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Thymidylate Synthase with a C-Terminal Deletion Catalyzes Partial Reactions but Is Unable To Catalyze Thymidylate Formation†

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Received January 17, 1992; Revised Manuscript Received March 18, 1992

ABSTRACT: The V316Am mutant of *Lactobacillus casei* thymidylate synthase has a single amino acid deletion at the C-terminus which abolishes catalysis of dTMP formation. However, V316Am catalyzes two partial reactions which require covalent catalysis: a CH_2H_4folate-dependent exchange of the 5-hydrogen of dUMP for protons in water and a thiol-dependent dehalogenation of 5-bromo- and 5-iodo-dUMP. These reactions proceed with k_{cat} and K_m values similar to those of the wild-type TS-catalyzed reactions. dUMP, dTMP, and FdUMP are competitive inhibitors of the debromination reaction with K_i values similar to those obtained with wild-type enzyme. These results show that removal of the terminal valine does not alter the ability of the enzyme to bind to or form covalent bonds with nucleotide ligands. V316Am also forms a covalent ternary complex with FdUMP and CH_2H_4folate. However, the affinity of the TS-FdUMP complex for the cofactor is reduced, and the rate of covalent ternary complex formation and its stability are significantly lower than with wild-type TS. These results allow us to place the major defects of the mutation on steps that occur subsequent to initial CH_2H_4folate binding.

Thymidylate synthase (TS,[1] EC 2.1.1.45) catalyzes the reductive methylation of dUMP by CH_2H_4folate to produce dTMP and H_2folate. The mechanism of TS has been extensively characterized, and three-dimensional structures of free and bound forms of the enzyme have been determined (Hardy et al., 1987; Perry et al., 1990; Montfort et al., 1990; Matthews et al., 1990). There is much interest in correlating enzyme structure and function using mutagenesis, and to that end several hundred mutants of *Lactobacillus casei* and *Escher-*

† This work was supported by Public Health Service Grant CA-14394 from the National Institutes of Health. C.W.C. was the recipient of a Research Supplement for Minority Graduate Research Assistants.
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[1] Abbreviations: TS, thymidylate synthase; V316Am, mutant *L. casei* TS lacking a C-terminal Val; dUMP, 2′-deoxyuridine 5′-monophosphate; CH_2H_4folate, 5,10-methylene-5,6,7,8-tetrahydrofolate; dTMP, thymidine 5′-monophosphate; H_2folate, 7,8-dihydrofolate; CB3717, 10-propargyl-5,8-dideazafolate; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; BrdUMP, 5-bromo-2′-deoxyuridine 5′-monophosphate; IdUMP, 5-iodo-2′-deoxyuridine 5′-monophosphate; TES, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid; DTT, dithiothreitol.

0006-2960/92/0431-6038$03.00/0

ichia coli TS have been produced (Climie et al., 1990) (Michaels et al., 1990). The mutants are first screened for activity by genetic complementation in a TS-deficient *E. coli* host, and mutants of interest are characterized in detail.

We are also interested in understanding why inactive mutants are inactive. The hope is that some mutations will produce identifiable defects in the normal catalytic pathway. Such mutants may permit the study of isolated "partial" reactions preceding the defect and may result in the accumulation of intermediates amenable to study. In the process of analyzing the mutants described by Climie et al. (1990), many were screened for their ability to form tight complexes with FdUMP and CH_2H_4folate. Several inactive mutants were found that bound the inhibitor, including the V316Am mutant which lacks the C-terminal valine found in the wild-type enzyme.

The C-terminal valine is conserved among 17 of the 23 known TS sequences (Perry et al., 1990; unpublished data) and has been implicated in catalysis by studies of carboxypeptidase inactivation (Aull et al., 1974). Although removal of the terminal valine from one subunit of *L. casei* TS leads to complete loss of activity, dUMP still binds with its normal affinity (Galivan et al., 1976). Likewise, removal of the C-terminal residue from both subunits of either *L. casei* or *E. coli* TS by mutagenesis also renders the enzyme unable to complement TS-deficient *E. coli* hosts (Climie et al., 1990; Michaels et al., 1990). Crystallographic studies show that, upon folate binding, the C-terminus undergoes a large conformational change to form an active site "lid" and part of the folate binding site (Montfort et al., 1990). In this paper, we show that although the V316Am mutant TS is unable to catalyze the formation of dTMP, it does catalyze the dehalogenation of 5-Br(I)dUMP and the exchange of tritium from [5-^{3}H]dUMP. V316Am also forms a covalent ternary complex with FdUMP and CH_2H_4folate.

Materials and Methods

Materials. E. coli strain χ2913 (ΔthyA572) was a gift from Russell Thompson, University of Glaslow. The plasmids pSCTS9 and pSCTS-V316Am have been described (Climie et al., 1990). Phosphocellulose P11 and DEAE-cellulose (DE52) were obtained from Whatman, and hydroxyapatite (Bio-Gel HTP) was purchased from Bio-Rad. [5-^{3}H]dUMP (13.2 Ci/mmol), [6-^{3}H]dUMP (15 Ci/mmol), [6-^{3}H]FdUMP (20 Ci/mmol), and [2-^{14}C] dUMP (56 mCi/mmol) were from Moravek Biochemicals. (6*R*)-CH_2H_4folate was a generous gift from SAPEC S.A. (Lugano, Switzerland), and (6*R*)-[6-^{3}H]CH_2H_4folate (26.6 mCi/mmol) was as described (Bruice & Santi, 1982). Nucleotides were obtained from Sigma and were purified by DEAE-cellulose chromatography (Wataya & Santi, 1977). Formaldehyde was obtained from Mallinckrodt and quantitated with the Nash reagent (Nash, 1953). All other materials were obtained from commercial sources and used without purification.

Protein Purification. V316Am was purified from the *E. coli* strain χ2913 (thy$^-$) containing pSCTS-V316Am (Climie et al., 1990). Three 1-L flasks of LB medium supplemented with 50 μg/mL thymidine and 50 μg/mL ampicillin were each innoculated with 25-mL overnight cultures of χ2913/pSCTS-V316Am and grown for 24 h at 37 °C. Cells were harvested by centrifugation, washed with 500 mL of 150 mM NaCl, and stored at −80 °C. All subsequent steps were performed at 4 °C. Cells were resuspended in 75 mL of 10 mM KH_2PO_4, pH 6.8 (buffer A), and lysed by passing through a French pressure cell at 18 000 psi. Cellular debris was removed by centrifugation, and the supernatant was loaded onto a phosphocellulose column (2.5 cm × 15 cm) equilibrated with buffer A. The column was washed with 1 L of buffer A before eluting TS with a 500-mL linear gradient from 0 to 600 mM KCl in buffer A. TS-containing fractions eluting between 100 and 290 mM KCl were pooled and loaded onto a hydroxyapatite column (2.5 cm × 8 cm) equilibrated with 10 mM KH_2PO_4, pH 7.0 (buffer B). The column was washed with 100 mL of buffer B before eluting with a 350-mL linear gradient to 350 mM KH_2PO_4, pH 7.0. TS-containing fractions eluted between 20 and 190 mM KH_2PO_4 and were concentrated to 10 mg/mL using an Amicon ultrafiltration device equipped with a YM-10 membrane. The purification was followed by SDS–PAGE on 12% gels (Laemmli, 1970) and by monitoring debromination of BrdUMP (see below). The enzyme was stored at −80 °C until use.

Enzyme Assays. TS activity was monitored spectrophotometrically at 340 nm as described (Pogolotti et al., 1986). The standard TES/DTT assay buffer contained 50 mM TES, pH 7.4, 25 mM $MgCl_2$, 6.5 mM formaldehyde, 1 mM EDTA, and 10 mM DTT.

TS-catalyzed exchange of the 5-hydrogen of dUMP for solvent protons was monitored at ambient temperature by the decrease in ^{3}H/^{14}C of [2-^{14}C,5-^{3}H]dUMP during the course of the reaction (Pogolotti, et al., 1979). Reaction mixtures contained 0.7–3.5 μM V316Am and 90 μM [2-^{14}C,5-^{3}H]-dUMP (55 mCi ^{3}H/mmol, 8 mCi ^{14}Cmmol) in standard TES/DTT assay buffer. When indicated, (6*R*)-CH_2H_4folate or folic acid was included at 100 μM. Duplicate 50-μL aliquots were removed and quenched with 0.3 mL of MeOH, and the solution was evaporated to dryness at 45 °C using a heat lamp. The residue was twice redissolved in 0.3 mL of MeOH and evaporated, dissolved in 0.2 mL of water, and counted in 4 mL of Aquasol II. Sufficient counts were collected to obtain counting errors of <1%.

TS-catalyzed dehalogenation of BrdUMP and IdUMP was monitoried at 25 °C by the decrease in absorbance which accompanies dehalogenation ($\Delta\epsilon_{285}$ = 5320 M^{-1} cm^{-1} for BrdUMP or $\Delta\epsilon_{290}$ = 6520 M^{-1} cm^{-1} for IdUMP; Garrett et al., 1979). Reaction mixtures (1 mL) contained standard TES/DTT assay buffer, 6–200 μM BrdUMP or IdUMP, and 2.8 μM V316Am or wild-type TS. Kinetic constants were determined by a nonlinear least-squares fit to the Michaelis–Menten equation using the program Kaleidagraph (Abelbeck Software, 1989) run on a Macintosh II computer. One unit of activity is defined as the amount of enzyme that will debrominate 1 μmol of BrdUMP per minute in a 1-mL reaction mixture.

K_i values for dUMP, dTMP, and FdUMP were determined by monitoring enzymatic debromination of BrdUMP in reaction mixtures containing the standard TES/DTT assay buffer, 2.8 μM enzyme, 90 μM BrdUMP, and concentrations of the inhibitory nucleotides between 50 and 250 μM. K_i values were calculated from a nonlinear least-squares fit to eq III-5 described by Segel [Segel (1975), p 105]. In the case of inhibition by dUMP, Lineweaver–Burke analysis was also used.

HPLC Analysis of Nucleotide Products. HPLC was performed using a Rainin HPLC equipped with a Hewlett Packard 1040A diode array detector. Isocratic separation of dUMP and dTMP was accomplished on an Altex Ultrasphere IP column (4.6 mm × 25 cm) using 5 mM KH_2PO_4, pH 7.0, 5 mM tetra-*n*-butylammonium sulfate, and 5% (v/v) acetonitrile as the eluant with a flow rate of 1 mL/min. Retention volumes for dUMP and dTMP were 20 and 29 mL, respectively. Samples containing enzyme were deproteinized prior

to HPLC using Centricon 10 ultrafiltration devices. For analysis of radioactive reactants and products, dUMP and dTMP UV markers (15 nmol each) were added to samples prior to chromatography. Fractions (1 mL) were collected and counted in 6 mL of Aquasol II. For TS-catalyzed dehalogenation of BrdUMP, the concentration of acetonitrile in the mobile phase was 10% (v/v); dUMP and BrdUMP had retention volumes of 10 and 16.5 mL, respectively.

SDS–PAGE of the Enzyme–FdUMP–CH_2H_4folate Complex. Covalent complexes were formed by incubating a mixture containing 1.8 μM V316Am, 50 μM [6-^{3}H]FdUMP (830 mCi/mmol), and 0–1.7 mM (6*R*)-CH_2H_4folate in standard TES/DTT buffer at room temperature. Aliquots (10 μL) were removed over a 6-h period, quenched with 10 μL of 10% 2-mercaptoethanol and 1% SDS, and boiled for 2 min before loading onto 12% SDS–PAGE. The TS–FdUMP–(6*R*)-[6-^{3}H]CH_2H_4folate complex was formed by incubating 4.5 μM V136Am, 10.8 μM FdUMP, and 1.5 mM (6*R*)-[6-^{3}H]CH_2H_4folate as described above; control reactions lacked FdUMP. The gels were stained with Coomassie blue and prepared for fluorography as described (Chamberlain, 1979). For quantitation of protein bound radioactivity, Coomassie-stained bands were excised, solubilized using Solvable (NEN), and counted in 10 mL of Atomlight (NEN).

Trichloroacetic Acid Precipitation of the Enzyme–FdUMP–CH_2H_4folate Complex. Complexes were formed as described above, and controls omitted CH_2H_4folate. Aliquots were removed and protein was precipitated using 10% trichloroacetic acid as described (Cisneros & Dunlap, 1990).

Ultraviolet Difference Spectroscopy of the V316Am–FdUMP–Ch_2H_4folate Complex. UV spectra were measured using a Hewlett Packard 8452A diode array spectrophotometer. Reaction mixtures (1 mL) contained 6.5 μM V316Am and 50–130 μM (6*R*)-CH_2H_4folate in the standard TES/DTT assay buffer and were titrated with 2-nmol additions of FdUMP. After additions of FdUMP, spectra were recorded until no further increase in A_{330} occurred. Appropriate corrections were made for dilution and light scattering, and difference spectra were calculated by subtracting the spectrum of enzyme plus CH_2H_4folate from spectra obtained after incubation with FdUMP.

The $\Delta\epsilon_{330}$ for complex formation was obtained from the total ΔA_{330} after saturating 7.6 nmol of FdUMP with 1.4-nmol additions V316Am in the presence of 130 μM CH_2H_4folate.

Psuedo-first-order rate constants for the spectral change at 330 nm were determined under conditions of saturating ligand concentrations. A reaction containing 6.5 μM V316Am and 122 μM (6*R*)-CH_2H_4folate in the standard TES/DTT assay buffer was made 170 μM in FdUMP, and the absorbance change at 330 nm as a function of time was fit to the first-order rate equation.

Results

Enzyme Purification. V316Am was expressed in *E. coli* χ2913 as approximately 25% of the total soluble protein and was purified to over 95% homogeneity as determined by densitometric scanning of SDS–PAGE (Figure 1). The specific activity of the purified enzyme for BrdUMP dehalogenation was 0.014 unit/mg.

V316Am Does Not Catalyze Formation of dTMP. Previously, we reported that pSCTS-V316Am did not complement *E. coli* χ2913 cells deficient in TS, indicating that pSCTS-V316Am produced less than 1% of the TS activity expressed by the wild-type parent plasmid, pSCTS9 (<0.002 unit/mg in crude extract) (Climie et al., 1990). To ascertain whether V316Am catalyzed any conversion of dUMP to dTMP, we

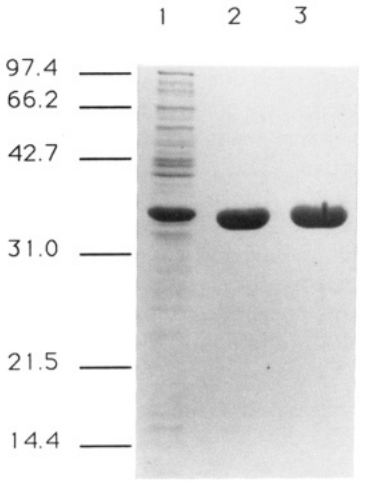


FIGURE 1: 12% SDS–PAGE of samples from V316Am purification. Lane 1, crude extract; lane 2, phosphocellulose chromatography pool; lane 3, hydroxyapatite chromatography pool. Molecular weights ($\times 10^{-3}$) are given.

performed increasingly sensitive assays. First, we tried to detect TS activity using the spectrophotometric assay, which has a sensitivity of about 10^{-4} unit/mL (25 mAU/h at 340 nm). With crude extracts or with purified enzyme, up to 0.25 mg of protein/mL showed no activity for as long as 1 h. Second, we repeated the assay using 0.25 mg/mL pure V316Am and [2-^{14}C,5-^{3}H]dUMP and analyzed for [2-^{14}C]-dTMP. After incubation for 22 h, HPLC separation of the reaction mixture (79 000 dpm ^{14}C, 4.5 nmol dUMP) did not reveal the presence of [^{14}C]dTMP (<200 dpm, <12 pmol). When wild-type TS was subsequently added to the reaction, complete conversion of [2-^{14}C,5-^{3}H]dUMP to [2-^{14}C]dTMP was observed, showing that the substrate and cofactor had not deteriorated. These experiments demonstrate that V316Am has a specific activity of less than 4×10^{-8} unit/mg, at least 10^8-fold lower than wild-type TS.

CH_2H_4folate-Dependent Tritium Release from [2-^{14}C,5-^{3}H]dUMP. In the presence of CH_2H_4folate, V316Am catalyzes a slow exchange of tritium from [2-^{14}C,5-^{3}H]dUMP for protons of water. Separation of the products of a reaction mixture containing 3.5 μM V316Am, 100 μM [2-^{14}C,5-^{3}H]-dUMP, and 100 μM (6*R*)-CH_2H_4folate by HPLC showed the time-dependent generation of a volatile, UV-transparent, tritium-containg material which eluted in the void volume. We concluded that the material was ^3H_2O. No new ^{14}C-containing peaks were detected, and the recovered dUMP had the same ^{3}H/^{14}C ratio as observed after drying of the reaction mixture prior to HPLC. Tritium release followed first-order kinetics for approximately 12 h (Figure 2). Approximately 60% of the tritium was released during 18 h, corresponding to about 16 turnovers per V316Am dimer. The rate of tritium release increased linearly with V316Am concentration up to 3.5 μM. V316Am showed a specific activity for tritium release of 1.4×10^{-4} μmol/(min·mg), the same value obtained with wild-type TS (Pogolotti et al., 1979), and more than 5×10^4 fold higher than was necessary to detect dTMP formation. [2-^{14}C,5-^{3}H]dUMP in reaction mixtures which lacked either V316Am or CH_2H_4folate, or in which folic acid was substituted for CH_2H_4folate, had less than 1.5% of the tritium released during the same time period.

Dehalogenation of BrdUMP and IdUMP. As described for wild-type TS (Garrett et al., 1979), V316Am catalyzes the thiol-dependent dehalogenation of BrdUMP and IdUMP to produce dUMP. Figure 3 shows the spectral changes which occur when BrdUMP is reacted with V316Am and 10 mM

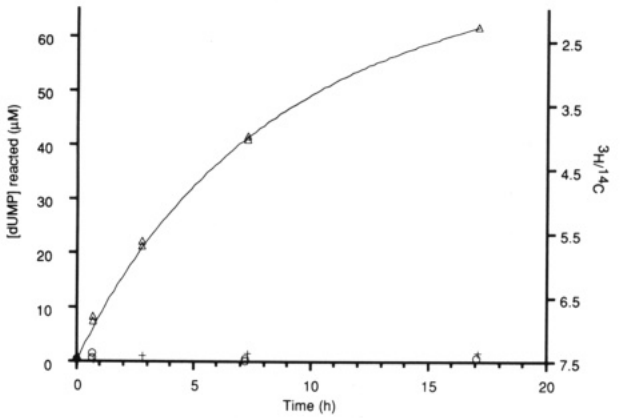


FIGURE 2: V316Am-catalyzed tritium release from [2-^{14}C,5-^{3}H]-dUMP. This reaction contained 3.5 μM V316Am, 100 μM [2-^{14}C,5-^{3}H]dUMP, and either 100 μM (6*R*)-CH_2H_4folate (Δ), 100 μM folic acid (+), or no folates (O) in the standard TES/DTT buffer. The curve is described by a double-exponential equation which accounts for the first order inactivation of the enzyme (Pogolotti et al., 1979).

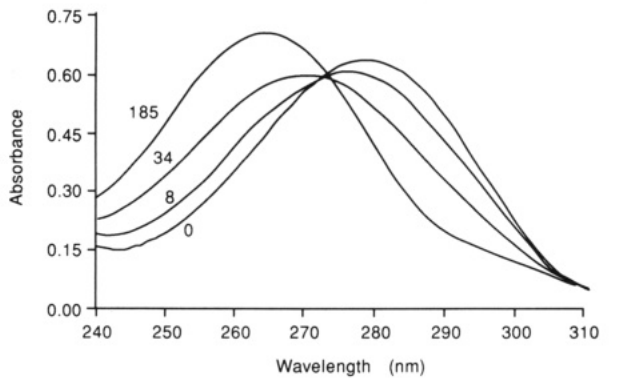


FIGURE 3: Spectral changes that occur upon V316Am-catalyzed conversion of BrdUMP to dUMP. The numbers shown indicate the reaction time in minutes. Reactions contained 3.5 μM V316Am and 36 μM BrdUMP in the standard TES/DTT buffer.

Table I: Kinetic Constants for V316Am-Catalyzed Dehalogenation Reactions[a]

nucleotide (kinetic constant)	wild-type	V316Am
BrdUMP (K_m)	10 ± 4 μM	18 ± 2 μM
IdUMP (K_m)	10 ± 5 μM	19 ± 4 μM
BrdUMP (k_{cat})	1.2 ± 0.1 min^{-1}	1.2 ± 0.1 min^{-1}
IdUMP (k_{cat})	1.6 ± 0.2 min^{-1}	1.7 ± 0.2 min^{-1}
dUMP (K_i)[b]	5 ± 1 μM	11 ± 1 μM
dTMP (K_i)[b]	22 ± 2 μM	32 ± 3 μM
FdUMP (K_i)[b]	36 ± 3 μM	43 ± 5 μM

[a] Values given include standard errors from nonlinear least-squares fit of kinetic models. [b] Inhibition of TS-catalyzed debromination of BrdUMP.

DTT. Over time, the spectrum of BrdUMP is converted to one identical to that of dUMP, and HPLC analysis confirmed that the major product was dUMP. The rate of the debromination of BrdUMP increased linearly between 0.14 and 3.5 μM V316Am. The reaction requires an exogenous thiol, and HPLC analysis showed that dUMP was formed when 10 mM DTT, 2-mercaptoethanol, or cysteine was included in the reaction mixture. Under the conditions used, V316Am catalyzed dehalogenation of BrdUMP and IdUMP had the same k_{cat} and K_m values as wild-type TS. K_i values for dUMP, dTMP and FdUMP are also similar to those obtained for the wild-type enzyme (Table I).

Complex with FdUMP and CH_2H_4folate. We have used three different methods to show that V316Am forms a ternary

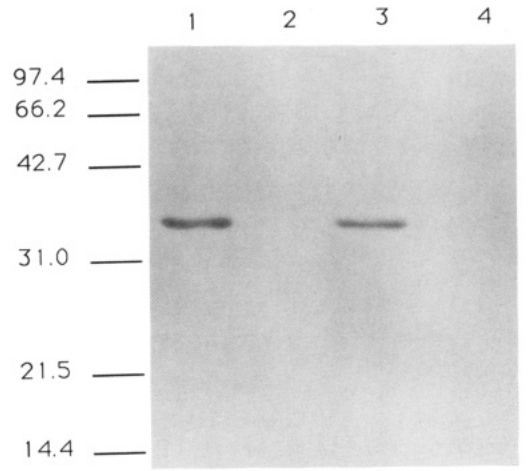
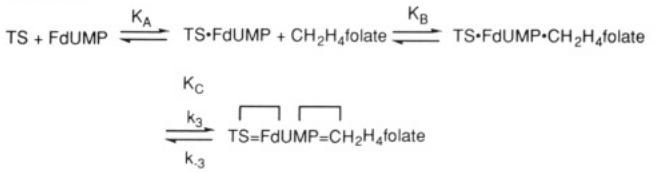


FIGURE 4: Autoradiogram of SDS–PAGE showing the covalent complex between V316Am, FdUMP, and [6-^{3}H]CH_2H_4folate. Lanes 1 and 2 are V316Am and lanes 3 and 4 are wild-type TS. Reaction mixtures are described in the text; lanes 2 and 4 omit FdUMP. Molecular weights (×10^{-3}) are given.

Scheme I



complex with FdUMP and (6*R*)-CH_2H_4folate. First, when 1.8 μM V316Am was incubated with 50 μM [6-^{3}H]FdUMP and 50 μM to 1.7 mM (6*R*)-CH_2H_4folate, tritium-labeled enzyme was observed on SDS–PAGE. The complex migrated with an apparent molecular mass of 36 kDa, just above the free enzyme. Formation of the complex required both ligands, and a tritium label on either ligand resulted in labeling of the complex (Figure 4). By quantitating the tritiated covalent complex, we followed the time course of its formation in the presence of excess FdUMP and found that equilibrium was reached by 3 h. The fraction of V316Am that was covalently bound increased with CH_2H_4folate concentration until all of the enzyme was converted to the covalent complex. When [6-^{3}H]dUMP was substituted for FdUMP, V316Am migrated the same as free enzyme on SDS–PAGE, and tritium was not detected in protein bands.

Next, we isolated V316Am–FdUMP–CH_2H_4folate complexes by precipitation with trichloroacetic acid. When complexes were formed as described above, precipitation of protein resulted in the CH_2H_4folate-dependent coprecipitation of [6-^{3}H]FdUMP. When [6-^{3}H]dUMP was substituted for FdUMP, no radioactivity precipitated in the presence or absence of CH_2H_4folate.

Finally, ternary complex formation was monitored spectrophotometrically (Santi et al., 1976). When FdUMP was added to a mixture of V316Am and (6*R*)-CH_2H_4folate, the UV-difference spectrum showed a slow increase in A_{330} ($\Delta\lambda_{max}$); the initial increase in A_{262} due to the addition of FdUMP was followed by a slow decrease in absorbance at this wavelength. Saturation was achieved after addition of 2 mol of FdUMP per mol of enzyme dimer, and $\Delta\epsilon_{330}$ for formation of the bivalent complex was 20 900 M^{-1} cm^{-1}, calculated per mol of enzyme subunit. When 7.6 μM FdUMP was titrated with V316Am in the presence of an excess of CH_2H_4folate, $\Delta\epsilon_{330}$ was 21 570 M^{-1} cm^{-1}. These values are comparable to the $\Delta\epsilon_{330}$ of 17 700 M^{-1} cm^{-1} observed for the wild-type enzyme (Santi et al., 1976). When dUMP was substituted for FdUMP

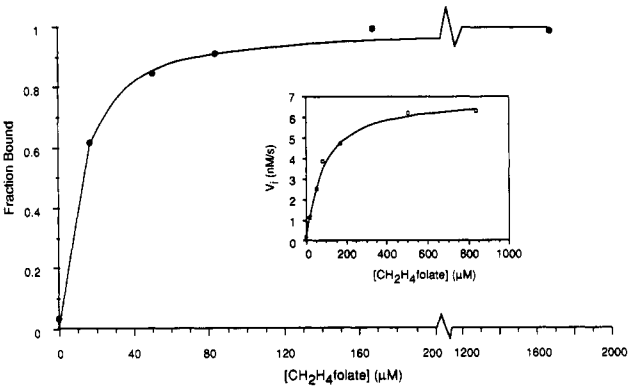


FIGURE 5: Formation of the V316Am–FdUMP–CH_2H_4folate complex as measured by SDS–PAGE. Reactions contained 1.8 μM V316Am and 50 μM [6-^{3}H]FdUMP. (Main plot) Titration of V316Am with CH_2H_4folate. Points are experimental, and the line is a best fit to eq II-54 from Segel (1975; p 74), which yields a value for K_BK_C of 8 μM. (Inset) Initial rates of complex formation. Points were experimentally determined, and the line is a best fit of these points to eq 1, which yields K_B = 44 μM and k_3 = 0.24 min^{-1}.

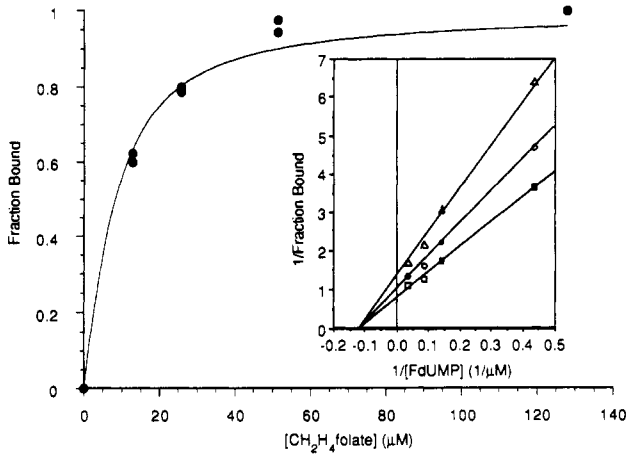


FIGURE 6: Spectrophotometric titrations of 6.5 μM V316Am with CH_2H_4folate and FdUMP. (Main plot) Titration of V316Am with CH_2H_4folate in the presence of 186 μM FdUMP. Points are experimental, and the line is a best fit of eq II-54 from Segel (1975; p 74), which yields a value for K_BK_C of 5 μM. (Inset) Double-reciprocal plot of titrations with FdUMP at 13 (△), 26 (○), and 52 μM (□) CH_2H_4folate.

in similar experiments, no spectral changes were observed.

Kinetics and Equilibria for Formation of the Covalent V316Am–FdUMP–CH_2H_4folate Complex. As depicted in Scheme I, the interaction of TS with FdUMP follows an ordered mechanism with nucleotide binding first (K_A). Subsequently, CH_2H_4folate binds (K_B) to form a reversible ternary complex which is then converted to the covalent ternary complex (K_C) (Santi et al., 1987; Danenberg & Danenberg, 1978).

Initial rates of formation of the covalent complex from the binary V316Am–FdUMP complex were determined by SDS–PAGE using [6-^{3}H]FdUMP and varying CH_2H_4folate concentration. Assuming a rapid preequilibrium formation of the noncovalent complexes, eq 1 describes the rate of formation of the covalent comlex. A fit of the data shown in the inset of Figure 5 to eq 1 gave values for K_B = 44 μM and k_3 = 3.5 × 10^{-3} s^{-1}. The value for k_3 determined by monitoring

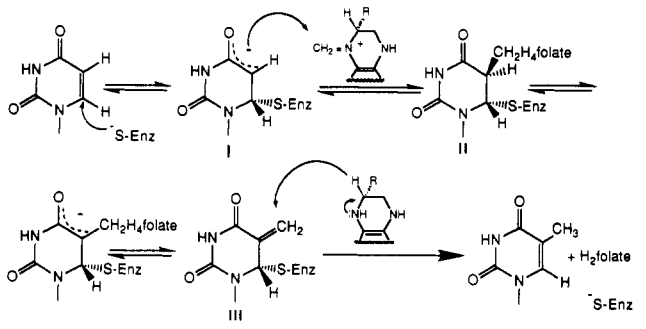
$$V_i = \frac{k_3[\text{V316Am}][\text{CH}_2\text{H}_4\text{folate}]}{K_B\left(\frac{K_A}{[\text{FdUMP}]} + 1\right) + [\text{CH}_2\text{H}_4\text{folate}]} \quad (1)$$

Table II: Equilibrium and Kinetic Constants Describing the Reaction of V316Am with FdUMP and CH_2H_4folate

	wild-type[a]	V316Am[b]
K_A	10 μM	43 ± 5 μM
K_B	1 μM	44 ± 4 μM
K_C	5.7 × 10^{-5}	0.15[c]
K_BK_C	5.7 × 10^{-5} μM	6.5 ± 1 μM[d]
k_3	0.586 s^{-1}	(3.5 ± 0.1) × 10^{-3} s^{-1} [d]
k_{-3}	3.3 × 10^{-5} s^{-1}	5 × 10^{-4} s^{-1} [e]

[a] Values for wild-type TS are taken from Santi et al. (1987). [b] Values given include standard errors from nonlinear least-squares fit of kinetic models. [c] Calculated using $K_C = K_BK_C/K_B$. [d] Average of values obtained using spectrophotometric and SDS–PAGE methods. [e] Calculated using $k_{-3} = K_Ck_3$.

Scheme II



the rate of absorbance change at 330 nm under saturating conditions for both ligands was 3.0 × 10^{-3} s^{-1}, in agreement with the value obtained by the SDS–PAGE method.

Equilibrium concentrations of covalent complexes under conditions of varying ligands were determined by both SDS–PAGE and UV-difference spectroscopy. Figure 6 shows the results of spectrophotometric titration of V316Am with CH_2H_4folate. High concentrations of CH_2H_4folate caused saturation of the enzyme's sites in the presence of a subsaturating concentration of FdUMP; however, high concentrations of FdUMP were not sufficient to cause saturation at subsaturating concentrations of CH_2H_4folate (Figure 6, inset). These data rule out a random order of binding and an ordered mechanism with CH_2H_4folate binding first [Segel (1975), pp 325]; they are in accord with the ordered mechanism with FdUMP binding first that has been previously reported for the wild-type enzyme (Santi et al., 1987; Danenberg & Danenberg, 1978).

The apparent dissociation constant obtained when V316Am is titrated with CH_2H_4folate in the presence of a high concentration of FdUMP corresponds to K_BK_C. Least-squares fit of an equilibrium binding equation which corrects for ligand depletion by enzyme [Segel (1975), pp 74] to plots of fraction of bound enzyme versus CH_2H_4folate (Figures 5 and 6) gave values for K_BK_C of 5 and 8 μM using spectroscopic and SDS–PAGE data, respectively. From K_B = 44 μM as determined above, K_C ($K_C = K_{app}/K_B$) is 0.15. From the values obtained for K_C and k_3, we calculated k_{-3} ($k_{-3} = K_Ck_3$) to be 5.25 × 10^{-4} s^{-1}. A summary of these data appears in Table II.

Discussion

The chemistry of the TS-catalyzed conversion of dUMP and CH_2H_4folate to dTMP and H_2folate is well understood (Scheme II). After binding of dUMP and CH_2H_4folate to TS, nucleophilic attack by a conserved thiol (Cys 198 in *L. casei* TS) at the 6-position of dUMP converts the 5-carbon to a nucleophilic enol or enolate (I). This is followed by

covalent bond formation between the 5-position of the bound dUMP and the one-carbon unit of the cofactor. Next, the 5-proton of II is abstracted, followed by β-elimination of H_4folate to give the exocyclic intermediate III and H_2folate. Finally, hydride transfer and β-elimination of the enzyme occur to provide products.

TS also participates in several reactions which utilize important catalytic features of the enzyme. First, TS catalyzes a thiol-mediated dehalogenation of Br(I)dUMP which does not require cofactor. Here, protonation of a covalent binary complex analogous to I activates the halogen for removal by thiol. Second, TS catalyzes the exchange of tritium from [5-^{3}H]dUMP for protons of water. Here, either I or III undergoes protonation by solvent and reversal. Finally, the mechanism-based inhibitor FdUMP undergoes reactions in the pathway up to and including formation of the corresponding analog of II. At this stage, the fluorine atom cannot be removed, and the fluorinated analog of the steady-state intermediate accumulates.

The C-terminal valine of *L. casei* TS has been implicated to be important for folate binding and catalytic function. Carboxypeptidase removal of a single C-terminal Val led to inactive enzyme which bound dUMP with normal affinity (Aull et al., 1974; Galivan et al., 1976). Removal of the C-terminal Val from TS by mutagenesis also led to inactive enzyme, confirming the importance of this residue (Climie et al., 1990; Michaels et al., 1990). Crystallographic studies of TS provide insight into how the C-terminal residue may be involved in function (Hardy et al., 1987; Perry et al., 1990; Montfort et al., 1990; Matthews et al., 1990). The structures of free TS[2] and the binary TS–dUMP complex are very similar, and high *B* factors (40–50 Å^2 for residues of the C-terminal tetrapeptide) suggest that C-terminal residues are flexible (Hardy et al., 1987; Montfort et al., 1990). Upon formation of the ternary TS–dUMP–CB3717 complex, a large conformational change occurs in which the C-terminus moves about 4 Å to cover the active site and form a tight lid over the bound substrate and cofactor. Crystallographic *B* factors for the C-terminal tetrapeptide in this structure are 16–33 Å^2, reflecting the reduced flexibility of these residues following the conformational change. In the ternary complex, the carboxylate of the C-terminal residue forms a hydrogen-bond network with conserved Trp 85 and Arg 23, which moves to interact with the phosphate of dUMP. The Val 316 side chain becomes buried against hydrophobic atoms of Thr 24, and residues 312–315 also experience new interactions.

We have examined the V316Am mutant of *L. casei* TS with the goal of understanding why this single amino acid deletion had such a pronounced effect on activity. Initially, we confirmed that the mutant was truly defective in the catalysis of dTMP formation. Using highly sensitive assays, we could detect no conversion of dUMP to dTMP. We showed that the mutant either is completely inactive or is less than 10^{-8}-fold as active as wild-type TS, with calculated half-life for turnover of over 6 months. The inability of V316Am to catalyze dTMP formation can be rationalized by the anticipated effects of the mutation on the structure of the ternary complex. First, the position of the C-terminal carboxylate would change by about 3–4 Å, precluding the formation of a "lid" over the active site and the hydrogen-bond network that accompanies CH_2H_4-folate binding. Second, the absence of Val 316 would abolish side-chain-mediated hydrophobic interactions of the C-terminal side chain that contribute to the conformational change (Climie et al., 1992).

Unlike the C-terminus in the structure of the TS–dUMP–CB3717 complex, the C-terminus in the dUMP–TS structure is fully exposed and plays no apparent role in binding to the nucleotide (R. Stroud, in preparation). Together with the observation that carboxypeptidase-inactivated TS binds dUMP (Galivan et al., 1977), this led us to suspect that the mutant might undergo one or more of the aforementioned partial reactions of TS.

First, as with wild-type and catalytically active mutants of TS (Garrett et al., 1979; Climie et al., 1992), V316Am catalyzes dehalogenation of 5-Br(I)dUMP in the absence of CH_2H_4folate. We have previously reported that k_{cat} and K_m for this partial reaction are not altered by mutations which affect folate binding and subsequent steps in the pathway (Climie et al., 1992) and proposed that the conformational change of the C-terminus is not involved in the dehalogenation reaction. All that seems to be required of the enzyme is the ability to bind nucleotide and form a covalent adduct between the catalytic thiol of Cys 198 and the nucleotide. Our observation that steady-state kinetic parameters of the V316Am-catalyzed dehalogenation reactions are very similar to those of wild-type TS supports this conclusion. In addition, dUMP, dTMP, and FdUMP are competitive inhibitors of debromination with binding constants similar to those observed with wild-type TS (Table I). The observation that removal of Val 316 does not significantly alter nucleotide binding or nucleophilic attack by the catalytic thiol supports the proposal that these steps do not require a conformational change of the C-terminus. Defects caused by this mutation must therefore occur subsequent to these events.

Second, V316Am catalyzes exchange of the 5-protons of dUMP, but only in the presence of cofactor. Since the TS-catalyzed 5-H exchange reaction requires nucleophilic attack at C-6 dUMP (Pogolotti et al., 1979), this supports the previous conclusion that the nucleophilic attack does not require an intact C-terminus. The CH_2H_4folate dependence of this reaction demonstrates that V316Am is capable of binding CH_2H_4folate in the presence of dUMP. It also places the insurmountable defect of the mutation at a step of the pathway after dUMP has lost its C-5 proton. The cofactor may simply cause an environmental change which assists the formation of I (Scheme II), or it may chemically participate in the exchange by forming the covalent intermediate III. Reversal of a ternary complex containing either I or III would result in the observed exchange.

Third, formation of the covalent V316Am–FdUMP–CH_2H_4folate complex shows that the mutant enzyme is capable of binding CH_2H_4folate, forming a covalent adduct with FdUMP, and causing carbon–carbon bond formation between C-5 of the FdUMP adduct and the one-carbon unit of CH_2H_4folate to yield an analog of II. A close agreement between values obtained for k_3 using SDS–PAGE and spectrophotometric methods suggests that the spectral change and covalent bond formation share the same rate-limiting step. The rate of conversion of the reversible ternary complex to the covalent complex is about 150-fold slower for the V316Am mutant than for wild-type enzyme, and we calculate a rate of breakdown for this complex that is 16 times faster for V316Am than for wild-type TS. This may reflect the difficulty the mutant enzyme has undergoing the conformational change at the C-terminus associated with catalysis (Santi et al., 1987;

[2] We refer to the structures of *L. casei* and *E. coli* TS which have inorganic phosphate bound at the active site as free enzyme, the TS–dUMP binary complex from *L. casei*, and the *E. coli* TS–dUMP–CB3717 ternary complex. We assume relevant features of the ternary complex are similar in the *L. casei* and *E. coli* enzymes.

Climie et al., 1992) or an unproductive mode of binding of the cofactor which requires isomerization before covalent bonds can form. In either case, it is clear that the difference in free energy between the covalent ternary complex and the noncovalent ternary complex is much less for V316Am than for wild-type enzyme.

Removal of Val 316 also decreased the affinity of the binary V316Am–FdUMP complex for CH_2H_4folate (K_B), and the stability of the covalent versus noncovalent complex (K_C) has been decreased more than 5000-fold. Overall, removal of Val 316 decreased the equilibrium constant between the binary V316Am–FdUMP complex and the covalent ternary V316Am–FdUMP–CH_2H_4folate complex by about 10^5. Why a similar covalent ternary complex is not isolable with dUMP remains an enigma. Perhaps it is too unstable to detect by the methods used.

In summary, we have shown that the V316Am mutant of TS is capable of carrying out several partial reactions but has defects which prevent it from completing the normal catalytic cycle. The mutant binds to nucleotide substrates which may undergo covalent bond formation with the catalytic thiol of TS in an apparently unaltered fashion. The defects in the V316Am mutant are manifested in folate binding, and in catalyzing chemistry subsequent in binding the cofactor. The first defect in the pathway is in cofactor binding, which is diminished about 40-fold. The second defect appears to be in the rate of formation of the ternary complex II (Scheme II) and probably results from the inability of the mutant to undergo a conformational change of the C-terminus. Finally, there is an insurmountable defect which prevents the intermediate from proceeding further through the pathway. This could be removal of a proton from C-5 of dUMP in intermediate II or the hydride transfer step that normally follows.

Acknowledgments

We thank V. J. Davisson, J. Kealey, K. Perry, R. Reid, and P. Greene for helpful discussions and members of R. M. Stroud's lab for sharing unpublished structures.

Registry No. CH_2H_4folate, 3432-99-3; Val, 72-18-4; TS, 9031-61-2; dUMP, 964-26-1.

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