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Trapping of an intermediate in the reaction catalyzed by flavin-dependent thymidylate synthase (FDTs)

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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. Recently it has been shown that FDTs catalysis does not rely on an enzymatic nucleophile and 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 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 the efforts to rationally design 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 *thyA* gene (the *TYMS* gene in humans) are referred to here as classical TSases. These have been extensively studied, leading to the established kinetic and chemical mechanisms. Classical TSase enzymes use N⁵,N¹⁰-methylenetetrahydrofolate (CH₂H₄folate) 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 *thyX*-encoded proteins utilize a non-covalently-bound flavin adenine dinucleotide (FAD) prosthetic group to catalyze the redox chemistry and use CH₂H₄folate only as a methylene donor. These flavin-dependent thymidylate synthases (FDTs) are

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found primarily in prokaryotes, including several pathogens and biological warfare agents.^{3,4} The structural and mechanistic differences between FDTS and classical TSase present an enticing new avenue for the development of antibiotics with a potential for minimal toxicity to humans.

Several mechanism-based drugs targeting classical TSase are available today (e.g. 5-fluorouracil, 5-trifluoromethyl 2'-deoxyuridine, raltitrexed, etc.).⁵⁻⁷ 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 with an active site cysteine covalently activating dUMP via Michael addition (step 2), which then undergoes a Mannich-type condensation with the methylene of CH₂H₄folate (step 3). The resulting bridged intermediate eliminates H₄folate (step 4) to form an enzyme-bound exocyclic methylene intermediate, which accepts a hydride from H₄folate (step 5) producing H₂folate and the product dTMP.^{1,2} Step 3 of this mechanism is supported by the quenching experiments with wild-type *Lactobacillus casei* TSase⁸, the crystal structure of the covalent complex of wild-type *Escherichia coli* enzyme with 5F-dUMP and CH₂H₄folate, and the isolation on SDS-PAGE of the enzyme-bound bridged intermediate (between steps 3 and 4) in reactions of E60A and E60L mutants of *Lc*TSase with radiolabeled substrates.⁹ The existence of the exocyclic methylene intermediate (between steps 4 and 5) was supported by experiments with a 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 nor 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. P.D.B accession 1tIs) present non-covalently bound complexes that do not provide significant information about the catalytic mechanism or intermediate structures.^{13,14}

One of the most convincing evidence for any chemical mechanism is 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 been previously unavailable.¹³⁻¹⁹ In this article we show that a reaction intermediate can be chemically trapped and isolated during a single-turnover oxidative half-reaction^Ψ of FDTS. 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 CH₂H₄folate. This finding together with the time course of accumulation and decay of this intermediate limits the options for potential mechanisms and provides a timeframe for key chemical events of FDTS catalysis.

Results and Discussion

Identification of reaction intermediates is key to elucidation of any chemical reaction's mechanism. Our recent spectroscopic findings (ref 17) suggested 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.

^ΨThe conversion of dUMP to dTMP only occurs during the oxidative half-reaction where FADH₂ reacts to form FAD.

Acid-quenching of the oxidative half-reaction of FDTS

In order 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 *T. maritima* and carried out the reactions at room temperature – significantly below *T. maritima*'s physiological 80 °C – enhancing the duration and magnitude of intermediate accumulation. Briefly, the FAD bound to *T. maritima* FDTS enzyme was stoichiometrically reduced with dithionite under anaerobic conditions, allowing FADH₂ 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 pre-reduced enzyme prior to the reaction since in the catalytic turnover this substrate binds before CH₂H₄folate and probably even prior to the flavin reduction.^{17,20,21} Oxidative FDTS half-reactions were then initiated by rapid mixing with CH₂H₄folate and quenched with 1 M HCl at various reaction times (for details see Materials and Methods).

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, dTMP, and their sum (which represents the total amount of material accounted for by these species). It was noticed that for time points between ~ 0.5–10 seconds the sum of the ion counts for dUMP and dTMP was substantially less than at the beginning and the end of the reaction. This observation suggests that a reaction intermediate has accumulated during this time period. This finding is in accordance with the lag in product formation we reported recently,¹⁷ but does not reveal the identity of the intermediate, leading to the next set of experiments.

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, ¹⁴C-radiolabeled substrates were used where the labeled carbon was either on the dUMP or the methylene of the CH₂H₄folate. The oxidative turnover of FDTS with the radiolabeled nucleotide, [2-¹⁴C]-dUMP, was quenched with acid at various times, as described above and under the Materials and Methods. Figure 2a shows an HPLC-radiochromatogram of a reaction quenched at 2 seconds, where under the reaction conditions about 80% of the total radioactivity was in the form of the trapped intermediate. All the initial radioactivity was accounted for 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 originates from a radiogram like the one presented in Figure 2a), suggesting that all the missing nucleotide observed in the LC-MS analysis above was being accounted for by this single newly developing radioactive peak. It is also noteworthy that the fraction of total radioactivity associated with the new radioactive material accumulates and decays during the course of the single-turnover half-reaction, a behavior typical of enzymatic intermediates (Figure 2c).

To test whether the trapped species already contains the methylene from the cofactor CH₂H₄folate, we performed another crucial experiment wherein the enzyme mixed with non-labeled dUMP was reacted with [11-¹⁴C]-CH₂H₄folate under the same conditions as above. By following the radiolabeled methylene we found that when quenching at 2 seconds a new radioactive peak developed that had the same retention time as the peak observed when starting with [2-¹⁴C]-dUMP (Figure 2b). This clearly shows that the intermediate nucleotide that is being chemically trapped during the acid-quenching has already undergone the condensation with CH₂H₄folate, and the carbon-carbon bond between the C5 of dUMP and the methylene has 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 Materials and Methods). In accordance with the fact that all radioactivity was accounted for 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).

Characterization and identification of the acid-trapped intermediate

Once the chromatographic elution time of the trapped intermediate was known, non-radioactive FDTS reactions were quenched at ~2 seconds, which produced the largest amount of trapped intermediate, and were purified by HPLC. The purified trapped intermediate was analyzed by ESI-MS and high-resolution MS and was found to have an m/z of 337.0432 (M-1 ion), which is consistent with the exact mass and atomic composition of the product dTMP plus a hydroxyl group (i.e. 17 a.m.u.). The possibility of the trapped intermediate being 5-hydroxymethyl-dUMP was tested by the matching of HPLC retention time, HRMS, and MS-MS of the purified trapped intermediate compared to the synthesized 5-hydroxymethyl-dUMP (see Materials and Methods), as shown in Figure 3. LC-MS analyses of reactions quenched at short (2 msec) or long (400 sec) times did not contain 5-hydroxymethyl-dUMP indicating it is 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 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 Materials and Methods), and all the radiolabeled trapped material was accounted for in the reactant, product, and a single, soluble intermediate. 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 thioether.¹⁰ Our efforts to use thiols as trapping reagents (see Materials and Methods) 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 that did not support a nucleophilic attack on dUMP by any enzymatic residue.¹³ In this mechanism, dUMP accepts a hydride from the N5 of FADH₂ (step 1) generating an enolate that attacks the iminium form of CH₂H₄folate (step 2). Elimination of tetrahydrofolate (H₄folate, 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 under acidic conditions to the isomer of dTMP proposed in Scheme 1b. Notably, if 5-hydroxymethyl-dUMP does originate from treating this isomer of dTMP with acid, it requires 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 mechanism 2a because quenched reactions are exposed to oxygen immediately after quenching.

More recent studies, following flavin absorbance in an oxidative half-reaction by 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 H₄folate (step 3). The resulting exocyclic-methylene cation is then reduced by a hydride from FADH₂ (step 4) to form the exocyclic isomer of dTMP (if reduced at the C6 as suggested in ref 14 based on 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 acidic media 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 attempt to further distinguish between these two mechanisms, we repeated quenching experiments without pre-reducing the flavin, i.e., the enzyme had bound FAD rather than FADH₂, and [2-¹⁴C]-dUMP prior to mixing with CH₂H₄folate. We found no intermediates that accumulate, 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 H₄folate (as suggested in Scheme 1c), it does play a role in a conformational change in the enzyme, which is needed to bring both substrates in a reactive configuration.

Spectral evidence has also suggested that more than one reaction intermediate may accumulate during the FDTs reaction.¹⁷ Yet, at all reaction times analyzed here, only one acid-trapped species was identified. Furthermore, in reactions using radiolabeled substrates, the total radioactive counts are always conserved amongst dUMP, 5-hydroxymethyl-dUMP, and dTMP, and the reaction time-course fits 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, e.g., bridged dUMP-CH₂H₄folate 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, which was not covalently bound to the enzyme and was identified as 5-hydroxymethyl-dUMP. This provides evidence for the existence of non-covalently bound intermediates (Schemes 1b and 1c) and indicates the timing of carbon bond formation between dUMP and CH₂H₄folate. 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 mechanism and eliminates several mechanisms proposed in the past.^{15,16,18,19,22} The identified timing of intermediate(s) accumulation will be crucial for future efforts to characterize the intermediate following different chemical trapping agents, or the unmodified intermediate(s). 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 Schemes 1b and 1c are 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 FDTs from mesophilic organisms and pathogens. This will test whether these enzymes follow the same catalytic mechanism as *Tm*FDTs, where for these FDTs at room temperature an intermediate is expected to accumulate to a lesser extent and at much earlier time points.

Experimental

Materials

All chemicals were reagent grade and used as purchased without further purification, unless specified. 2'-deoxyuridine 5'-monophosphate (dUMP), 5-hydroxymethyl-2'-deoxyuridine (5-hydroxymethyl-dU), glucose oxidase powder, D-glucose, D₂O, and formaldehyde solution (36.5% by weight) were obtained from Sigma. N⁵,N¹⁰-methylene-5,6,7,8-tetrahydrofolate (CH₂H₄folate) was provided by Eprova Inc. (Schaffhausen, Switzerland). Radiolabeled [2-¹⁴C]-dUMP was purchased from Moravsek Biochemicals. Radiolabeled [11-¹⁴C]-CH₂H₄folate was prepared according to the previously developed chemoenzymatic synthesis procedure.²³ Sodium dithionite powder was purchased from J.T. Baker, and tris(hydroxymethyl)aminomethane was obtained from Research Products International Corp. The FDTs from *Thermatoga maritima* (TM0449, GenBank accession number NP228259) was expressed and purified as previously described.²⁴ 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 ²⁵. More specifically, the 5-hydroxymethyl-dUMP was synthesized by phosphorylation of commercial 5-hydroxymethyl-dU at 37 °C in a 100 mM Tris, 10 mM MgCl₂, 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 deoxycytidine kinase (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 UV/vis diode array detector and 500TR series Packard flow scintillation analyzer. An analytical reverse phase Supelco column (Discovery series 250 mm × 4.6 mm) was used with 50 mM KH₂PO₄ 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 ($\epsilon = 11,300 \text{ cm}^{-1}\text{M}^{-1}$). Liquid chromatography-mass spectrometry (LC-MS) analysis was performed on UltiMate 3000 Dionex LC system, using an eluent gradient of water and acetonitrile containing 0.1% formic acid, followed by a Finnigan LCQ deca mass spectrometer. High-resolution mass analysis was done on a Waters Q-TOF mass spectrometer.

Purification Methods

The trapped intermediate and 5-hydroxymethyl-dUMP were purified by HPLC using an analytical (Discovery series 250 mm × 4.6 mm) or a semipreparative (Discovery series 250 mm × 10 mm) reverse phase Supelco columns, respectively. Mobile phase used for separation was a gradient of 50 mM KH₂PO₄ at pH 6 (for purification of the intermediate) or 100 mM KH₂PO₄ 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 H₂O for LC-MS, MS-MS, high-resolution-MS analyses.

Acid quenching of FDTS during the oxidative half-reaction

A solution of oxidized FDTS (100 μ M) 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 μ M) from a side-arm of the tonometer and loaded on a KinTek Chemical Quench-Flow instrument (model RQF-3), which had been previously scrubbed of oxygen with a glucose/glucose oxidase solution (50 units/mL). An anaerobic 400 μ M CH₂H₄folate solution was prepared containing 50 units/mL glucose oxidase, 10 mM glucose (to assure anaerobic conditions) and 30 mM formaldehyde (to stabilize CH₂H₄folate). FDTS reactions were initiated by rapid mixing of the enzyme/dUMP and CH₂H₄folate 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, radioactivity flow detection and by LC-MS.

Searching for an enzyme-bound intermediate(s)

The acid-quenched oxidative half-reactions of FDTS with [2-¹⁴C]-dUMP were spun in a microcentrifuge, and the radioactivity in 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 re-suspended 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 μ M FDTS, 100 μ M dUMP, 500 μ M NADPH, 500 μ M CH₂H₄folate and a trace of [2-¹⁴C]-dUMP were incubated at 37°C. β -mercaptoethanol (3 M) was added at 1 minute and aliquots were withdrawn at 1.5, 3 and 5 min for analysis. Single turnover FDTS (20 μ M) reactions with 10 mM dithionite (excess to ensure a high quantity of reactive thiol), 500 μ M CH₂H₄folate and limiting [2-¹⁴C]-dUMP (10 μ M) were manually quenched with 1 M HCl at 1–2 seconds. The aliquots from both experiments were dried by speed-vacuum and re-suspended 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):

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

$$\begin{aligned}\frac{d}{dt}[\text{dUMP}] &= -k_1 \cdot [\text{dUMP}] \\ \frac{d}{dt}[\text{Intermediate}] &= k_1 \cdot [\text{dUMP}] - k_2 \cdot [\text{Intermediate}] \\ \frac{d}{dt}[\text{dTMP}] &= k_2 \cdot [\text{Intermediate}]\end{aligned}$$

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References

1. Carreras CW, Santi DV. *Annu Rev Biochem.* 1995; 64:721–762. [PubMed: 7574499]
2. Finer-Moore JS, Santi DV, Stroud RM. *Biochemistry.* 2003; 42:248–256. [PubMed: 12525151]
3. Myllykallio H, Lipowski G, Leduc D, Filee J, Forterre P, Liebl U. *Science.* 2002; 297:105–107. [PubMed: 12029065]
4. Lesley SA. *Proc Natl Acad Sci U S A.* 2002; 99:11664–11669. [PubMed: 12193646]
5. Sotelo-Mundo RR, Ciesla J, Dzik JM, Rode W, Maley F, Maley GF, Hardy LW, Montfort WR. *Biochemistry.* 1999; 38:1087–94. [PubMed: 9894005]
6. Takemura Y, Jackman AL. *Anticancer Drugs.* 1997; 8:3–16. [PubMed: 9147608]
7. Touroutoglou N, Pazdur R. *Clin Cancer Res.* 1996; 2:227–243. [PubMed: 9816165]
8. Moore MA, Ahmed F, Dunlap RB. *Biochemistry.* 1986; 25:3311–3317. [PubMed: 3089276]
9. Huang W, Santi DV. *J Biol Chem.* 1994; 269:31327–31329. [PubMed: 7989294]
10. Barrett JE, Maltby DA, Santi DV, Schultz PG. *JACS.* 1998; 120:449.
11. EsraÖnen F, Boum Y, Jacquement C, Spanedda MV, Jaber N, Scherman D, Myllykallio H, Herscovici J. *Bioorg Med Chem Lett.* 2008; 18:3628–3631. [PubMed: 18513963]
12. KoÅgler M, Vanderhoydonck B, De Jonghe S, Rozenski J, Van Belle K, Herman J, Louat T, Parchina A, Sibley C, Lescrinier E, Herdewijn PJ. *Med Chem.* 2011; 54:4847–4862.
13. Koehn EM, Fleischmann T, Conrad JA, Palfey BA, Lesley SA, Mathews II, Kohen A. *Nature.* 2009; 458:919–923. [PubMed: 19370033]
14. Koehn EM, Kohen A. *Arch Biochem Biophys.* 2010; 493:96–102. [PubMed: 19643076]
15. Agrawal N, Lesley SA, Kuhn P, Kohen A. *Biochemistry.* 2004; 43:10295–10301. [PubMed: 15301527]
16. Chernyshev A, Fleischmann T, Kohen A. *Appl Microbiol Biotechnol.* 2007; 74:282–289. [PubMed: 17216455]
17. Conrad JA, Ortiz-Maldonado M, Hoppe SW, Palfey BA. *J Am Chem Soc.* 2011 accepted for publication pending revision, and enclosed for reviewers only.
18. Graziani S. *J Biol Chem.* 2006; 281:24048–24057. [PubMed: 16707489]
19. Griffin J, Roshick C, Iliffe-Lee E, McClarty G. *J Biol Chem.* 2005; 280:5456–5467. [PubMed: 15591067]
20. Chernyshev A, Fleischmann T, Koehn EM, Lesley SA, Kohen A. *Chem Commun.* 2007:2861–2863.
21. Wang Z, Chernyshev A, Koehn EM, Manuel TD, Lesley SA, Kohen A. *FEBS Journal.* 2009; 276:2801–2810. [PubMed: 19459936]
22. Leduc D. *Proc Natl Acad Sci USA.* 2004; 101:7252–7257. [PubMed: 15123820]
23. Agrawal N, Mihai C, Kohen A. *Anal Biochem.* 2004; 328:44–50. [PubMed: 15081906]
24. Kuhn P, Lesley SA, Mathews II, Canaves JM, Brinen LS, Dai X, Deacon AM, Elslinger MA, Eshaghi S, Floyd R, Godzik A, Grittini C, Grzechnik SK, Guda C, Hodgson KO, Jaroszewski L, Karlak C, Klock HE, Koesema E, Kovarik JM, Kreusch AT, McMullan D, McPhillips TM, Miller MA, Miller M, Morse A, Moy K, Ouyang J, Robb A, Rodrigues K, Selby TL, Spraggon G, Stevens RC, Taylor SS, van den Bedem H, Velasquez J, Vincent J, Wang X, West B, Wolf G, Wooley J, Wilson IA. *Proteins: Struct Funct Genet.* 2002; 49:142–145. [PubMed: 12211025]
25. Hazra S, Ort S, Konrad M, Lavie A. *Biochemistry.* 2010; 49:6784–6790. [PubMed: 20614893]

Figure 1. Single-turnover FDTs reaction. Total ion counts for dUMP and dTMP determined by LC-MS analysis are given for dUMP (in purple) and dTMP (in blue). Notably, the sum of the counts for dUMP and dTMP (in Black) is not conserved during the reaction, suggesting the accumulation of an intermediate (in red).

Figure 2.

Intermediate trapping using ^{14}C -labeled substrates. HPLC radiograms for FDTS reactions quenched at 2 seconds with 1M HCl starting with either (a) ^{14}C -labeled dUMP, or (b) ^{14}C -labeled $\text{CH}_2\text{H}_4\text{folate}$. The labeled carbon is shown in red. In both cases, ^{14}C -containing trapped intermediate elutes at ~11 minutes, representing the same trapped species. Panel (c) presents single-turnover FDTS reaction with $[2-^{14}\text{C}]$ -dUMP as a radiotracer as a function of oxidative half-reaction time. Each time point results from a radiogram like 2a (the 2 sec. point). The curves represent dUMP (red), intermediate (blue), and dTMP (black) kinetics globally fitted to a mechanism with one intermediate, i.e., minimal model, as described under Materials and Methods. The total radioactive counts (dUMP, dTMP and the intermediate combined) are shown in green.

Figure 3. LC-ESI data for the synthesized 5-hydroxymethyl-dUMP standard (top panel) and the trapped intermediate (bottom panel). Shown are the chromatograms (**a and d**), MS spectra (**b and e**) and MS-MS spectra (**c and f**) 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. By these analyses, the synthesized standard was indistinguishable from the trapped intermediate.

Scheme 1.

Thymidylate synthase chemical mechanisms. 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 H₄folate is green, and the hydride from FADH₂ is red. R=2'-deoxyribose-5'-phosphate; R'=(p-aminobenzoyl)-glutamate; R''=adenosine-5'-pyrophosphate-ribityl.

Scheme 2.

Possible mechanisms for acid trapping of the intermediates proposed in Scheme 1b (a) and c (b).