneling of lighter isotope than the heavier isotope and in the moderate tunneling regime of the Arrhenius plot for hydride transfer.
Figure 3 shows the Temperature dependence of both primary and intrinsic KIEs of H147V and WT TSase. The temperature dependence of intrinsic KIEs together with the activation energies in the physiological temperature range, suggested that a Marcus-like model is required to explain the findings of H147V and the WT. Tunneling in such a model is dominated by the “rearrangement term” including reorganization energy $\lambda$ and driving force $\Delta G^\circ$ and by the fluctuations of the distance between the donor and acceptor molecule.\textsuperscript{16}

The H147V mutant shows a steeper temperature dependence of the KIEs compared to WT TSase. The lack of temperature dependence of the KIEs for the WT TSase could rationalize as a perfect rearrangement of the active site with an average donor-acceptor distance (DAD) is optimized for tunneling. Therefore no thermal fluctuations (termed “gating”) are needed for the optimized hydride transfer. On the other hand the H147V mutant has a system with a poorly organized reaction coordinate for tunneling. Therefore H147V system shows a substantial need for gating in order to tunnel in the physiological temperature range. This is strongly supported by the increase in the activation energy of H147V relative to WT TSase.

H147 is involved in H bonding with water being a part of the H-wire between TSase and O4 of the substrate, dUMP.\textsuperscript{5} This hydrogen bond network could help to stabilize any negative charge developed on O4 of dUMP during the formation of intermediates. Therefore it is possible the hydrogen network is interrupted in H147V creating an unfavorable situation for the developing charge on O4 of dUMP during the hydride transfer step according to the stepwise chemical mechanism (scheme 2B) of TSase. This is consistent with formation of an intermediate anion on C4 carbonyl during the hydride transfer step in H147V mutant although our QM/MM calculations suggested that the hydride transfer and the thiol abstraction at C146 take place in a concerted way by 1,3-S$_\text{N}$2 fashion for the WT(scheme 2A).
CONCLUSIONS

Although our knowledge of the chemical mechanism of TSase has improved over the past decade, there is still much to be learned as evidenced by this paper which would help in designing more specific chemotherapeutic drugs. Here, the proton abstraction step and the hydride transfer step in the H147V ecTSase reaction were examined by different tools such as KIE in this work to draw significant conclusions about the reaction mechanism. Interestingly the findings of the hydride transfer step of H147V mutant present new insight to the chemical mechanism. The significant change in the temperature dependence of hydride transfer implicates the highly altered protein motions at the transition state. This suggests the mutation deteriorates the protein motions at the active site needed for the hydride transfer. The outcome of the research indicate that the disruption of the H-bond network due to substitution of histidine by valine at 147 position causes higher penalty for reorganization of protein environment for the hydride transfer, being H147 as one of the main contributor to the H-bond network in the active site. These findings suggest that the hydride transfer involves the formation of thiol intermediate at C4 position of dUMP (scheme 2B) rather than 1,3S_N2 substitution. We hope that the new findings will enable to further investigation on QM/MM studies on this H147V mutant.

However we were unable to expose the proton abstraction step of H147V due to the observation of rapid release of tritium compared to dTMP formation as described above. Therefore we cannot distinguish the two chemical mechanisms proposed for the proton abstraction step (scheme 1, step 4). But, the experiments suggest the H147V mutation mostly affects a step between the proton transfer and the hydride transfer. This could be the deprotonation of the enol at C4 of dUMP or the protonation of N5 of tetrahydrofolate (H₄folate) in the reaction mechanism.
**Scheme 1.** The proposed chemical mechanism for TSase

**Scheme 2.** The possible mechanisms proposed for the hydride transfer step: (A) concerted mechanism; (B) stepwise

**Figure 1:** Structure of WT ec TSase (PDB entry 2KCE)

**Figure 2:** (A) Steady state initial velocities of H147V vs the concentration of CH$_2$H$_4$folute at 200 µM dUMP. (B) The Eyring plot of $k_{cat}$

**Figure 3:** Arrhenius plots of the KIEs on the hydride transfers catalyzed by the WT (blue, from Ref$^1$) and H147V (red) ecTSase. The lines represent the least-squares nonlinear regression of the intrinsic KIEs. The empty circles represent the observed KIEs, and filled circles represent the intrinsic KIEs.
Table 1. Steady state kinetic parameters of the WT and H147V ecTSase at 25 °C.

<table>
<thead>
<tr>
<th>TSase</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_{m}^{dUMP}$</th>
<th>$K_{m}^{CH_{2}H_{4}folate}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT w/ß-Mercaptoethanol$^a$</td>
<td>8.8</td>
<td>4.1 ± 0.4</td>
<td>13.6 ± 0.2</td>
</tr>
<tr>
<td>H147V w/TCEP</td>
<td>0.6 ± 0.04</td>
<td>4.03 ± 1.26</td>
<td>24.8 ± 3.5</td>
</tr>
</tbody>
</table>

$^a$ Data from Ref.12 A comparison of WT activity with ß-Mercaptoethanol and with TCEP found no differences.

Table 2. Activation parameters of the steady state initial velocities of WT and H147V TSase.

<table>
<thead>
<tr>
<th>TSase</th>
<th>temperature range</th>
<th>$\Delta H^\ddagger$ kcal/mol</th>
<th>$T \Delta S^\ddagger$ at 25 °C kcal/mol</th>
<th>$\Delta G^\ddagger$ kcal/mol</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td>5-35 °C</td>
<td>3.4 ± 0.2</td>
<td>-12.8 ± 0.2</td>
<td>16.2 ± 0.4</td>
</tr>
<tr>
<td>H147V</td>
<td>5-25 °C</td>
<td>5.2 ± 0.4</td>
<td>-12.6 ± 0.4</td>
<td>17.8 ± 0.6</td>
</tr>
</tbody>
</table>

Table 3. Isotope effects on Arrhenius parameters of the WT and H147V TSase on hydride transfer.

<table>
<thead>
<tr>
<th>$A_H/A_T$</th>
<th>H147V</th>
<th>WT$^a$</th>
<th>S.C. $A_I/A_T$$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.015 ± 0.009</td>
<td>6.8 ± 2.8</td>
<td>0.3 – 1.7</td>
</tr>
<tr>
<td>$\Delta E_a$</td>
<td>3.72 ± 0.38</td>
<td>0.02 ± 0.25</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Data from Ref.1
$^b$ Semi-classical limits of isotope effects on the preexponential factor.16
REFERENCES


