Effects of Subunit Occupancy on Partitioning of an Intermediate in Thymidylate Synthase Mutants

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ABSTRACT: Experimental evidence for a 5-exocyclic methylene-dUMP intermediate in the thymidylate synthase reaction was recently obtained by demonstrating that tryptophan 82 mutants of \textit{Lactobacillus casei} enzyme produced 5-(2-hydroxyethyl)thiomethyl-dUMP (HETM-dUMP) (Barret, J. E., Maltby, D. A., Santi, D. V., and Schultz, P. G. (1998) J. Am. Chem. Soc. 120, 449–450). The unusual product was proposed to emanate from trapping of the intermediate with \(\beta\)-mercaptoethanol in competition with hydride transfer from \(\mathrm{H}_2\)folate to form dTMP. Using mutants of the C-terminal residue of thymidylate synthase, we found that the ratio of HETM-dUMP to dTMP varies as a function of \(\mathrm{CH}_2\mathrm{H}_2\)folate concentration. This observation seemed inconsistent with the conclusion that both products arose from a common intermediate in which \(\mathrm{CH}_2\mathrm{H}_2\)folate was already bound to the enzyme. The enigma was resolved by a kinetic model that allowed for differential partitioning of the intermediate formed on each of the two subunits of the homodimeric enzyme in forming the two different products. With three C-terminal mutants of \textit{L. casei} TS, HETM-dUMP formation was consistent with a model in which product formation occurs upon occupancy of the first completely bound subunit, the rate of which is unaffected by occupancy of the second subunit. With one analogous \textit{E. coli} TS mutant, HETM-dUMP formation occurred upon occupancy of the first subunit, but was inhibited when both subunits were occupied. With all mutants, dTMP formation occurs from occupied forms of both subunits at different rates; here, binding of cofactor to the first subunit decreased affinity for the second, but the reaction occurred faster in the enzyme form with both subunits bound to dUMP and \(\mathrm{CH}_2\mathrm{H}_2\)folate. The model resolves the apparent enigma of the cofactor-dependent product distribution and supports the conclusion that the exocyclic methylene intermediate is common to both HETM-dUMP and dTMP formation.

Thymidylate synthase (TS, EC 2.1.1.45) catalyzes the conversion of dUMP and 5,10-methylene-5,6,7,8-tetrahydrofolate (CH\(_2\)H\(_2\)folate) to dTMP and 7,8-dihydrofolate (H\(_2\)folate). In all cases examined, TS is a highly conserved dimer of identical subunits; the enzyme has been extensively studied in terms of structure, mechanism, and mutagenesis (1).

1 Abbreviations: TS, thymidylate synthase; CH\(_2\)H\(_2\)folate, 5,10-methylene-5, 6,7,8-tetrahydrofolate; H\(_2\)folate, 7,8-dihydrofolate; dUMP, 2′-deoxyuridine \(\delta\)-monophosphate; dTMP, thymidine \(\delta\)-monophosphate; HETM-dUMP, 5-(2-hydroxyethyl)thiomethyl-dUMP; TES, N-[tris(hydroxymethyl)ethyl]\(\delta\)-aminoethanesulfonic acid; \(\beta\)ME, \(\beta\)-mercaptoethanol; HPLC, high-performance liquid chromatography; TBAHS, tetra-\(\delta\)-butylammonium hydrogen sulfate; TFA, trifluoroacetic acid.
The salient features of the chemical pathway of the TS reaction are shown in Scheme 1. Within the reversible TS–dUMP–CH₃H₄folate complex, nucleophilic attack by the thiol of a cysteine residue (Cys 198 in Lactobacillus casei TS) at C-6 of dUMP converts the 5-carbon of dUMP to the enol I. This is followed by covalent bond formation between C-5 of dUMP and the one-carbon unit (C-11) of CH₃H₄folate to produce intermediate II. The C-5 proton of II is removed, leading to the β-elimination of H₂folate to give the intermediate III. Finally, hydride transfer from H₂folate to the exocyclic methylene group of III and β-elimination of the enzyme result in the products H₂folate and dTMP.

Direct evidence for the exocyclic intermediate III was recently obtained by studies of L. casei TS W82 mutants (2). These mutants produced 5-(2-hydroxyethyl)thiomethyl-dUMP (HETM-dUMP) in addition to dTMP. It was proposed that the unusual product was derived from trapping of intermediate III with β-mercaptoethanol, a component of the assay buffer, in direct competition with the reaction of hydride from H₂folate (Scheme 2). In the course of studies directed at understanding how extensive this reaction was in our library of TS mutants, we observed that the ratio of HETM-dUMP to dTMP varied as a function of CH₃H₄folate concentration. Since this seemed inconsistent with the previous conclusion that HETM-dUMP and dTMP arose from partitioning of the common intermediate III, we undertook a more detailed study of the reaction.

In the present work, we investigated the effect of CH₃H₄folate concentration on the kinetics of HETM-dUMP and dTMP formation by three C-terminal mutants of L. casei TS and one C-terminal mutant of E. coli TS. The enigma of cofactor-dependent product distribution was resolved by a model, in which the two subunits of the homodimeric enzyme catalyze formation of the two products at different rates. That is, there is a cofactor-induced nonequivalency of sites that results in different partitioning of intermediate III at each of the two subunits.

**MATERIALS AND METHODS**

Materials. (6R)-CH₃H₄folate was a gift from EPROVA AG (Schaffhausen, Switzerland). pThyA containing the E. coli TS gene was originally a gift from Frank Maley, New York Department of Public Health, Albany, NY. (3). Unless otherwise specified, all materials were obtained from commercial sources.

TS Mutants. The L. casei TS mutants V316Am, V316G, V316R, and W82Y have been previously described (4, 5). E. coli I264Am was prepared by mutagenesis of pThyA using the QuikChange site-directed mutagenesis kit (Strategene, La Jolla, CA) with 5′-GGCATTAAGCAGCCCGTGCTTAGATAATTACGAAACATCC-3′ and its reverse complement as mutagenic primers; the underlined TAG amber codon replaces the wild-type ATC. The resultant pThyA (I264Am) was used to transform E. coli strain γ2913recA (4), and the colonies resistant to ampicillin were selected (6). The mutation was confirmed by sequencing.

For expression and purification of I264Am, a single colony was inoculated into 100 mL of LB containing 50 μg/mL ampicillin, and after growth at 37 °C overnight, the culture was transferred to 4 L of LB containing 50 μg/mL ampicillin. Cells were harvested by centrifugation at 5000 rpm for 10 min and lysed by sonication, and the protein was purified by a modification of the procedure described for wild-type E. coli TS (3). After the phenyl sepharose procedure, about 5 mg of the total protein was applied to a POROS 20 HQ column (4.6 × 50 mm) previously equilibrated with 25 mM phosphate, pH 7.5, containing 20 mM βME. The enzyme was eluted using an 18 mL gradient of 0–0.6 M NaCl in 25 mM phosphate containing 20 mM βME, pH 7.5. The recovered protein (~4 mg) was >95% homogenous by SDS–PAGE. The total yield of pure I264Am from 4 L of culture was ~100 mg.

**TS HPLC Assay.** Reaction mixtures (500 μL) contained 250 μM dUMP, varying concentrations of (6R)-CH₃H₄folate, and 4.0 μM L. casei TS V316Am or a 1.0 μM concentration of other mutants in TES buffer (50 mM TES, 25 mM MgCl₂, 6.5 mM formaldehyde, 1 mM EDTA, 75 mM βME, pH 7.4). After 150 min for L. casei mutants or 60 min for E. coli I264Am, reactions were quenched by the addition of 30 μL of 20% TCA (w/v). Following centrifugation at 10 000 rpm for 10 min, the supernatant was neutralized by vortexing with an equal volume of 0.5 M tri-n-octylamine in dichloromethane (7). The aqueous layer was collected, and a 200 μL aliquot was chromatographed on a Beckman Ultrasphere IP column (4.6 × 250 mm) using a Hewlett-Packard 1090 HPLC equipped with a diode array detector. Solvent A was 5 mM KH₂PO₄ (pH 7.0) containing 5 mM TBAHS, and solvent B was a 1:1 mixture of 10 mM KH₂PO₄ (pH 7.0) containing 10 mM TBAHS and 100% acetonitrile. A gradient of 0–20% B was achieved in 30 min and 20–30% B in

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2 TS amino acids are numbered appropriately for each source; V316 of L. casei TS corresponds to I264 of E. coli TS.
FIGURE 1: Rate of formation of dTMP (▲) and HETM-dUMP (○) versus the concentration of CH₂H₄ folate at 250 μM dUMP for V316G, R, and Am. Data were fit using the Henri–Michaelis–Menten equation for a single-site system.

Data Processing. Kinetic and thermodynamic parameters were determined by nonlinear least-squares fits of data to the appropriate equations using the program KaleidoGraph 3.0.2 (Abelbeck Software) run on a Macintosh Power PC.

RESULTS AND DISCUSSION

L. casei C-Terminal Mutants. Development of the Model. Three TS V316 mutants were chosen for the present work: V316G, V316R, and V316Am. dTMP and HETM-dUMP, and only these products, were produced by each of the mutants, but in different relative amounts. Under saturating concentrations of dUMP and CH₂H₄folate, HETM-dUMP represents a small amount of the total product with V316G (34%), a moderate amount with V316R (44%), and a large amount with V316Am (78%).

In early experiments we demonstrated that the HETM-dUMP/dTMP formed was constant over time and varying dUMP concentration (data not shown). However, the HETM-dUMP/dTMP was affected by CH₂H₄folate concentration. Figure 1 shows the initial rates of formation of dTMP and HETM-dUMP for the three mutants studied under conditions of saturating dUMP concentration and varying concentrations of CH₂H₄folate. In all cases, the apparent CH₂H₄folate Kₘ values for HETM-dUMP formation are lower than those for dTMP formation, and the rate of formation of HETM-dUMP achieved saturation at lower cofactor concentration than that of dTMP. Further, in all mutants the initial rate of HETM-dUMP formation at low cofactor concentrations appears to be greater than that of dTMP formation, although with two mutants (V316G, V316R) the Vₘₐₓ is lower. Interestingly, V316Am, which produces dTMP very slowly, produces a relatively large amount of HETM-dUMP.

The formation of dTMP and H₂folate is energetically favorable and essentially irreversible (1, 9). However, there is no a priori reason to believe that the reaction of intermediate III with βME to give HETM-dUMP and H₂folate would be irreversible. It was considered possible that at low cofactor concentrations, H₂folate would irreversibly escape from the enzyme after HETM-dUMP formation, whereas with high cofactor concentrations, the increased H₂folate present in equilibrium with CH₂H₄folate might effectively reverse the reaction and result in an increased partitioning of III to dTMP. If correct, the folate dependence of the HETM-dUMP/dTMP might simply be explained by product inhibition. To test this possibility, we prepared HETM-dUMP and examined its ability to undergo a reverse reaction with H₂folate to form dUMP or dTMP. When 200 μM HETM-dUMP and 200 μM H₂folate were treated with V316G for 20 h, HPLC analysis showed no new nucleotide peaks. Thus,

another 10 min. Each peak was scanned from 220 to 400 nm and identified by its retention time (dUMP, 17.5 min; dTMP, 21 min; HETM-dUMP, 27 min), lamax (dUMP, 263 nm; dTMP, 269 nm; HETM-dUMP, 270 nm), and comigration with authentic standards. Nucleotide concentrations were determined by integrating the area under the peak at 265 nm and comparison to calibration standards.

The CH₂H₄folate concentration (S) at the time of measurement (t) was calculated by subtracting the dTMP formed from the initial cofactor concentration (S₀). Note that HETM-dUMP formation is not accompanied by CH₂H₄folate consumption; rather, H₂folate is formed, which is recycled to CH₂H₄folate in the presence of the excess formaldehyde. The average CH₂H₄folate concentration over the rate measurement was calculated as (S₀ + S)/2, and the overall rate was calculated as (S₀ − S)/t. For kinetic analysis, average CH₂H₄folate concentrations and overall rates were calculated for each data point and used in fitting the data to the appropriate equation (8).

Preparation of HETM-dUMP. A solution (8 mL) containing TES buffer, 1.25 mM dUMP, 1.25 mM CH₂H₄folate, and 13.5 μM W82Y was incubated at room temperature overnight. After TCA precipitation of protein, the products were isolated as described above. The aqueous layer was applied to a DE52 column (20 mL) equilibrated with 5 mM ammonium bicarbonate. After the column was washed with the same buffer, nucleotides were eluted with a 200 mL linear gradient of 10–100 mM ammonium bicarbonate. HETM-dUMP was further purified by chromatography on a Vydac C-18 HPLC column (10 × 250 mm) with solvent A as 0.1% TFA in H₂O and solvent B as 80% acetonitrile plus 20% A, using a linear gradient of 0–20% B in 20 min. The 1H NMR of the purified HETM-dUMP was identical to that reported (2).

The concentration of HETM-dUMP was determined spectrophotometrically, assuming a εₘₐₓ identical that of to dTMP (ε₂₅₀ = 9650 M⁻¹ cm⁻¹). Known concentrations of HETM-dUMP (0.050–6.4 nmol in 200 μL) were injected onto a Beckman Ultralphase IP column (4.6 × 250 mm) and the peak areas at 265 nm determined. A calibration curve of HETM-dUMP versus peak area served to estimate the concentration of nucleotides in the samples following HPLC separations.

Stability of HETM-dUMP in the Presence of V316G and H₂folate. A solution (500 mL) containing TES buffer, 200 mM HETM-dUMP, 200 mM H₂folate, and 10 mM V316G was incubated at room temperature for 16 h. After removal of the protein and neutralization as described above, the products were subjected to HPLC analysis.
under the experimental conditions used here, the formation of HETM-dUMP and H4folate is effectively irreversible, and the reverse reaction does not account for the cofactor-dependent change in HETM-dUMP/dTMP.

It has been well established that the two subunits of the homodimeric TS are occupied by both substrates, although binding to the first decreases, and under some conditions prevents, binding of the second (9–14). Regardless, others and we have usually assumed a conventional single (or independent equivalent) site system for kinetic analysis because the different sites are sufficiently similar that kinetic differences are not easily resolved. Scheme 2 shows the two-subunit model pathway we have used to describe the TS-catalyzed formation of dTMP and HETM-dUMP; the model is depicted and analyzed under conditions of saturating dUMP concentration and varying concentrations of CH2H4folate. E–dUMP2 represents the heterodimer saturated with dUMP but not containing CH2H4folate, E–dUMP2–CH2H4folate is the form with both subunits occupied by dUMP and a single subunit site occupied by CH2H4folate, and E–dUMP2–CH2H4folate2 is the form with both sites occupied by both dUMP and CH2H4folate. In this model, K1 and K2 are K_m values assumed to be dissociation constants for the referred-to enzyme forms, and k1 and k2 represent the k_cat values for the formation of each product by E–dUMP2–CH2H4folate and E–dUMP2–CH2H4folate2, respectively. Figure 2 shows simulations of concentrations of the three enzyme forms as a function of CH2H4folate concentration assuming K1 = K2, K1 = 3K1, and K2 = 10K1. Here, E–dUMP2–CH2H4folate increases to a maximum when K1 < [CH2H4folate] < K2, then decreases, and approaches zero as [CH2H4folate] > K2, and E–dUMP2–CH2H4folate2 predominates over other enzyme forms. It can be seen from Figures 1 and 2 that E–dUMP2–CH2H4folate is not the sole reactive form for dTMP or HETM-dUMP formation; otherwise, the V_{max} values would decrease at high cofactor concentrations.

The assumptions and tenets used in the ensuing analysis are as follows. First, as previously proposed (2) both dTMP and HETM-dUMP emanate from partitioning of the covalent ternary complex III, so the formations of both products have the same pathway up to and including formation of the intermediate.\(^{(3)}\) Second, both bound enzyme forms are active in producing either product; thus, the apparent K_m and k_cat values obtained by a single-site model are each composed of two unresolved microscopic constants. Third, we assume that k_cat for either product is rate determining (i.e., pre-equilibrium formation of reversibly bound enzyme forms), as shown for dTMP formation with wild-type E. coli enzyme (1, 9). Thus, the rate of formation of either product as described by eq 1 expands into one for the rapid-equilibrium mechanism in eq 2. Here, the formation of products is described as a function of the dissociation (K) and rate (k) constants, and K_m values reflect dissociation constants of the cofactor.

\[

v = k_1[E–dUMP_2–CH_2H_4folate] + k_2[E–dUMP_2–CH_2H_4folate_2] \quad (1)
\]

\[

v = \frac{k_1}{K_1/[CH_2H_4folate] + 1 + [CH_2H_4folate]/K_2} + \frac{k_2}{K_1K_2/[CH_2H_4folate]^2 + 1 + K_2/[CH_2H_4folate]}[E_i] \quad (2)
\]

Since the K1 and K2 values for cofactor binding must be the same for both reactions, and since the HETM-dUMP reaction shows a lower apparent K_m than dTMP formation, we conclude that the HETM-dUMP K_m reflects K1 and the apparent dTMP K_m reflects a composite of and lies between K1 and K2. Importantly, rate saturation for HETM-dUMP formation occurs in apparent accord with occupancy of a single site; however, as previously argued, the reaction cannot

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\(^{(3)}\) A reviewer suggested a model in which H4folate dissociates from the enzyme–exocyclic methylene intermediate before 2-mercaptoethanol enters to react with it. The increased H4folate present in equilibrium at higher cofactor concentrations would result in higher occupancy of the complex, which might explain increased partitioning of the intermediate toward dTMP at high cofactor concentration. If correct, exogenous H4folate would have the same effect. TS W82A was treated with 30 mM cofactor, 250 mM dUMP, and varying H4folate concentration without added formaldehyde, and the dTMP and HETM-dUMP were measured. With increasing H4folate concentration, the rate of product formation decreased in accord with competitive inhibition with a K_i of about 150 mM. However, over a range of 15–125 mM H4folate the dTMP/HETM-dUMP remained constant at 1.25. This result argues against the suggestion that increased partitioning toward dTMP results from loss of dUMP from the enzyme–exocyclic methylene intermediate.
occurred solely from the enzyme form with single-subunit occupancy; otherwise, the concentration of E–dUMP–CH₂H₄folate and therefore the reaction rate would decrease at high cofactor concentration. To explain this apparent enigma in context of the enzyme forms present under varying cofactor concentration (Figure 2, Scheme 2), k₁ must be equal to k₂ for HETM-dUMP formation; in effect, occupancy of the second subunit by both substrates does not affect the rate of HETM-dUMP formation. As indicated in Figure 2, if k₂ were lower than k₁, at high concentrations of CH₂H₄folate, E–dUMP–CH₂H₄folate₂ would predominate and the rate would decrease; if k₂ were greater than k₁, at high concentrations of CH₂H₄folate, E–dUMP₂–CH₂H₄folate₂ would predominate and the rate would increase. Since neither is the case, we conclude that k₁ = k₂ for HETM-dUMP formation.

From the rate dependence of HETM-dUMP formation versus CH₂H₄folate (Figure 1), we directly obtained K₁ (Kₘ) and k₂ (from Vₘₐₓ). With these constants as constraints, best fits of the data for HETM-dUMP formation to eq 2 showed that k₃ values were essentially identical to k₂ (Table 1). As expected, since k₁ = k₂, k₃ values were insensitive to k₂ values used in eq 2.

In dTMP formation, both sites are active and the apparent Kₘ for the cofactor is a composite of K₁ and K₂. To fit the experimental data to eq 2, we used the K₁ value measured from HETM-dUMP formation and k₂ from the Vₘₐₓ of dTMP formation (Figure 1). Using k₁ and k₂ as constraints, best fits of the data to eq 2 yielded the values for k₁ and k₂ given in Table 1. Experimentally determined values of k₃ obtained by extrapolating plots of S/ν versus S to zero cofactor concentration (8) were in excellent agreement with those obtained from eq 2. It is noted that the data for dTMP formation can be fit equally well (R² = 0.99) to eq 2 or the Henri–Michaelis–Menten equation for a single (or two equivalent independent) site(s) (R² = 0.99), and in the absence of other information the differential activity of the two sites cannot be resolved.

The partition coefficient of intermediate III, kₚ[HETM-dUMP]/(kₚ[HETM-dUMP] + kₚ[dTMP]), from either of the two reactive enzyme forms may be calculated from the values in Table 1. The partitioning of III in the fully occupied enzyme can also be experimentally determined as the fraction of HETM-dUMP (i.e., HETM-dUMP/[HETM-dUMP + dTMP]), formed at saturating cofactor concentrations where all enzyme is present as E–dUMP₂–CH₂H₄folate₂. Finally, the fraction of III simultaneously formed from all enzyme forms at any concentration of the cofactor may be calculated by using eq 3 and the constants given in Table 1. As shown in Figure 3, there is excellent agreement between values calculated from eq 3 and the experimental data obtained at varying cofactor concentrations.

\[
\frac{[\text{dTMP}]/([\text{HETM-dUMP}] + [\text{dTMP}])} = \frac{[\text{K}_1 \cdot \text{CH}_2\text{H}_4\text{folate}] \cdot k_1(\text{dTMP}) + [\text{CH}_2\text{H}_4\text{folate}] \cdot k_2(\text{dTMP})}{[\text{K}_1 \cdot \text{CH}_2\text{H}_4\text{folate}] \cdot k_1(\text{HETM-dUMP}) + \text{CH}_2\text{H}_4\text{folate} \cdot k_2(\text{HETM-dUMP}) + \text{CH}_2\text{H}_4\text{folate} \cdot k_2(\text{dTMP}) + [\text{HETM-dUMP}] + [\text{dTMP}])}
\]

E. coli I264Am. Verification of the Model. We felt that structural studies of a mutant that produced HETM-dUMP might reveal information regarding how this unusual product
was formed. Of the mutants described thus far, L. casei TS V316Am produced the largest amount of HETM-dUMP over dTMP, and thus seemed an appropriate candidate for structure determination. We prepared the corresponding E. coli TS I264Am since ternary complexes of the E. coli enzyme have been found to be more amenable to crystallization (15). However, kinetic characterization of the mutant revealed properties different from those observed with the corresponding L. casei mutant.

At saturating cofactor concentration, E. coli TS I264Am was ∼30-fold more active at dTMP production than L. casei TS V316Am but much less efficient at HETM-dUMP production (Table 1). Moreover, with varying cofactor concentration the rate of HETM-dUMP formation increased to a maximum at about 100 μM CH₂H₄folate and then decreased with increasing concentrations (Figure 4). Comparison of Figure 4 to Figure 2 shows that varying cofactor concentration in the E. coli TS I264Am-catalyzed formation of HETM-dUMP tracks the enzyme form occupied at a single subunit, E−(dUMP)₂−CH₂H₄folate. The data were fit to eq 2 by constraining K₂ over the range of 0−0.07 min⁻¹; the latter is the last data point sampled, and represents an upper limit. The best fits (R² ≥ 0.99) occurred with k₁ = 0.34, k₂ = 0−0.003, K₁ = 29−30 μM, and K₂ = 270 to 280 μM. The k₁ obtained is in excellent agreement with the experimentally determined value obtained by a plot of S/ν vs S (0.38 min⁻¹). Thus, with E. coli I264Am, E−(dUMP)₂−CH₂H₄folate is active in the formation of HETM-dUMP, but in contrast to the corresponding L. casei TS mutant, full occupancy of both subunits causes a complete or near-complete inhibition of the reaction (i.e., k₂ = 0−0.003). The differential behavior of the fully occupied enzyme forms of the E. coli and L. casei C-terminal deletion mutants in HETM-dUMP formation, together with their excellent fits to eq 2, adds credence to the model proposed in Scheme 2.

dTMP formation by E. coli TS I264Am behaved as did the L. casei TS mutants, with both sites active in forming product. The rate data were fit to eq 2 by constraining K₁ to the range obtained for HETM-dUMP formation and to the k₂ for dTMP formation (R² = 0.99; Figure 4). The k₂ and k₁ values thus obtained were 216−220 μM and 0.34−0.35 min⁻¹, respectively. When eq 2 was constrained by K₁ and K₂ obtained from HETM-dUMP formation, the fit was also excellent (R² = 0.99) and the calculated values were in agreement with those reported above (k₁ = 0.4, k₂ = 1.4 min⁻¹; Figure 4). The apparent Kₘ for dTMP using a single-site model (144 μM) lies intermediate to the two microscopic dissociation constants K₁ and K₂. Finally, for E. coli I264Am, the partitioning of III to products at any cofactor concentration calculated from eq 3 is in excellent agreement with the experimental data (Figure 3, panel B).

**SUMMARY**

Using data obtained from three C-terminal L. casei TS mutants and one analogous E. coli TS mutant, we constructed a congruent model for the reaction pathways leading to the formation of HETM-dUMP and dTMP. The model embraces the original proposal that the two products emanate from a common steady-state intermediate (2). It explains the cofactor dependence of the relative amounts of products formed by different partitioning of the steady-state intermediate III in forms of the homodimeric enzyme that are occupied by substrates at one εFᵣᵣᵣS two subunits. In the case of L. casei V316 mutants, HETM-dUMP formation can be explained by a model in which the unusual product forms upon occupancy of the first bound subunit, and the rate is unaffected by occupancy of the second. For E. coli TS I264Am, HEMT-dUMP is formed upon occupancy of the first bound subunit, but is inhibited by occupancy of the second. For all mutants studied, dTMP formation emanates from occupied forms of both subunits at different rates; there is a cooperative effect in which binding of cofactor at the first subunit decreases binding to the second, and the reaction occurs ∼4−8-fold faster in the completely occupied enzyme form. Utilizing the k₁ values obtained from HETM-dUMP formation with these TS mutants enabled resolution of differences in the kinetic parameters of the two subunits in dTMP formation that are not easily determined by conventional steady-state kinetic analysis. Indeed, we can confidently assign the different rate and cofactor binding constants associated with the singly and fully occupied subunit forms of the homodimeric enzyme. Further, the approach should be generally applicable to other heterodimeric systems (e.g., wild-type TS and other mutants), provided an independent method is available to determine K₁. With the ability to dissect the dependency of product formation εFᵣᵣᵣS cofactor concentration, we are now in a

These expanded equations were substituted into the rate expression

\[ v = k_1[E - dUMP_2 - CH_2H_4 folate] + k_2[E - dUMP_2 - CH_2H_4 folate_2] \] (1)

to give eq 2.

\[ v = \frac{k_1}{K_1([CH_2H_4 folate] + 1 + [CH_3H_2 folate]/K_2)} + \frac{k_2}{K_1K_2/[CH_2H_4 folate]^2 + 1 + K_2/[CH_2H_4 folate]} [E_1] \] (2)

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APPENDIX

The derivation of eq 2 is based on the pathway shown in Scheme 2 and assumes a rapid equilibrium of enzyme forms prior to a rate-determining catalytic reaction. The potentially reactive enzyme forms were defined in terms of dissociation constants

\[ K_1 = \frac{[E - dUMP_2][CH_2H_4 folate]}{[E - dUMP_2 - CH_2H_4 folate]} \]
\[ K_2 = \frac{[E - dUMP_2 - CH_2H_4 folate][CH_2H_4 folate]}{[E - dUMP_2 - CH_2H_4 folate_2]} \]

and the total enzyme was described as the sum of all enzyme forms

\[ [E_1] = [E - dUMP_2] + [E - dUMP_2 - CH_2H_4 folate] + [E - dUMP_2 - CH_2H_4 folate_2] \]

The potentially reactive enzyme forms were described in terms of the total enzyme, dissociation constants, and cofactor concentration

\[ [E - dUMP_2 - CH_2H_4 folate]/[E_1] = \frac{1}{K_1/[CH_2H_4 folate] + 1 + [CH_3H_2 folate]/K_2} \] (4)
\[ [E - dUMP_2 - CH_2H_4 folate_2]/[E_1] = \frac{1}{K_1K_2/[CH_2H_4 folate]^2 + 1 + K_2/[CH_2H_4 folate]} \] (5)

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