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Hydride Transfer versus Hydrogen Radical Transfer in Thymidylate Synthase

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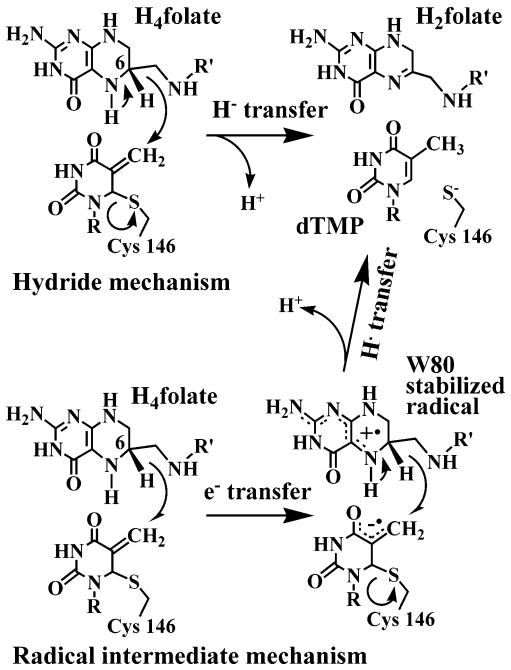
Thymidylate synthase (TS) (EC 2.1.1.45) catalyzes the last committed step in the de novo biosynthesis of 2′-deoxythymidine-5′-monophosphate (dTMP, one of the four building blocks of DNA). The enzyme catalyzes the reductive methylation of 2′-deoxyuridine-5′-monophosphate (dUMP) to dTMP. Two independent reactions are involved in which the cofactor N^5,N^{10}-methylene-5,6,7,8-tetrahydrofolate (CH_2H_4folate) serves first as a donor of a methylene group and second as a donor of a hydride ion.[1−3] The hydride transfer takes place between the C6 hydrogen of H_4folate and the dUMP exocyclic methylene intermediate (Scheme 1). While the existence of the exocyclic methylene intermediate has been reasonably supported by its trapping with β-mercaptoethanol ($HSCH_2$-CH_2OH),[4] the H-transfer step that follows has been a matter of some controversy. Different experimental data have supported two different and distinct mechanisms. Schultz and co-workers[5] used unnatural amino acids substituting W82 of *L. casei* TS[6] and found a correlation between the steady-state k_{cat} for tritium release (from C5 of 5T-dUMP) and the theoretical ability of tryptophan (W) and phenylalanine (F) substituents of W82 to bind Na^+ in the gas phase. These researchers concluded that this correlation indicates that W82 stabilizes a H_4folate cation radical intermediate or transition state. They proposed that the H-transfer is initiated by an electron transfer from H_4folate to the dUMP exocyclic methylene intermediate to form anion and cation radicals (bottom of Scheme 1). Since crystal structures have suggested that W82 (W80 in *ec*TS) cannot stabilize such a cation at its ground state (Figure 1), it was proposed that the pterin ring flips closer to the tryptophan (forming charge stabilizing "stacking interactions"), and then flips back to transfer a hydrogen radical leading to products (right-hand of Scheme 1). Stroud and co-workers[7] examined the crystal structures of the wild-type (wt) and W80G mutant of *ec*TS together with other crystal structures of the enzyme with various ligands. They concluded that the flip proposed by ref 5 is unlikely and proposed that the role of W80 is to orient L143 and the reactants, thereby protecting the exocyclic methylene intermediate from nucleophilic attack by solutes, such as β-mercaptoethanol. They further concluded that the mechanism of the H-transfer is likely to be a one-step hydride transfer (top of Scheme 1).

The shortcoming of the methods used in refs 5 and 7 is that they could not examine the nature of the H-transfer[8] step per se in the complex kinetic cascade of TS. Recently, we developed a method to study the nature of that step by means of intrinsic kinetic isotope effects (KIEs)[9] and their temperature dependence and other activation parameters.[10] This methodology is very sensitive even to minor changes in the reaction's potential surface and dynamics[11−13] and has been used to detect effects of mutations close and far from the active site on the nature of the H-transfer.[14−18] Thus, the temperature dependence of intrinsic KIEs provides a way to examine whether a two-step radical mechanism or a one-step hydride transfer mechanism is more likely. The radical mechanism[5] would imply that replacing W with M, which cannot stabilize a cation radical nearly as well as the aromatic W, would substantially change the nature of the H-transfer.

The hydride transfer mechanism,[7] on the other hand, would not require significant alteration of the nature of the H-transfer even though k_{cat} is reduced.[19,20] Steady-state parameters (e.g., k_{cat} and K_M) of several mutants of W82 *lc*TS and W80 *ec*TS have been examined in the past.[4,7,19] W80M *ec*TS was chosen in this work because it is still quite active relative to mutations with a smaller

Scheme 1. Two Proposed Mechanisms for the H-Transfer from C6 of H_4folate to the Exocyclic Methylene[a]



[a] At the top, a one-step hydride transfer as proposed by refs 2, 3, and 7. At the bottom, the two-step radical mechanism proposed by ref 5. Full arrows symbolize transfer of a pair of electrons, and half arrows symbolize transfer of a single electron.

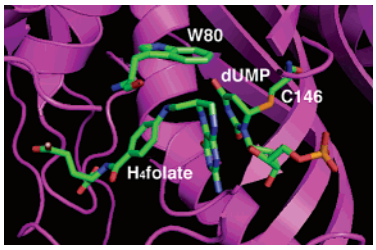


Figure 1. Crystal structure of *ec*TS with dUMP and H_4folate (PDB entry 1KZI[7]). This structure best mimics the conformation of the system prior to the H-transfer step.[2] The C146 bound dUMP, the H_4folate, and W80 are highlighted.

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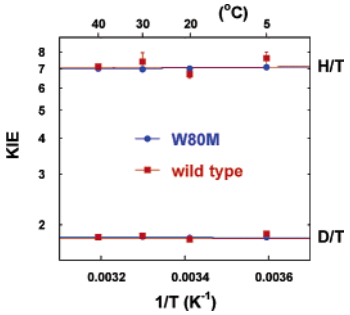

Figure 2. Arrhenius plots of H/T and D/T intrinsic KIEs with the wt (red squares) and W80M (blue circles) *ec*TS.[28] The lines are the exponential root mean square fitting of the intrinsic KIEs to the Arrhenius equation using the package KaleidaGraph.

side chain and it is less likely to alter the entrance to the active site,[7] but its side chain cannot stabilize a cation radical as aromatic residues can (e.g., W and F[5]). The ability of M to stabilize a cation radical was evaluated by the same method used by Schultz and co-workers[5] and extensively used to assess the ability of protein residues to stabilize cation radicals in general.[21] We found that the binding energy of Na^+ to dimethylthioether (the side chain of M) is 10.9 kcal/mol lower than that to indole (the side chain of W), which is a most significant difference.[22]

To compare the nature of the H-transfer between the wt and W80M *ec*TSs, we measured the KIEs of hydrogen to tritium (H/T) and deuterium to tritium (D/T) using a competitive method as described in detail elsewhere.[23] In this method, two isotopically labeled CH_2H_4folates at position C6 compete for the active site and yield a very accurate KIE on the second-order rate constant k_{cat}/K_M.[24] If the relation between H/T and D/T follows the semiclassical Swain−Schaad relationship,[25] it serves as a strong indication that the observed KIE is also the intrinsic KIE.[14,26] If, on the other hand, the ratio of ln(H/T) to ln(D/T) is smaller than its semiclassical value, the Northrop method[24,27] can be used as described previously.[10] For the wt *ec*TS, it was found that the observed and intrinsic KIEs are the same between 20 and 30 °C, but some kinetic complexity masked the intrinsic KIE at elevated and reduced temperatures so that the observed KIE appeared smaller.[20]

The current results for W80M indicate that the observed and intrinsic KIEs are identical from 5 to 40 °C, indicating no kinetic complexity on k_{cat}/K_M, but some on k_{cat}. Figure 2 compares the intrinsic KIEs of the wt and W80M *ec*TSs at temperature range of 5−40 °C (see data in Table S1[20]). Both enzymes appear to have very similar KIEs at all temperatures (within experimental error). The different theoretical models[11−13] that address that phenomenon would all agree that the temperature dependence of intrinsic KIEs is a very sensitive probe for the nature of the H-transfer, and that the data presented in Figure 2 indicate no measurable change in the nature of the H-transfer between wt and W80M *ec*TS.

The steady-state rates of the wt and W80M mutant were also compared across the same temperature range (see Table S2[20]). The data indicated that the wt enzyme was 1−2 orders of magnitude faster at all temperatures, and that their activation parameters (both entropy and enthalpy) were quite different ($\Delta T\Delta S^{\ddagger}_{20°C} = 2.2 \pm 0.9$ kcal/mol and $\Delta\Delta H^{\ddagger} = 3.8 \pm 0.9$ kcal/mol).[20] These data suggest that, in contrast to the H-transfer step, parameters affecting k_{cat} (protein rearrangements, product release, etc.) have been altered by the W to M mutation, in accordance with the conclusions of ref 7.

In summary, a sensitive probe of the nature of the H-transfer step has been applied here in search of the effect of W80 substitution to M in *ec*TS. The findings are in accordance with a alteration of the pre- and reorganization of the system toward and after the H-transfer step, but no significant change in the H-transfer step per se. Since M cannot stabilize cation radical as well as W, the findings support a hydride transfer mechanism (top of Scheme 1) dominating the reaction even for the wt enzyme. We hope that clarifying the chemical mechanism to TS will aid in rational drug design.

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Supporting Information Available: Experimental information and tables with the kinetic and activation data, and an Eyring plot. This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) KIE is the ratio of rates of two isotopically labeled reactants.
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