Concerted versus Step-wise Mechanism in Thymidylate Synthase

Zahidul Islam, Timothy S. Struzenberg, Ilya Gurevic and Amnon Kohen

Department of Chemistry, The University of Iowa, Iowa City, Iowa,

Materials and Instruments

[6-³H]dUMP (390-470 mCi/mmol) and [2-¹⁴C]dUMP (specific radioactivity of 52 Ci/mol) were purchased from Moravek Biochemicals. [³H]NaBH₄ (15 Ci/mmol) and [²H]NaBH₄ were from American Radiolabeled Chemicals and Cambridge Isotopes, respectively. EPLOVA (Switzerland) provided the unlabeled CH₂H₄folate as a generous gift. Ultima Gold liquid scintillation cocktails and vials were purchased from Packard Bioscience and Research Products International Corp, respectively. All of the other chemicals such as tris-(hydroxymethyl)-aminomethane (Tris), tris(2-carboxyethyl)phosphine (TCEP), unlabeled dUMP and folate were purchased from Sigma. Dihydrofolate (H₂folate) was synthesized following a published procedure. Tritiated [2-³H]- and deuterated [2-²H]- isopropanol were synthesized by reducing acetone with [³H]NaBH₄ and [²H]NaBH₄, respectively. Wild-type E.coli TSase and mutant R166K were expressed and purified according to the procedure from ref 2. A Hewlett-Packard Model 8452A diode-array spectrophotometer with a temperature-controlled cuvette assembly was used for the steady-state kinetic experiments. A Supelco Discovery C18 reverse model 1100 HPLC system was used for all analytical separations for the competitive KIE experiments. The radioactive samples were analyzed using a liquid scintillation counter (LSC).

Synthesis of [6R-²⁻H]CH₂H₄folate

Synthesis of [6R-²⁻H]CH₂H₄folate was carried out following previously published procedures. Briefly, a one-pot synthesis of [6R-²⁻H]CH₂H₄folate was performed in strict anaerobic conditions using a glucose/glucose oxidase in situ oxygen scavenging system. In this one-pot synthesis, alcohol dehydrogenase from Thermoanaerobium brockii (tbADH) reduces NADP to [4R-²⁻H]NADPH using [2-²⁻H]PrOH as a reducing agent, and then DHFR catalyzes the conversion of H₂folate to [6S-²⁻H]-H₄folate using in situ synthesized [4R-²⁻H]NADPH. Finally, HCHO is added to the reaction mixture to react with [6S-²⁻H]-H₄folate, thus forming [6R-²⁻H]-CH₂H₄folate. The reaction was performed in a water bath at 40 °C. The synthesized [6R-²⁻H]-CH₂H₄folate was purified by RP HPLC, then lyophilized and stored at -80 °C.

Steady State Kinetic Experiments.

The increase of absorbance at 340 nm (Δε_{340nm} = 6.4 mM⁻¹ cm⁻¹) indicating the conversion of CH₂H₄folate to H₂folate was followed to measure the steady-state initial
velocities for R166K. The reaction mixture contained 2 mM tris-(2-carboxyethyl)phosphine (TCEP), 1 mM ethylenediaminetetraacetate (EDTA), and 5 mM formaldehyde (HCHO) in 100 mM tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.5). Michaelis parameters for dUMP and CH₂H₄folate were measured with 3 mM CH₂H₄folate and 5 mM dUMP, respectively, at room temperature (25°C). Analysis of the steady-state kinetic data was carried out employing the nonlinear regression available in KaleidaGraph (version 4.03). The initial velocities against varying concentrations of dUMP were fit into the standard Michaelis-Menten equation:

\[
\frac{v}{[E]_t} = \frac{k_{cat}[S]}{k_{cat}+[S]}
\]  

(S1)

In wild-type TSase and some of its mutants, high concentration of CH₂H₄folate was reported to inhibit the enzyme’s activity. R166K, however, did not display such a deleterious effect on enzyme’s function with high concentration of CH₂H₄folate within the saturation limit of spectrophotometer’s detector, which allowed us to fit the initial velocities for different concentration of CH₂H₄folate into the same standard Michaelis-Menten equation (S1).

Figure S1: Initial velocities with varying concentration of CH₂H₄folate for R166K.

**Competitive Primary (1°) KIE Experiments.**

Primary (1°) H/T and D/T KIEs on the hydride transfer from the C6 of H₄folate to the exocyclic methylene intermediate for R166K were measured competitively, using the same method used for the WT enzyme by Agrawal et al. Briefly, the reactions were carried out in 100 mM Tris buffer at 5, 15, 25, and 35°C (pH adjusted to 7.5 at the desired temperature). The reaction mixtures contained 1 mM EDTA, 5 mM formaldehyde, 2 mM TCEP, [2-¹⁴C]dUMP, trace (R)-[6-³H]CH₂H₄folate in protiated or deuterated CH₂H₄folate for H/T or D/T KIE experiments, respectively. The concentration of dUMP was taken in ~25-30 % excess to CH₂H₄folate to follow the fractional conversion. The ratio of (R)-[6-³H]CH₂H₄folate to [2-¹⁴C]dUMP was kept above 6.0 in order to increase accuracies in analysis by LSC. The reaction was initiated by adding R166K mutant. While the reaction was running, around five to six aliquots of reaction mixture at different time points (t) were removed and quenched by adding an excess of 5F-dUMP,
which is a specific inhibitor of TSase with $K_i$ of 1nM. Three infinity time points ($t_\infty$) were prepared by adding concentrated wild-type TSase to make the reaction go to completion. The reaction products were separated from the reactants by RP HPLC, followed by LSC analysis as described elsewhere.\textsuperscript{5} The observed KIEs were calculated from three measured-values for each time point: fractional conversion ($f$), $R_t$ (the ratio of $^3$H/$^{14}$C in the product at the time points) and $R_\infty$ (the ratio of $^3$H/$^{14}$C in the product at the infinity time points):

\[
\text{KIE} = \frac{\ln (1-f)}{\ln [1-f(R_t/R_\infty)]}
\]

(S2)

The fractional conversion $f$ for all time points was calculated using the following equation (S3)

\[
f = \frac{[^{14}\text{C}]\text{dUMP}}{(100-%\text{excess})([^{14}\text{C}]\text{dUMP}+[^{14}\text{C}]\text{dTMP})}
\]

(S3)

Intrinsic KIEs on Hydride Transfer

Intrinsic KIEs were obtained from the observed competitive H/T and D/T KIEs, which yields the isotope effect on $k_{cat}/K_m (V/K)$ by following the Northrop method: \textsuperscript{7,8}

\[
\frac{T(V/K)_{H_{obs}}^{1}}{T(V/K)_{D_{obs}}^{1}} - 1 = \frac{(k_H/k_T)^{-1}-1}{(k_H/k_T)^{-1/3.34}-1}
\]

(S4)

where $k_H/k_T$ represents the intrinsic H/T KIE, and $T(V/K)_{H_{obs}}$ and $T(V/K)_{D_{obs}}$ are the observed H/T and D/T KIEs, respectively. The above equation was numerically solved for the intrinsic KIEs using a program available on our web site under “Tools” \url{http://www.chem.uiowa.edu/kohen-research-group}. All the observed and intrinsic H/T and D/T KIEs are provided in Table S2. We fit all intrinsic KIEs into the following Arrhenius equation (S5) to obtain the isotope effects on Arrhenius parameters for the hydride transfer in R166K:

\[
\text{KIE} = \frac{k_L}{k_H} = \frac{A_L}{A_H} \exp \left( - \frac{\Delta E_a}{RT} \right)
\]

(S5)

where the subscripts L and H refer to light and heavy isotopes of hydrogen, respectively; $\frac{A_L}{A_H}$ and $\Delta E_a$ represents the isotope effect on the Arrhenius preexponential factor and activation energy, respectively; $\frac{k_L}{k_H}$ represents the KIE for the hydride transfer; $R$ is the gas constant; and $T$ is absolute temperature. KaleidaGraph (version 4.03) was used in carrying out the analysis as the root-mean-square fit exponential regression.

Since the hydride transfer is rate-limiting on $k_{cat}/K_m$ in wild-type near room temperatures, the observed KIEs follow the Swain-Schaad relationships, i.e., the observed and the intrinsic KIEs are the same.\textsuperscript{5} However, the intrinsic KIEs for the R166K are much higher than the observed KIEs at all temperatures due to kinetic complexity,\textsuperscript{7,8} making the hydride transfer no longer rate-limiting on $k_{cat}/K_m$ (Figure 2 in the main text). Since the residue R166 actively contributes to the binding of dUMP as well as to the
formation of non-covalent ternary complex (TSase-dUMP-CH$_2$H$_4$folate), it would not be surprising for any steps before the chemical steps to become rate-limiting because of the mutation.

**Competitive Secondary (2°) KIE Experiments.**

Competitive α-2° H/T KIE on C6 of dUMP (i.e. on $k_{cat}/K_m$) was performed using commercially available [2-$_{14}$C]dUMP as a tracer and [6-$_3$H]dUMP with the wild-type TSase at 298K. The experimental conditions were identical to competitive 1° KIE experiments except the tritium labeling was on C6 of dUMP, instead of CH$_2$H$_4$folate.

**Calculation (B3LYP) of Equilibrium maximal α-2° H/T KIE (EIE)**

*Gaussian09* was used to perform the calculation. The initial geometries for the calculations were the structures of dUMP, exocyclic methylene intermediate (compound D, Scheme 1 in the main text) and the dTMP with the ribose ring replaced by a methyl group. The thiol side-chain of C146 was included from structure of PDB ID 1KZI. All of the structures were optimized at B3LYP with the basis set 6-311+(d,p). The vibrational frequencies of dUMP, exocyclic methylene intermediate (compound D, Scheme 1) and dTMP were calculated using their optimized structures with the same level of theory and basis set, B3LYP/6-311+(d,p). The vibrational frequencies of the reactants and products obtained from *Gaussian* calculations were fed into the program *ISOEFF07* that calculated the EIE using the Bigeleisen equation.\(^9\)

**Figure S2:** Optimized structures of free dUMP, cysteine-bound exocyclic methylene intermediate (1) and free dTMP showing all of the atoms used for the calculation of equilibrium maximal secondary KIE at B3LYP/6-311+(d,p).
Figure S3: Snapshots of the average structures obtained from QM/MM calculations. Note, at the transition state, R166 reaches closer to the thioether. Reproduced from ref 11 with permission from ACS.

Table S1: Kinetic parameters and their comparisons from initial velocity studies for wild-type and mutants of all arginines.

| TSase  | $k_{\text{cat}}$(s$^{-1}$) | $K_{m}^{\text{dUMP}}$(µM) | $K_{m}^{\text{folate}}$(µM) | $k_{\text{cat}}/K_{m}^{\text{dUMP}}$(µM$^{-1}$s$^{-1}$) | $k_{cat}$ (wt/mt)$^d$ | $K_{m}^{\text{dUMP}}$(mt/wt)$^d$ | $K_{m}^{\text{folate}}$(mt/wt)$^d$ |
|--------|-----------------|-----------------|-----------------|-----------------|----------------|-----------------|-----------------|
| wt$^a$ | 2.3±0.1         | 2.4±0.1         | 15±1.0          | 0.95            | 1              | 1               | 1               |
| R21K$^b$ | 0.16           | 44              | 224             | 0.013           | 32.5           | 2.2             | 10.2            |
| R127K$^b,c$ | 3.2           | 8               | 21              | 0.4             | 1.1            | 2.66            | 1               |
| R126K$^b,c$ | 8             | 130             | 60              | 0.061           | 1.8            | 43              | 4               |
| R166K$^e$ | 0.024±0.0004   | 816±66          | 280±20          | 2.94×10$^{-5}$  | 95.8           | 340             | 18.6            |

$^a$From ref 5. $^b$Equivalent residues in L. casei and collected from ref 12. $^c$From the other subunit, $^d$Comparisons between the mutants (mt) and the respective wild-type(wt), $^e$the relative discrepancy in turnover rates for the R166K in E.coli to the one mentioned in ref 12 for equivalent residue in L. casei is probably due to the presence of Mg$^{2+}$ in the reaction assay in the later. Mg$^{2+}$ has been reported to increase the turnover rate.$^f$

Table S2: Observed and Intrinsic KIEs on hydride transfer catalyzed by R166K.

|         | H/T$^\text{obs}$ | D/T$^\text{obs}$ | H/T$^\text{int}$ | D/T$^\text{int}$ |
|---------|-----------------|-----------------|-----------------|-----------------|
| 5 °C    | 5.74±0.02       | 2.00±0.02       | 16.99±1.06      | 2.33±0.04       |
| 15 °C   | 6.43±0.10       | 1.97±0.01       | 13.50±0.58      | 2.17±0.02       |
| 25 °C   | 6.31±0.10       | 1.89±0.009      | 10.66±0.37      | 2.03±0.02       |
| 35 °C   | 6.09±0.02       | 1.80±0.04       | 9.19±1.22       | 1.93±0.08       |
Table S3: Isotope Effects on Arrhenius Parameters for the Hydride Transfer in wild-type and R166K TSases.

| TSase      | wild-type<sup>a</sup> | R166K    |
|------------|------------------------|----------|
| ΔE<sub>a(T-H)</sub> (kcal/mol) | -0.02±0.25 | 3.6±0.1  |
| A<sub>H</sub>/A<sub>T</sub> | 6.8±2.8    | 0.023±0.003 |

<sup>a</sup>ref 5

References

(1) Blakley, R. L. Nature (London, U. K.) 1960, 188, 231.
(2) Changchien, L. M.; Garibian, A.; Frasca, V.; Lobo, A.; Maley, G. F.; Maley, F. Prot. Express. Pur. 2000, 19, 265.
(3) Agrawal, N.; Mihai, C.; Kohen, A. Anal. Biochem. 2004, 328, 44.
(4) Wang, Z.; Abeyesinghe, T.; Finer-Moore, J. S.; Stroud, R. M.; Kohen, A. J. Am. Chem. Soc. 2012, 134, 17722.
(5) Agrawal, N.; Hong, B.; Mihai, C.; Kohen, A. Biochemistry 2004, 43, 1998.
(6) Wang, Z.; Sapienza, P. J.; Abeyesinghe, T.; Luzum, C.; Lee, A. L.; Finer-Moore, J. S.; Stroud, R. M.; Kohen, A. J. Am. Chem. Soc. 2013, 135, 7583.
(7) Cook, P. F.; Cleland, W. W. In Enzyme kinetics and mechanism; Garland Science: London ; New York, 2007, p 253.
(8) Roston, D., Islam, Z., Kohen, A. Arch. Biochem. Biophys. 2014, 544, 96.
(9) Anisinov, V.; Paneth, P. Lodz, Poland, 2007.
(10) Bigeleisen, J.; Mayer, M. G. J. Chem. Phys. 1947, 15, 261.
(11) Kanaan, N.; Ferrer, S.; Marti, S.; Garcia-Viloca, M.; Kohen, A.; Moliner, V. J. Am. Chem. Soc. 2011, 133, 6692.
(12) Kawase. S., C., S.W.Rozelle, J., Stroud, M. R., Finer-Moore, J. and Santi, V. D. Protein Eng. 2000, 13, 557.