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Trapping of an Intermediate in the Reaction Catalyzed by Flavin-Dependent Thymidylate Synthase

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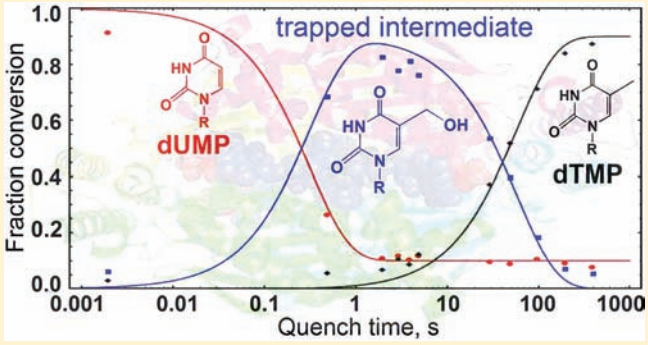
Supporting Information

ABSTRACT: Thymidylate is a DNA nucleotide that is essential to all organisms and is synthesized by the enzyme thymidylate synthase (TSase). Several human pathogens rely on an alternative flavin-dependent thymidylate synthase (FDTS), which differs from the human TSase both in structure and molecular mechanism. It has recently been shown that FDTS catalysis does not rely on an enzymatic nucleophile and that the proposed reaction intermediates are not covalently bound to the enzyme during catalysis, an important distinction from the human TSase. Here we report the chemical trapping, isolation, and identification of a derivative of such an intermediate in the FDTS-catalyzed reaction. The chemically modified reaction intermediate is consistent with currently proposed FDTS mechanisms that do not involve an enzymatic nucleophile, and it has never been observed during any other TSase reaction. These findings establish the timing of the methylene transfer during FDTS catalysis. The presented methodology provides an important experimental tool for further studies of FDTS, which may assist efforts directed toward the rational design of inhibitors as leads for future antibiotics.



INTRODUCTION

Thymidylate synthases (TSases) catalyze the last step in the de novo biosynthesis of the DNA nucleotide 2′-deoxythymidine-5′-monophosphate (dTMP) by reductively methylating the uracil moiety of 2′-deoxyuridine-5′-monophosphate (dUMP).[1,2] There are two currently known classes of TSases that differ in structure, sequence, and cofactor requirements.[3] The homodimeric enzymes encoded by the *thy*A gene (the *TYMS* gene in humans) are here termed classical TSases. These have been extensively studied, leading to the established kinetic and chemical mechanisms. Classical TSase enzymes use N^5,N^{10}-methylenetetrahydrofolate (CH_2H_4folate) for both the one-carbon methylene and the reducing hydride to form the C7 methyl of the dTMP product (Scheme 1a).[2] The more recently discovered *thy*X-encoded proteins utilize a noncovalently bound flavin adenine dinucleotide (FAD) prosthetic group to catalyze the redox chemistry and use CH_2H_4folate only as a methylene donor. These flavin-dependent thymidylate synthases (FDTSs) are found primarily in prokaryotes, including several pathogens and biological warfare agents.[3,4] The structural and mechanistic differences between FDTSs and classical TSases present an enticing new avenue for the development of antibiotics with a potential for minimal toxicity to humans.

Several mechanism-based drugs targeting classical TSases are available today (e.g., 5-fluorouracil, 5-trifluoromethyl-2′-deoxyuridine, raltitrexed, etc.).[5−7] The development and identification of these compounds relied greatly on the knowledge of the molecular mechanism of catalysis and identification of reaction intermediates for the classical TSase reaction. To emphasize the differences in catalysis from the FDTS enzymes under study here, we present the established chemical mechanism of the classical TSase (Scheme 1a). In short, the classical TSase reaction begins when an active-site cysteine covalently activates dUMP by Michael addition (step 2), which then undergoes a Mannich-type condensation with the methylene of CH_2H_4folate (step 3). The resulting bridged intermediate eliminates tetrahydrofolate (H_4folate) (step 4) to form an enzyme-bound exocyclic methylene intermediate, which accepts a hydride from H_4folate (step 5) to produce H_2folate and the product dTMP.[1,2] Step 3 of this mechanism is supported by the results of quenching experiments with wild-type *Lactobacillus casei* TSase (*Lc*TSase),[8] the crystal structure of the covalent complex of wild-type *Escherichia coli* enzyme with 5F-dUMP and CH_2H_4folate, and the SDS-PAGE isolation

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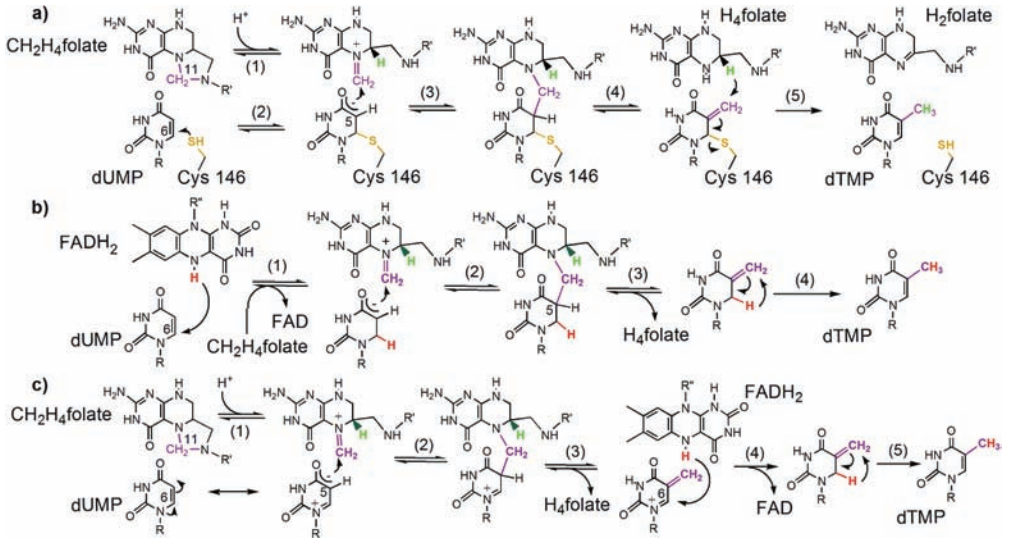
Scheme 1. Thymidylate Synthase Chemical Mechanisms[a]



[a](a) The mechanism for classical TSase.[1,2] (b) A mechanism proposed for FDTS where dUMP reduction occurs prior to methylene transfer.[13] (c) An alternative mechanism proposed for FDTS where reduction happens after methylene transfer.[17] The enzymatic nucleophile is orange, the methylene is purple, the reducing hydride from H_4folate is green, and the hydride from $FADH_2$ is red. R = 2′-deoxyribose-5′-phosphate; R′ = (*p*-aminobenzoyl)glutamate; R″ = adenosine-5′-pyrophosphate−ribityl.

of the enzyme-bound bridged intermediate (between steps 3 and 4) in reactions of the E60A and E60L mutants of *Lc*TSase with radiolabeled substrates.[9] The existence of the exocyclic methylene intermediate (between steps 4 and 5) is supported by the results of experiments with the W82Y mutant of *Lc*TSase, which allowed chemical trapping with β-mercaptoethanol under steady-state conditions.[10]

A few moderate inhibitors of FDTS enzymes have been developed, none of which are mechanism-based or have shown highly specific inhibition of FDTS over classical TSase.[11,12] Potent inhibitors of classical TSase, such as 5F-dUMP, produce moderate reversible inhibition of FDTS, and crystal structures of FDTS with 5F-dUMP (e.g., PDB entry 1tls) present noncovalently bound complexes that do not provide significant information about the catalytic mechanism or intermediate structures.[13,14]

One of the most convincing types of evidence for any chemical mechanism is provided by the identification and characterization of reaction intermediates. Although several chemical mechanisms have been proposed for FDTS, direct evidence of the identity of any of the proposed reaction intermediates has previously been unavailable.[13−19] In this work, we have shown that a reaction intermediate can be chemically trapped and isolated during a single-turnover oxidative half-reaction of FDTS.[20] The identification of the trapped intermediate described below indicates that it is not covalently bound to the enzyme and already includes the methylene originally carried by the CH_2H_4folate. This finding, together with the time course of accumulation and decay of this intermediate, limits the options for potential mechanisms and provides a time frame for key chemical events in FDTS catalysis.

RESULTS AND DISCUSSION

The identification of reaction intermediates is key to elucidating any chemical reaction's mechanism. Our recent spectroscopic findings[17] suggested a possible accumulation of intermediate(s) in the oxidative half-reaction of FDTS. Below we present the chemical trapping of such a reaction intermediate(s) and the identification of the trapped species.

Acid Quenching of the Oxidative Half-Reaction of FDTS. To determine whether any intermediate(s) accumulate during the FDTS-catalyzed reaction, a series of quench-flow experiments were performed. To increase the chance for intermediate trapping, we used a hyperthermophilic FDTS from *Thermotoga maritima* (*Tm*FDTS) and carried out the reactions at room temperature (significantly below *T. maritima*'s physiological 80 °C) to enhance the duration and magnitude of intermediate accumulation. Briefly, the FAD bound to *Tm*FDTS was stoichiometrically reduced with dithionite under anaerobic conditions, allowing $FADH_2$ to serve as the limiting reactant in the oxidative half-reaction under study (i.e., conversion of dUMP to dTMP). The dUMP was bound to the prereduced enzyme prior to the reaction, since in the catalytic turnover this substrate binds before CH_2H_4folate and probably even prior to the flavin reduction.[17,21,22] Oxidative FDTS half-reactions were then initiated by rapid mixing with CH_2H_4folate and quenched with 1 M HCl at various reaction times (for details, see the Experimental Section).

By quantitatively tracking the substrate dUMP and product dTMP by LC−MS, we were able to construct a time course for the oxidative FDTS half-reaction. Figure 1 shows the total ion counts measured at various reaction times for dUMP and dTMP along with their sum (which represents the total amount of material due to these species). It was noticed that for time points between ∼0.5 and 10 s, the sum of the ion counts for dUMP and dTMP was substantially less than those at the beginning and the end of the reaction. This observation suggests that a reaction intermediate accumulated during this time period. This finding was in accordance with the lag in product formation that we reported recently[17] but did not reveal the identity of the intermediate, leading to the next set of experiments.

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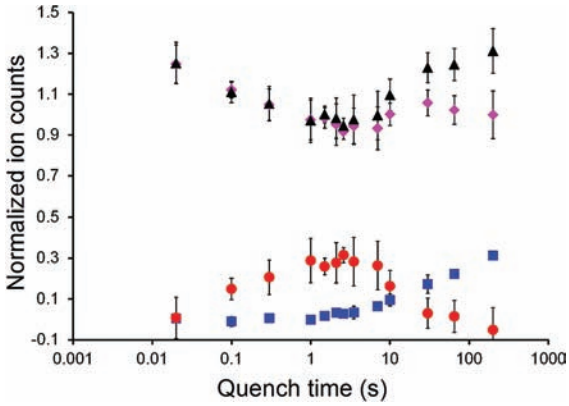

Figure 1. Single-turnover FDTS reaction. Total ion counts for dUMP and dTMP determined by LC−MS analysis are given for dUMP (purple ◆) and dTMP (blue ■). Notably, the sum of the counts for dUMP and dTMP (black ▲) was not conserved during the reaction, suggesting the accumulation of an intermediate (red ●).

Following Intermediate Formation Using Radiolabeled Substrates. To characterize the acid-trapped intermediate, it was first necessary to identify this material in the chromatographic analysis. To do so, ^{14}C-radiolabeled substrates were used in which the labeled carbon was on either the dUMP or the methylene of the CH_2H_4folate. The oxidative turnover of FDTS with the radiolabeled nucleotide, [2-^{14}C]dUMP, was quenched with acid at various times, as described above and in the Experimental Section. Figure 2a shows an HPLC radiochromatogram of a reaction quenched at 2 s, where under the reaction conditions ~80% of the total radioactivity was in the form of the trapped intermediate. It was possible to account for all of the initial radioactivity in the radiograms, and the total radioactive counts (dUMP, dTMP, and the newly identified peak) remained constant at all quenched reaction times (Figure 2c, in which each time point originated from a radiogram like the one presented in Figure 2a), suggesting that this single newly developing radioactive peak accounted for all of the missing nucleotide observed in the LC−MS analysis. It is also noteworthy that the fraction of total radioactivity associated with the new radioactive material accumulated and decayed during the course of the singe-turnover half-reaction, a behavior typical of enzymatic intermediates (Figure 2c).

To test whether the trapped species already contains the methylene from the cofactor CH_2H_4folate, we performed another crucial experiment wherein the enzyme mixed with nonlabeled dUMP was reacted with [11-^{14}C]CH_2H_4folate under the same conditions as above. By following the radiolabeled methylene, we found upon quenching at 2 s that a new radioactive peak had developed that had the same retention time as the peak observed when starting with [2-^{14}C]dUMP (Figure 2b). This clearly shows that the intermediate nucleotide being chemically trapped during the acid quenching had already undergone the condensation with CH_2H_4folate and that the carbon−carbon bond between the C5 of dUMP and the methylene had been formed prior to the formation of that intermediate.

Since reactions of classical TSase with the radioactive starting materials above would have led to an enzyme-bound intermediate, we carefully checked for radioactivity bound to enzyme in our experiments (see the Experimental Section). In accordance with the fact that we could account for all of the radioactivity in the radiograms (Figure 2), no radioactivity was identified on the enzyme from the same quenched samples, providing no support for enzyme-bound intermediate(s).

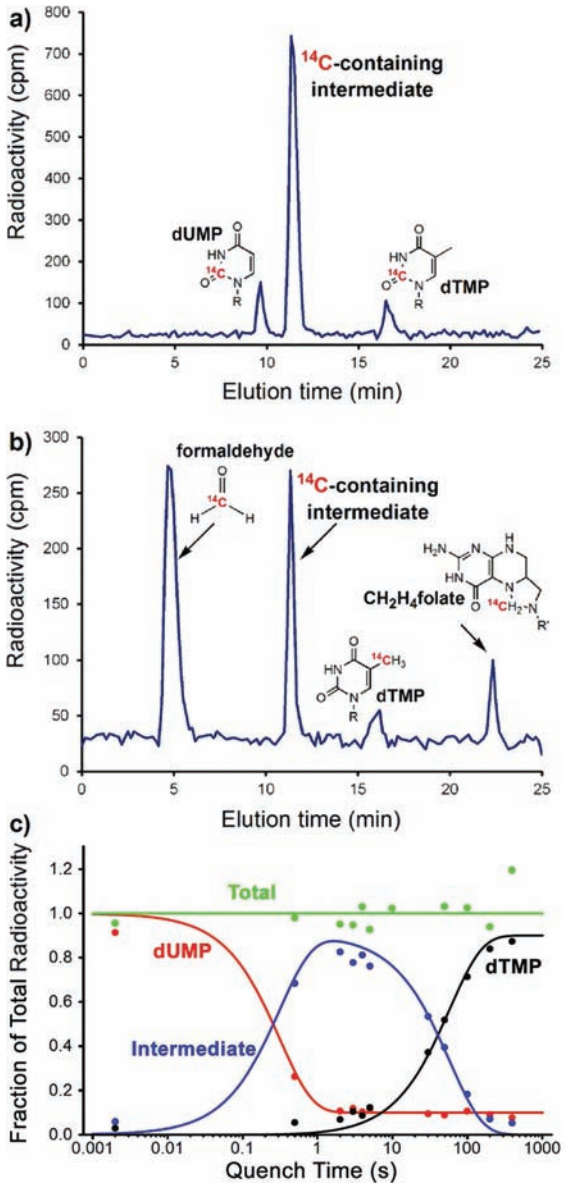

Figure 2. Intermediate trapping using ^{14}C-labeled substrates. (a, b) HPLC radiograms for FDTS reactions quenched at 2 s with 1 M HCl starting with either (a) ^{14}C-labeled dUMP or (b) ^{14}C-labeled CH_2H_4folate. The labeled carbon is shown in red. In both cases, the ^{14}C-containing trapped intermediate eluted at ~11 min, representing the same trapped species. (c) Single-turnover FDTS reaction with [2-^{14}C]dUMP as a radiotracer as a function of oxidative half-reaction time. Each time point results from a radiogram like the one shown in (a). The curves represent the kinetics of dUMP (red), intermediate (blue), and dTMP (black) globally fitted to a mechanism with one intermediate (i.e., a minimal model) as described in the Experimental Section. The total radioactive counts (dUMP, dTMP, and the intermediate combined) are shown in green.

Characterization and Identification of the Acid-Trapped Intermediate. Once the chromatographic elution time of the trapped intermediate was known, nonradioactive FDTS reactions were quenched at ~2 s, which produced the largest amount of trapped intermediate, and were purified by HPLC. The purified trapped intermediate was analyzed by electrospray ionization mass spectrometry (ESI-MS) and high-

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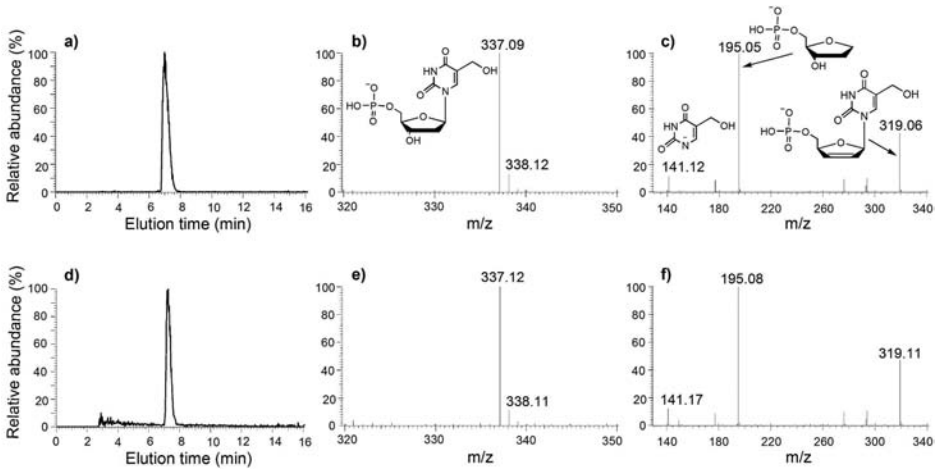
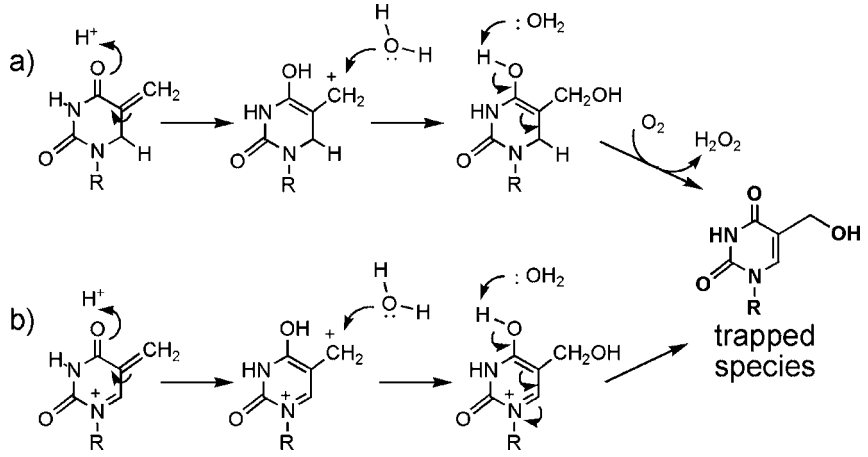


Figure 3. LC−ESI-MS data for the synthesized 5-hydroxymethyl-dUMP standard (top panels) and the trapped intermediate (bottom panels). Shown are (a, d) the chromatograms, (b, e) the MS spectra, and (c, f) the MS−MS spectra of the standard and the trapped intermediate, respectively. All spectra were collected in the negative-ion mode. The structures of the ions with the observed masses are shown. On the basis of these analyses, the synthesized standard was indistinguishable from the trapped intermediate.

Scheme 2. Possible Mechanisms for Acid Trapping of the Intermediates Proposed in (a) Scheme 1b and (b) Scheme 1c



resolution MS (HRMS), and its $[M - H^+]$ ion was found at *m/z* 337.0432, which is consistent with the exact mass and atomic composition of the product dTMP plus a hydroxyl group (i.e., 17 Da). The possibility of the trapped intermediate being 5-hydroxymethyl-dUMP was tested by comparing the HPLC retention time, HRMS data, and MS−MS results for the purified trapped intermediate to those for synthesized 5-hydroxymethyl-dUMP (see the Experimental Section), as shown in Figure 3. LC−MS analyses of reactions quenched at short (2 ms) or long (400 s) times did not contain 5-hydroxymethyl-dUMP, indicating that it was not a pre-existing contaminant. Furthermore, the accumulation and decay pattern observed when using radioactive substrates (Figure 2c) was consistent with the accumulation and decay of the intermediate as analyzed by LC−MS (Figure 1).

Mechanistic Implications. Reactions of classical TSase enzymes with the same radiolabeled substrates, as described above, led to the accumulation and isolation of the covalent bridged intermediate (Scheme 1a, between steps 3 and 4).[9] No such enzyme-bound species was found with FDTS (see the Experimental Section), and the reactant, product, and a single, soluble intermediate account for all of the radiolabeled trapped material. Thiol addition to the enzyme-bound exocyclic methylene intermediate in classical TSase mutants (Scheme 1a, between steps 4 and 5) resulted in chemical modification at C7 as a thioether.[10] Our efforts to use thiols as trapping reagents (see the Experimental Section) resulted in no trapped species during the FDTS-catalyzed reaction. These observations further emphasize the mechanistic differences between classical TSase and FDTS.

Substantial evidence has been published indicating that the FDTS-catalyzed reaction occurs without participation of an enzymatic nucleophile, a notable deviation from not only classical TSase but also other uridyl-methylating enzymes.[13,14] The mechanism for FDTS catalysis presented in Scheme 1b was proposed following mutagenesis and isotope-labeling studies with *Tm*FDTS, which did not support a nucleophilic attack on dUMP by any enzymatic residue.[13] In this mechanism, dUMP accepts a hydride from N5 of $FADH_2$ (step 1), generating an enolate that attacks the iminium form of CH_2H_4folate (step 2). Elimination of H_4folate (step 3) results in a putative exocyclic methylene intermediate, which would need to isomerize to form dTMP (step 4). In Scheme 2a, we suggest a mechanism for water addition to the isomer of dTMP proposed in Scheme 1b that could occur under acidic conditions. Notably, if 5-hydroxymethyl-dUMP does originate

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from treatment of this isomer of dTMP with acid, it would require oxidation (i.e., loss of a proton and two electrons) to form the acid-trapped species. Molecular oxygen is proposed as a likely hydride acceptor in the last step of the mechanism in Scheme 2a because the quenched reactions were exposed to oxygen immediately after quenching.

More recent studies that followed flavin absorbance in an oxidative half-reaction using the stopped-flow technique showed that flavin oxidation is not likely to be the initial step in FDTS catalysis and led to an alternative mechanistic option for FDTS catalysis, presented in Scheme 1c.[17] In this mechanism, dUMP is electronically polarized upon binding the enzyme, leading to nucleophilic attack from C5 of dUMP with no covalent activation at C6. Methylene transfer can then occur (step 2), followed by elimination of H_4folate (step 3). The resulting exocyclic methylene cation is then reduced by a hydride from $FADH_2$ (step 4) to form the exocyclic isomer of dTMP (if reduced at C6 as suggested in ref 14 on the basis of deuteration of C6), which can isomerize to form dTMP (step 5). The exocyclic methylene cation proposed to form after step 3 could readily undergo hydroxyl addition in an acidic medium to yield 5-hydroxymethyl-dUMP, as depicted in Scheme 2b. In this last case, water addition to the methylene of the cationic intermediate could also occur without prior protonation of the carbonyl oxygen, still resulting in 5-hydroxymethyl-dUMP.

The mechanism proposed in Scheme 1b requires a hydride transfer from the reduced flavin prior to the formation of a methylene-bridged intermediate, while the mechanism in Scheme 1c offers an option that does not require flavin redox chemistry until after the methylene transfer. In a further attempt to distinguish between these two mechanisms, we repeated the quenching experiments without prereducing the flavin (i.e., the enzyme had bound FAD rather than $FADH_2$) and [2-^{14}C]dUMP prior to mixing with CH_2H_4folate. We found no intermediates that accumulated, suggesting that the reduced flavin is required to form the intermediate that we have shown to contain the transferred methylene. While this observation is in accordance with the mechanism proposed in Scheme 1b, it does not eliminate the mechanism in Scheme 1c, because it is possible that even though the reduced flavin might not participate in redox chemistry until after the elimination of H_4folate (as suggested in Scheme 1c), it could play a role in a conformational change in the enzyme that would be needed to bring the two substrates into a reactive configuration.

Spectral evidence has also suggested that more than one reaction intermediate may accumulate during the FDTS reaction.[17] However, at all reaction times analyzed here, only one acid-trapped species was identified. Furthermore, in reactions using radiolabeled substrates, the total radioactive counts were always conserved among dUMP, 5-hydroxymethyl-dUMP, and dTMP, and the reaction time course fit reasonably well to a simple mechanism with one intermediate (Figure 2c). The two spectral species and the single acid-trapped intermediate could be consolidated if the acid modification of more than one intermediate leads to the formation of 5-hydroxymethyl-dUMP, for example, bridged dUMP–CH_2H_4folate species transiently accumulating prior to the formation of the exocyclic methylene intermediate.

CONCLUSIONS

Rapid acid-quenching experiments with *Tm*FDTS at room temperature resulted in chemical trapping of a reaction intermediate that was not covalently bound to the enzyme and was identified as 5-hydroxymethyl-dUMP. This provides evidence for the existence of noncovalently bound intermediates (Scheme 1b,c) and indicates the timing of carbon bond formation between dUMP and CH_2H_4folate. Importantly, this trapped species has not been isolated from any classical TSase, supporting the notion that the FDTS-catalyzed reaction proceeds via a unique chemical mechanism providing a new and unique target for mechanism-based antibiotic drug design. The identification of this acid-trapped intermediate adds new restrictions to possible mechanisms and eliminates several mechanisms proposed in the past.[15,16,18,19,23] The identified timing of intermediate(s) accumulation will be crucial for future efforts to characterize the unmodified intermediate(s) or the intermediate(s) following treatment with different chemical trapping agents. While the trapping of 5-hydroxymethyl-dUMP in the FDTS reaction emphasizes the mechanistic distinctions from classical TSase and eliminates some proposed mechanisms, further examination is needed before the mechanisms proposed in Scheme 1b,c can be distinguished and/or revised. Additionally, now that the acid-quenched intermediate has been identified, the synthesized 5-hydroxymethyl-dUMP can serve as a standard in acid-quenching experiments with FDTSs from mesophilic organisms and pathogens. This will test whether these enzymes follow the same catalytic mechanism as *Tm*FDTS, where for these FDTSs at room temperature an intermediate would be expected to accumulate to a lesser extent and at much earlier time points.

EXPERIMENTAL SECTION

Materials. All chemicals were reagent grade and used as purchased without further purification, unless otherwise specified. 2′-Deoxyuridine-5′-monophosphate (dUMP), 5-hydroxymethyl-2′-deoxyuridine (5-hydroxymethyl-dU), glucose oxidase powder, D-glucose, and formaldehyde solution (36.5% by weight) were obtained from Sigma. N^5,N^{10}-Methylene-5,6,7,8-tetrahydrofolate (CH_2H_4folate) was provided by Eprova Inc. (Schaffhausen, Switzerland). Radiolabeled [2-^{14}C]dUMP was purchased from Moravek Biochemicals. Radiolabeled [11-^{14}C]CH_2H_4folate was prepared according to the previously developed chemoenzymatic synthesis procedure.[24] Sodium dithionite powder was purchased from J. T. Baker, and tris(hydroxymethyl)-aminomethane was obtained from Research Products International Corp. The FDTS from *T. maritima* (TM0449, GenBank accession number NP228259) was expressed and purified as previously described.[25] Human deoxycytidine kinase mutant dCK-DM was a generous gift from Dr. Arnon Lavie at the University of Illinois-Chicago.

Synthesis of 5-Hydroxymethyl-2′-deoxyuridine-5′-monophosphate (5-Hydroxymethyl-dUMP). The synthesis procedure has been previously reported and was adapted from ref 26. More specifically, 5-hydroxymethyl-dUMP was synthesized by phosphorylation of commercial 5-hydroxymethyl-dU at 37 °C in a 100 mM Tris, 10 mM $MgCl_2$, 100 mM KCl buffer at pH 7.5. The final reaction mixture contained 2 mM 5-hydroxymethyl-dU, 4 mM ATP, and 10 units/mL of dCK-DM. The 5-hydroxymethyl-dUMP product was purified by HPLC–UV/vis and analyzed by LC–ESI-MS and MS–MS (Figure 3).

Analytical Methods. Separations were carried out on an Agilent series HPLC with a UV/vis diode array detector and 500TR series Packard flow scintillation analyzer. An analytical reversed-phase Supelco column (Discovery series 250 mm × 4.6 mm) was used with 50 mM KH_2PO_4 at pH 6 followed by a gradient of methanol. The concentration of enzyme for rapid-quenching experiments was determined by the 454 nm absorbance of bound FAD (ε = 11 300 cm^{-1} M^{-1}). Liquid chromatography–mass spectrometry (LC–MS) analysis was performed using a Dionex UltiMate 3000 LC system with an eluent gradient of water and acetonitrile containing 0.1% formic

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acid followed by a Finnigan LCQ Deca mass spectrometer. HRMS analysis was done on a Waters Q-TOF mass spectrometer.

Purification Methods. The trapped intermediate and 5-hydroxymethyl-dUMP were purified by HPLC using analytical (Discovery series 250 mm × 4.6 mm) and semipreparative (Discovery series 250 mm × 10 mm) reversed-phase Supelco columns, respectively. The mobile phase used for separation was a gradient of 50 mM KH_2PO_4 at pH 6 (for purification of the intermediate) or 100 mM KH_2PO_4 at pH 2 (for purification of synthesized 5-hydroxymethyl-dUMP) and methanol. Elution of the species of interest was followed by UV absorbance at 267 nm. Eluent containing the purified species was collected, lyophilized to dryness, and dissolved in H_2O for LC−MS, MS−MS, and HRMS analyses.

Acid Quenching of FDTS during the Oxidative Half-Reaction. A solution of oxidized FDTS (100 μM) was made anaerobic in a sealed tonometer by cycles of applied vacuum and equilibration with purified argon. The anaerobic enzyme was reduced stoichiometrically with a dithionite solution, as followed spectrophotometrically at 454 nm. The reduced FDTS was then mixed with dUMP (92 μM) from a side arm of the tonometer and loaded on a KinTek Chemical Quench-Flow instrument (model RQF-3), which had previously been scrubbed of oxygen with a glucose/glucose oxidase solution (50 units/mL). An anaerobic 400 μM CH_2H_4folate solution containing 50 units/mL glucose oxidase, 10 mM glucose (to ensure anaerobic conditions), and 30 mM formaldehyde (to stabilize CH_2H_4folate) was prepared. FDTS reactions were initiated by rapid mixing of the enzyme/dUMP and CH_2H_4folate solutions in the instrument and quenched at various time points with 1 M HCl. The quenched reactions were analyzed by HPLC with UV−vis diode array or radioactivity flow detection and by LC−MS.

Searching for an Enzyme-Bound Intermediate(s). The acid-quenched oxidative half-reactions of FDTS with [2-^{14}C]dUMP were spun in a microcentrifuge, and the radioactivity in the supernatant was quantified by liquid scintillation counting (LSC). The FDTS protein pellets (denatured enzyme) were analyzed on 10% SDS-PAGE. To test for protein-bound radioactive nucleotide, Coomassie-stained FDTS bands were excised, solubilized with 30% hydrogen peroxide, and counted by LSC. In a separate analysis, the enzyme pellets were resuspended in water and filtered to remove the residual soluble radioactivity. Both the filtrate and the washed pellets were then analyzed by LSC.

Searching for Intermediates by Thiol Trapping during the FDTS Reaction. Steady-state reactions containing 1 μM FDTS, 100 μM dUMP, 500 μM NADPH, 500 μM CH_2H_4folate, and a trace of [2-^{14}C]dUMP were incubated at 37 °C. β-Mercaptoethanol (3 M) was added after 1 min, and aliquots were withdrawn at 1.5, 3, and 5 min for analysis. Single-turnover FDTS (20 μM) reactions with 10 mM dithionite (in excess to ensure a high quantity of reactive thiol), 500 μM CH_2H_4folate, and limiting [2-^{14}C]dUMP (10 μM) were manually quenched with 1 M HCl at 1−2 s. The aliquots from both experiments were dried by speed-vacuum and resuspended in water or neutralized (for HCl samples) and analyzed by HPLC with a radioactivity flow detector.

Data Fitting for FDTS Reaction Kinetics. Mathematica was used to fit the data to a mechanism with one reaction intermediate (Figure 2c):

$$\text{dUMP} \xrightarrow{k_1} \text{Intermediate} \xrightarrow{k_2} \text{dTMP}$$

The following set of rate equations was used in the fitting:

$$\frac{d}{dt}[\text{dUMP}] = -k_1[\text{dUMP}]$$

$$\frac{d}{dt}[\text{Intermediate}] = k_1[\text{dUMP}] - k_2[\text{Intermediate}]$$

$$\frac{d}{dt}[\text{dTMP}] = k_2[\text{Intermediate}]$$

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Complete ref 25. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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