Complete Replacement Set of Amino Acids at the C-Terminus of Thymidylate Synthase: Quantitative Structure-Activity Relationship of Mutants of an Enzyme†

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ABSTRACT: The C-terminal residue of thymidylate synthase (TS) is highly conserved and has been implicated in cofactor binding, catalysis, and a conformational change. The codon for the C-terminal valine of *Lactobacillus casei* TS has been replaced with those for 19 other amino acids and the amber stop codon. Fourteen of the resulting mutant proteins were active by genetic complementation using a Thy⁻ strain of *Escherichia coli*, and 18 mutants were active by in vitro assay. Only the aspartate and amber mutations had undetectable activity. All of the mutants were expressed at high levels (5–30% of soluble protein) and were purified by phosphocellulose chromatography. In general, the alterations at position 316 led to little effect on the K_m for dUMP, an increase in K_m for the folate cofactor, and a decrease in k_{cat}. The observations show that TS can tolerate the substitution of most amino acids for valine at the C-terminus without a complete loss of activity, that hydrophobic substitutions are preferred, and that the C-terminal side chain is involved in both cofactor binding and catalysis. There was an excellent correlation between log k_{cat} and hydrophobicity of the side chain at position 316 and an inverse correlation between log K_m and the hydrophobicity of this residue. Kinetic parameters of the cofactor-independent TS-catalyzed dehalogenation of BrdUMP showed no variation with the side chain at position 316. In context of the structure of TS, it is proposed that binding of the cofactor triggers a conformational change in which the C-terminal side chain undergoes hydrophobic interactions that stabilize a rate-limiting transition state of the TS reaction.

Thymidylate synthase (TS,[1] EC 2.1.1.45) catalyzes the conversion of dUMP and CH_2H_4folate to dTMP and H_2folate. TS has been widely studied, and much is known about the structure and function of the enzyme [see Santi and Danenberg (1984), Santi et al. (1987), and Finer-Moore et al. (1990)]. The amino acid sequences of TS from 18 organisms are known, and the three-dimensional structures of TS from several sources have been determined (Perry et al., 1990; Hardy et al., 1987; Montfort et al., 1990; Matthews et al., 1990).

The C-terminal valine is conserved among 14 of the 17 known TS sequences (Perry et al., 1990) and has been implicated in folate binding and catalysis by studies of carboxypeptidase inactivation (Aull et al., 1974; Galivan et al., 1977). Removal of one C-terminal residue from the homodimer results in loss the enzyme activity. Crystallographic studies have shown that the C-terminus, which is proximal to the active site, undergoes a large conformational change upon folate binding to form an active site "lid" that makes up part of the cofactor-binding site (Montfort et al., 1990; Matthews et al., 1990).

As an approach to understanding the structure and function of TS, the construction and analysis of "replacement sets" of multiple amino acids at specific positions within the protein has been undertaken (Climie et al., 1990; Michaels et al., 1990). In this paper, we describe the construction, expression, purification, and steady-state kinetic characterization of a complete replacement set of 20 mutants at the C-terminal position of *Lactobacillus casei* TS. We correlate the kinetic parameters with the hydrophobicity of the side chains and interpret the results in the context of what is known about the structure and mechanism of TS.

Materials and Methods

Materials. Restriction endonucleases and T4 DNA ligase were purchased from Bethesda Research Laboratories and New England BioLabs. [α-^{35}S]dATP (1000 Ci/mmol) was from Amersham, and other reagents used for DNA sequencing were from U.S. Biochemical Corp. Oligonucleotides were synthesized and Nensorb (Du Pont) purified at the Biomolecular Resource Center at the University of California, San Francisco. Phosphocellulose (P11) was purchased from Whatman. H_4folate was obtained from Sigma and converted to (6*R*,6*S*)-CH_2H_4folate as previously described (Bruice & Santi, 1982). Each liter of minimal media contained 7 g of K_2HPO_4, 2 g of KH_2PO_4, 0.5 g of sodium citrate, 0.1 g of $MgSO_4$, 1 g of $(NH_4)SO_4$, 3% casamino acids, 2% glucose, and 50 μg/mL each of amino acids Pro, Arg, Met, Leu, His, and Thr. The sources of other materials have been reported (Climie et al., 1990) or were commonly available.

Bacterial Strains. Strain TB-1 [ϕ80*lac*ZΔM15; *ara*, Δ(*lac-proAB*), *rpsL*, *hsdR*] (provided by T. O. Baldwin, Texas A&M) and χ2913 (from Russell Thompson, University of Glasgow) were used as the host strain for plasmid-mediated transformations during the initial construction of the mutants. A Thy⁻, tetracycline-resistant *Escherichia coli* strain χ2913recA (*ΔthyA572*, *recA56*) was used to test plasmids for TS activity by genetic complementation and for the production

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[1] Abbreviations: TS, thymidylate synthase; dUMP, deoxyuridine monophosphate; CH_2H_4folate, 5,10-methylenetetrahydrofolate; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; bp, base pair; PAGE, polyacrylamide gel electrophoresis; CB3717, 10-propargyl-5,8-dideazafolate; P_c, the probability of finding a correlation r in a set of n random data points.

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of mutant TS. This strain was constructed by transferring the recA56 allele from strain Q8015 (*srlA::Tn10*, *recA56*; from F. Banuett, University of California, San Francisco) to χ2913 by P1 transduction (Miller, 1972). Transduction of *srlA::Tn10* to χ2913 was confirmed by tetracycline resistance (10 μg/mL), and cotransduction of the recA56 allele was tested by increased sensitivity to killing by ultraviolet light (Miller, 1972).

Construction of C-Terminal TS Mutants. Methods for plasmid purification, subcloning, and bacterial transformation were as described (Sambrook et al., 1989). Bacterial cultures containing mutant plasmids were grown in LB supplemented with 100 μg/mL ampicillin and 50 μg/mL thymidine. For complementation studies, χ2913 transformants were plated on minimal agar lacking thymidine but containing ampicillin.

The construction of 15 mutants at the C-terminus of *L. casei* TS using a synthetic gene has been described (Climie et al., 1990). The mutants were constructed in plasmid pSCTS9 by cassette mutagenesis using a synthetic 27 bp double-stranded DNA cassette with *Sfi*I and *Hin*dIII cohesive ends. The mutagenic DNA cassette contained an equal mixture of all four bases at the first and second positions of the target codon and an equal mixture of G and C at the third position. This combination contains a mixture of 32 possible codons that encode all 20 amino acids and an amber stop codon at position 316. Mutagenesis and expression were carried out using the same plasmid vector, pSCTS9, and the Thy⁻ *E. coli* strain χ2913 to produce 15 of the desired mutants (Climie & Santi, 1990; Climie et al., 1990). The remaining mutants were constructed using two additional DNA cassettes that were prepared and used as described above except that the strain χ2913recA was used for the complementation and expression. One of the DNA cassettes carried a mixture of two codons at position 316 [CA(G/C)] encoding His and Gln, and the other carried a mixture of four codons [G(A/T)(A/T)] encoding Ile, Phe, Tyr, and Asn at position 316. Mutagenic oligonucleotides were annealed in 10-μL reaction mixtures that contained either 1, 5, 10, 50, or 100 pmol of each unphosphorylated oligonucleotide in ligation buffer (66 mM Tris-HCl, pH 7.6, 6.6 mM $MgCl_2$, 10 mM DTT, and 0.4 mM ATP). The oligonucleotide mixtures were heated at 100 °C for 3 min and then allowed to cool to room temperature over a period of 30 min. The annealed fragments were ligated into pSCTS9 by adding 0.5 μg of *Sfi*I–*Hin*dIII digested pSCTS9, 5× ligation buffer, and 1 μL of 10 mM ATP in a final volume of 20 μL. Three units of T4 DNA ligase was added, and the reaction mixtures were incubated at room temperature for 2 h. Half of each ligation mixture was used to transform *E. coli* strain TB-1, and the cells were plated on LB agar containing 100 μg/mL ampicillin. The resulting colonies were pooled by flooding the plates with 5 mL of LB, and plasmid DNA was purified from the pooled cells (Birnboim & Doly, 1979). This DNA was used to transform the tetracycline-resistant *E. coli* strain χ2913recA, which was then plated on LB agar containing 50 μg/mL thymidine and 100 μg/mL ampicillin. DNA was prepared from individual transformants, and Val 316 mutations were identified by dideoxy-DNA sequencing using the plasmid DNA as a template and the M13 universal primer (Chen & Seeburg, 1985; Tabor & Richardson, 1987). The primer hybridizes 40 bp downstream of the synthetic gene, and entire inserts and flanking regions were sequenced. Plasmids with defined Val 316 mutations were then used to transform χ2913recA to ampicillin resistance, and the mutations were again confirmed by DNA sequencing.

Purification and Characterization of TS Mutants. Val 316 mutants were initially characterized by their ability to grow on minimal agar in the absence of thymidine. Mutant TS was then purified from 50 mL of cultured cells. The cultures were grown overnight at 37 °C in 200 mL of LB supplemented with 100 μg/mL ampicillin and 50 μg/mL thymidine. Cells were harvested by centrifugation, resuspended in 2 mL of 100 mM Tris, pH 7.0, and 1 mM EDTA and disrupted by sonication. Cell debris was removed by centrifugation at 10000*g* for 15 min. The crude supernatants (2 mL) were loaded onto phosphocellulose columns (0.9 cm × 2.5 cm) equilibrated with 5 mL of 10 mM KH_2PO_4, pH 7.0, and 0.1 mM EDTA. The columns were washed with 4.5 mL of equilibration buffer and then 4.5 mL of equilibration buffer containing 100 mM KCl. TS was eluted with 3 mL of equilibration buffer containing 350 mM KCl. Purified TS was dialyzed against 20 mM KH_2PO_4, pH 7.0, and 0.5 mM EDTA and concentrated by ultrafiltration (Amicon). Protein concentrations were determined using Coomassie Blue (Bradford, 1976) for crude preparations (using bovine serum albumin as a standard) or by using $\epsilon_{278} = 1.07 \times 10^5\ M^{-1}\ cm^{-1}$ for purified TS (Santi et al., 1974). Proteins were analyzed by SDS–PAGE using 12% gels (Laemmli, 1970) that were stained with Coomassie Blue R250. Levels of TS expression were determined by scanning gels using a Bio-Rad model 260 densitometer.

dTMP formation was assayed spectrophotometrically at 25 °C (Pogolotti et al., 1986). One unit of TS activity catalyzes the formation of 1 μmol of dTMP per minute in a 1-mL reaction mixture. When the concentration of dUMP was varied, (6*R*)-CH_2H_4folate was constant at 300 μM; when CH_2H_4folate was varied, the concentration of dUMP was fixed at 100 μM. Kinetic data were analyzed by a nonlinear least-squares method using the program ENZFITTER (Elsevier-Biosoft). The k_{cat} and K_m values for cofactor were obtained from experiments varying CH_2H_4folate in the presence of a large excess of dUMP (>20K_m). Because of the high K_m values for CH_2H_4folate exhibited by some mutants, saturating concentrations of the cofactor were often not feasible when measuring the kinetic parameters for dUMP. However, k_{cat} values obtained by varying dUMP and correcting for CH_2H_4folate K_m values using the equation for a sequential ordered mechanism, $V_{max} = V_{app}(1 + K_{mB}/[B])$ [Segel (1975), p 566] were in agreement with values obtained with varying cofactor concentration at saturating dUMP. Apparent K_m values for dUMP could not be corrected without K_{ia} values for each mutant. TS-catalyzed dehalogenation of BrdUMP was performed as described (Garrett et al., 1979). Log–log plots of kinetic parameters versus side-chain hydrophobicity (Cornette et al., 1987)[2] and volume (Chothia, 1984) were analyzed by linear regression analysis with and without omission of outliers. Statistical analysis of the data was performed using the program Data Desk (Odesta Corp.) for the Macintosh computer.

Results

Nineteen amino acids and an amber stop codon were substituted for the C-terminal valine of TS by site-directed mutagenesis using a synthetic gene and double-stranded DNA cassettes encoding multiple amino acid substitutions at the target codon (Wells et al., 1985; Climie et al., 1990). The same plasmid vector was used for the construction of mutants, DNA sequencing, and expression of proteins.

Mutants were constructed by cassette/mixture mutagenesis using the synthetic *L. casei* gene and degenerate oligo-

[2] We refer to the normalized hydrophobicity scales by the acronyms used in Cornette et al. (1987).

Table I: Steady-State Kinetic Constants for Val 316 Mutants of *L. casei* TS

		dTMP formation					BrdUMP dehalogenation	
			K_m (μM)		k_{cat}/K_m (s^{-1} μM^{-1})			
residue 316	Thy⁻ media[a]	k_{cat} (s^{-1})	CH_2H_4folate	dUMP	CH_2H_4folate	dUMP	k_{cat} (s^{-1})	K_m (μM)
V (wt)	+	5.5	14	2.9	0.40	1.9	0.013	12
I	+	3.8	35	2.2	0.11	1.7		
L	+	1.3	84	1.7	0.016	0.81		
F	+	1.3	65	2.2	0.020	0.59		
T	+	1.2	140	3.5	0.0081	0.34	0.010	15
C	+	1.1	77	1.6	0.015	0.72		
A	+	0.81	370	1.2	0.0022	0.69	0.015	24
M	+	0.65	120	2.5	0.0054	0.26		
P	+	0.60	570	4.2	0.0011	0.14		
H	+	0.55	50	1.6	0.011	0.34		
S	+	0.54	180	1.7	0.0030	0.32	0.008	12
N	+	0.39	170	1.4	0.0023	0.27		
Q	+	0.32	280	3.1	0.0011	0.10	0.013	15
Y	+	0.29	170	2.4	0.0017	0.12		
E	−	0.15	830	2.5	0.00018	0.060	0.010	22
K	+	0.12	85	1.2	0.0014	0.099	0.013	16
W	−	0.050	300	1.5	0.00017	0.034		
R	−	0.020	130	1.5	0.00015	0.014		
G	−	0.030	380	5.6	0.000078	0.0054	0.008	10
D	−	nd	nd	nd	nd	nd		
Am	−	nd	nd	nd	nd	nd		

[a] Complementation of χ2913 *E. coli* in Thy⁻ medium.

nucleotides (Climie et al., 1990). In initial experiments, we tried to isolate as many of the 20 possible Val 316 mutants as practical using a single mutagenic DNA cassette that encoded a mixture of 32 codons at position 316 (Figure 1). We obtained 15 different mutants after sequencing the DNA of 29 isolates with an apparent mutagenesis efficiency of 100%. All 7 of the 20 mutants in the mixture of 32 codons that were represented by two or more synonymous codons were isolated as were eight mutants that were each represented by a single codon. Mutants that were not isolated using the 32-codon DNA cassette (Gly, Ile, Phe, Tyr, His) were each represented by a single codon in the mixture. Rather than sequence large numbers of additional isolates (see below) to identify the remaining mutants, we constructed two additional mutagenic DNA cassettes that carried mixtures of two (His, Gln) and four (Ile, Phe, Tyr, Asn) codons (Figure 1). These DNA cassettes were used in separate experiments to isolate the five remaining mutants. Six isolates of the two-codon mix were sequenced, resulting in the identification of three His mutants, two Gln mutants, and one wild-type Val. Similarly, two Phe, five Tyr, six Ile, and a single Asn mutant were identified after sequencing 15 isolates from the four-codon mix.

As used here, mixture mutagenesis avoids the expense and efforts of multiple, single mutagenesis experiments at the cost of redundant DNA sequencing to identify the mutants. By computer simulation, we have found that, in order to obtain all 20 possible mutations of an amino acid residue from a 32 codon mixture, it would be necessary to randomly sequence some 170 clones (E. Fauman, unpublished results). As a compromise between the advantages of mixed versus multiple mutagenesis, our current strategy is to sequence about 30 random clones from a 32-codon mixture experiment, which yields about 15 unique mutants. Then we perform additional mutagenesis with oligonucleotide mixtures which are specifically biased toward codons of the remaining desired mutations. The use of three mutagenic DNA cassettes allowed us to isolate a complete replacement set of amino acid substitutions at Val 316 while reducing the number of isolates to be sequenced from the expected 170 to 49 (Climie et al., 1990).

All 20 of the Val 316 mutants were initially characterized by genetic complementation using a Thy⁻ strain of *E. coli*

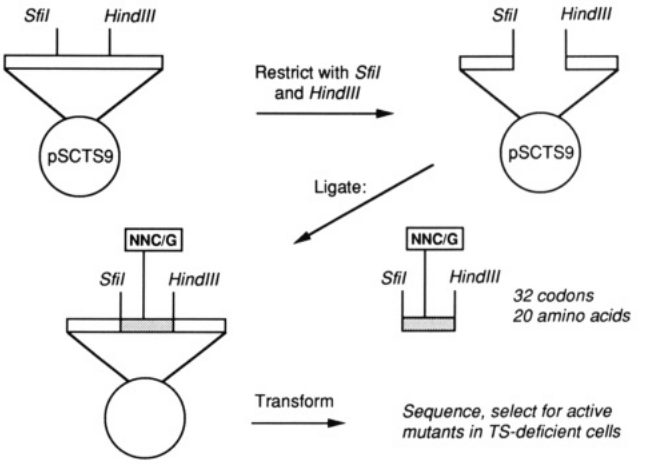

FIGURE 1: Construction of a replacement set of amino acids at the C-terminus of *L. casei* TS. Mutants were constructed by cassette mutagenesis using plasmid pSCTS9 which carries a synthetic *L. casei* TS gene flanked by *Eco*RI and *Hin*dIII restriction sites. Double-stranded mutagenic DNA cassettes that carry mixtures of 2, 4, and 32 possible codons are shown. The DNA cassettes contain *Sfi*I and *Hin*dIII cohesive ends to allow cassette mutagenesis. Boldface characters indicate positions encoding the C-terminal residue followed by a TAA stop codon (N, an equal mixture of all 4 bases; parentheses indicate equal mixtures of two bases at that position).

(χ2913) that carries a deletion of the *thyA* gene. Fourteen of the 20 mutants produced sufficient TS activity (>0.002 unit/mg) to allow growth of this strain on minimal agar in the absence of thymidine. TS mutants were expressed at levels ranging from 7% to 32% of the total cellular protein in soluble crude extracts, as determined by SDS–PAGE and comparison of specific activities to the purified proteins. Each mutant was purified by phosphocellulose chromatography to >90% homogeneity as assessed by SDS–PAGE.

Steady-state kinetic parameters of wild-type TS and the 18 purified active Val 316 mutants were determined (Table I); we could not detect TS activity with the V316D and V316Am mutants. The kinetic constants of active mutants were interpreted in the context of an ordered mechanism, with dUMP binding first, as observed in the wild-type enzyme (Danenberg & Danenberg, 1978; Daron & Aull, 1978). We also attempted

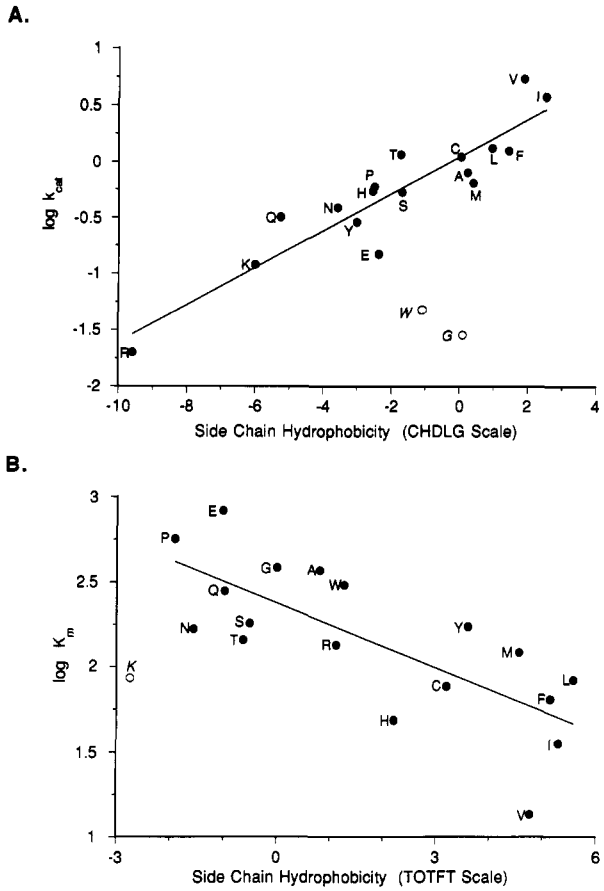

FIGURE 2: Linear free energy relationships of Val 316 mutant TSs. (A) Correlation of log k_{cat} with hydrophobicity of side-chain residues at position 316 of TS. The data are experimental, and the line is a best fit ($y = 0.0519 + 0.166x$; $r = 0.916$) to the normalized CHDLG hydrophobicity scale omitting Trp and Gly (Cornette et al., 1987). (B) Correlation of log K_m for CH_2H_4folate and the hydrophobicity of side-chain residues at position 316 of TS. The data are experimental, and the line is a best fit ($y = 2.376 - 0.127x$; $r = 0.745$) to the normalized TOTFL hydrophobicity scale omitting Lys (Cornette et al., 1987).

to correlate kinetic constants with parameters that describe the hydrophobicity and volume of the mutant side chains which replaced the C-terminal valine.

The k_{cat} values of wild-type TS and 18 active mutants varied over a 270-fold range. In general, hydrophobic residues as position 316 were associated with the highest k_{cat} values, hydrophilic replacements gave intermediate values, and charged residues resulted in the lowest k_{cat} values. Using some 40 reported scales of hydrophobicity of amino acid side chains (Cornette et al., 1987), we observed significant linear correlations with log k_{cat} of wild-type and mutant TS ($n = 19$). We chose the 10 scales with highest correlation ($r \geq 0.58$) and evaluated each independently. In these correlations Gly and Trp, and sometimes Arg, were outliers. When the outliers were omitted, the correlations of k_{cat} with hydrophobicity were remarkably good. Omission of Gly and Trp gave six scales with $r = 0.85$–0.92 ($n = 17$, $P_c < 0.001$); upon regression analysis, the best correlations were observed omitting Gly and Trp in the hydrophobicity scales WOLF ($r = 0.89$), EISEN ($r = 0.91$), and CHDLG ($r = 0.92$). The correlation of log k_{cat} with the CHDLG scale is shown in Figure 2A.

No linear relationship was seen between log k_{cat} and volume of the side chain, nor did multiple regression of log k_{cat} on hydrophobicity and volume improve the correlations. However, the most active mutants are clustered with side chains of intermediate volume. Trp and Arg, with highest side chain volumes, and Gly, with lowest volume, showed the lowest k_{cat} values and were also the outliers in the correlation with hydrophobicity. The variation in the apparent K_m values for dUMP was not considered significant since the sensitivity of the assay is low in the concentration range surrounding the apparent K_m values. Regardless, there was no significant correlation between the apparent K_m values of dUMP and physical properties of the side chains at position 316.

Apparent K_m values for CH_2H_4folate varied over a 60-fold range for the wild-type TS and 18 mutants (14–830 μM) (Table I). Qualitatively, mutants with hydrophobic side chains had the lowest apparent K_m values for CH_2H_4folate. Lack of a side chain at the C-terminus (V316G) resulted in an almost 30-fold increase in K_m for CH_2H_4folate. The introduction of a negative charge in the V316E mutant resulted in the highest K_m for CH_2H_4folate, while the K_m for the V316D mutant could not be determined because of low or nonexistent activity. For quantitative assessment, we correlated the log K_m values for 18 mutants and wild-type TS with the 42 normalized hydrophobicity scales tabulated by Cornette et al. (1987). Seven scales (MEIRO, POONU, PRIFT, PRILS, ALTFT, TOTLS, TOTFT) showed $r = 0.61$–0.66 ($n = 19$, $P_c = 0.005$–0.002). Expectedly, these scales are similar and well correlated ($r \geq 0.87$) with one another. Omission of Lys, which is an outlier in these plots, improves these correlations to $r = 0.71$–0.75 ($n = 18$, $P_c < 0.001$). The correlation of K_m for CH_2H_4folate with TOTFT is shown in Figure 2B. Interestingly, the scales which showed highest correlation with k_{cat} (see above) showed poor correlations with K_m ($r = 0.28$–0.32).

We also assessed whether kinetic parameters were themselves correlated. Log k_{cat} vs log K_m for CH_2H_4folate showed an inverse correlation with $r = -0.58$ ($n = 19$), with Arg, Gly, and Trp as apparent outliers; these are the same outliers observed in most log k_{cat} vs hydrophobicity plots examined here. Omitting these outliers, the parameters correlated with $r = -0.67$. Although k_{cat} is contained within the numerator of the K_m term for CH_2H_4folate, the inverse correlation obtained indicates that changes in k_{cat} do not dominate the correlation and that the k_{cat} and K_m terms behave independently. This conclusion is also supported by the observation that k_{cat} and K_m values are correlated with different hydrophobicity scales (see above). Log k_{cat} values also showed an excellent correlation with log k_{cat}/K_m for CH_2H_4folate ($r = 0.87$); however, the two parameters are not independent, and the correlation is dominated by changes in k_{cat}. When similar plots were made using randomly generated values for K_m, high correlations ($r = 0.87$–0.91) were obtained (Estell, 1987).

We determined steady-state kinetic properties for the TS-catalyzed dehalogenation of BrdUMP (eq 1) (Garrett et al.,

$$\text{BrdUMP} + 2\ \text{RSH} \rightarrow \text{dUMP} + (\text{RS})_2 + \text{Br}^- \quad (1)$$

1979) by wild-type TS and several mutants. The mutants chosen have a 183-fold range of k_{cat} values and a 27-fold range of K_m values for CH_2H_4folate in the normal reaction (Table I). In the dehalogenation of BrdUMP, both K_m and k_{cat} values were essentially the same for wild-type and all the mutants examined.

Discussion

The C-terminal valine (Val 316) of *L. casei* TS has been implicated to be important for folate binding and catalytic function (Aull et al., 1974; Galivan et al., 1977). Crystallographic studies of various forms of TS provide insight into how the C-terminal residue is involved in the TS reaction (Hardy

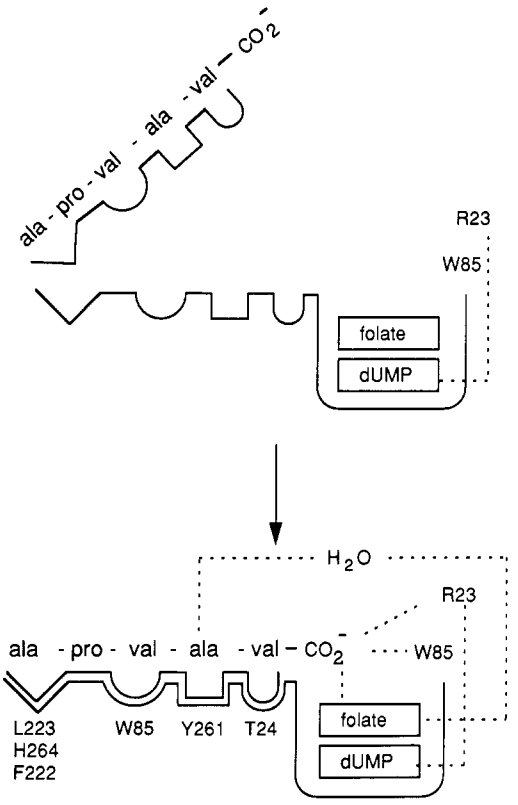

FIGURE 3: Depiction of interactions of the C-terminus after the conformational change. After binding of nucleotide and cofactor, C-terminal residues move to cover the active site and form new side-chain interactions and an extended hydrogen-bond network (dotted lines).

et al., 1987; Perry et al., 1990; Montfort et al., 1990; Matthews et al., 1990). The structures of free TS and the binary TS–dUMP complex are very similar. In both structures, C-terminal residues are highly exposed to solvent, and crystallographic *B* factors are high for these residues; but there are no large differences in the two structures. When the cofactor analog CB3717 is bound by the TS–dUMP complex, a large conformational change occurs in which up to seven residues at the C-terminus move to cover the active site and form a lid over the bound substrates (Figure 3) (Montfort et al., 1990; Matthews et al., 1990).

The TS reaction proceeds by an ordered mechanism (Danenberg & Danenberg, 1978; Daron & Aull, 1978), where dUMP binds first and forms part of the binding site for the folate cofactor. It has been proposed that the Michaelis complex undergoes a conformational change which contributes to (i.e., precedes or is involved in) a rate-determining step of the reaction (Santi et al., 1987). In the TS–dUMP–CB3717 and TS–FdUMP–CH_2H_4folate complexes,[3] a rapidly reversible ternary complex first forms, and then a slower conformational change occurs to give the stable, isolable complexes. We believe that the structure of the TS–dUMP–CB3717 complex resembles a steady-state intermediate formed after the conformational change rather than the initial Michaelis complex and that the conformational change observed in the structure is analogous to that proposed for the normal reaction (Santi et al., 1987). Available data suggest that, upon binding of the folate cofactor or analog to the TS–dUMP complex, new interactions occur which promote the conformational change and trigger catalysis. Features of the conformational change which are of interest in the context of this study are as follows (Figure 3).[4]

First, the C-terminal valine moves approximately 4 Å and experiences significant changes in its local environment. In the free enzyme and binary complex, side chains at the C-terminus and the completely conserved Thr 24 are both solvent accessible, with the hydroxyl group of Thr 24 pointing toward the exterior of the protein. In the ternary complex, the side chain of Thr 24 points toward the interior of the protein and is buried; the methyl groups of the C-terminal side chain abut the β and γ carbons of Thr 24, resulting in placement of the C-terminal side chain in a hydrophobic environment. The carboxylate of the C-terminal residue forms hydrogen bonds with the ring NH of Trp 85 which moves slightly over the folate, and with Arg 23 which moves to interact with the phosphate of dUMP (in eukaryotes and T4 phage Asn replaces Trp 85 of bacteria and could play a similar role in the hydrogen-bond network) (Montfort et al., 1990; Matthews et al., 1990).

Second, the remainder of the C-terminal arm experiences new interactions that may be relevant to the conformational change. In the ternary complex, the side chain of the conserved penultimate Ala 315 (Ser in *Bacillus subtilis* TsB and fungal TSs) is almost completely buried against the edge of the conserved Tyr 261, and the carbonyl oxygen of Ala 315 is hydrogen-bonded through a water molecule to the 2-NH_2 and 3-NH of the pyrimidine ring of the folate analog. At residue 314, *L. casei* TS and other bacteria have Val or Ile (Met in eukaryotes and T4 phage), which contacts the surface of the folate and Trp 85 (Gln in eukaryotes and T4 phage). Residue 312 is an Ala in bacteria (Met in eukaryotes) and fits into a pocket formed by conserved Leu 223, His 264, and Phe 222 (Gly 222 in eukaryotes); the side chain of residue 312 also moves from a solvent-accessible to a nearly inaccessible state upon formation of the ternary complex. It is interesting that residues involved in interactions of the C-terminus in the ternary complex are either completely conserved (residues 24, 223, 261, and 264) or undergo covariant changes in bacterial and eukaryotic TSs (residues 85, 222, 312, 314, and 315). This conservation and covariance points to functional roles for these residues that we speculate may be related to the conformational change necessary for catalysis.

We have used a combination of mutagenesis and steady-state kinetics to examine the role of the C-terminal valine of *L. casei* TS. Using a synthetic *L. casei* TS gene and cassette mutagenesis with degenerate oligonucleotides, we constructed a complete replacement set of mutants at position 316 (Figure 1). The mutants consisted of the 19 amino acid substitutions and the enzyme truncated by one residue (V316Am). Each mutant was expressed at high levels from the mutagenesis vector and purified, and its steady-state kinetic parameters were measured.

We could not detect TS activity in V316Am, which is in accord with the results obtained with carboxypeptidase truncation of TS (Aull et al., 1974). Since removal of the side chain alone (V316G) is sufficient to reduce k_{cat} by over 100-fold, it is not surprising that complete removal of the C-terminal residue should have a large effect. The loss of activity displayed by V316Am can be rationalized from knowledge of

[3] In comparison of structures, we use those of the *L. casei* and *E. coli* free TS, the TS–dUMP binary complex from *L. casei*, and the ternary *E. coli* TS–dUMP–CB3717 complex. The assumption is made that relevant features of the ternary complex are similar in the *L. casei* and *E. coli* TSs. Free enzyme is used to refer to the structure which has inorganic phosphate bound at the active site.

[4] Amino acid numbers refer to those of the *L. casei* enzyme.

the structure of the ternary TS–dUMP–CB3717 complex. First, the position of the C-terminal carboxylate in the ternary complex of V316Am would be moved by at least 3–4 Å, disrupting part of the hydrogen-bond network that facilitates CH_2H_4folate binding. Second, the loss of a residue at that position would abolish side-chain-mediated hydrophobic interactions that contribute to the conformational change. Third, the trunctuation would disrupt hydrogen bonding of the penultimate residue of TS to the folate. A complete study of the V316Am mutant (Carreras et al., 1992) and the crystal structure of this mutant (K. Perry, unpublished results) will be reported elsewhere.

Because of the putative interactions of the C-terminal side chain in the ternary complex, we attempted to correlate the steady-state kinetic parameters of the mutants with hydrophobicity and volume parameters of the side chains. Upon initial assessment, we could find no significant correlations of kinetic parameters with side-chain volume, but correlations with hydrophobicity appeared promising. Since the various hydrophobicity scales reported are not all well correlated, and since we had no objective reason for choosing one over another, we first evaluated our data against some 40 hydrophobicity scales tabulated by Cornette et al. (1987). Next, we chose the best correlations and evaluated each independently. When outliers in the correlations were apparent, we removed them from the data set and reevaluated the correlation.

Using several commonly used hydrophobicity scales, there was a very good correlation ($r = 0.92$) between log k_{cat} of the mutants and the hydrophobicity of the side chain at position 316 (Figure 2). This indicates that hydrophobic interactions stabilize an intermediate prior to the rate-determining step or the transition state of the rate-determining step in the TS reaction. In the context of what is known about TS structure and mechanism, it is proposed that hydrophobic interactions between the side chain of the C-terminal residue and Thr 24 are involved in inducing the conformational change which places the C-terminal lid over the substrates. Why Trp and Gly, and sometimes Arg, are outliers in the correlations is not clear. Possible explanations include suboptimal packing of the largest and smallest side chains, disparity in the hydrophobicity values of these residues, or partial inactivation of these mutants.

The apparent K_m values for dUMP were similar in wild-type TS and 18 mutants, indicating that the binding of dUMP is not significantly affected by mutations in the C-terminal residue. This is consistent with crystallographic studies which show that the free enzyme and the binary dUMP–TS complex have similar structures and that the C-terminus is not directly involved in nucleotide binding in the binary complex (Hardy et al., 1987; Perry et al., 1990; Montfort et al., 1990; Matthews et al., 1990; R. M. Stroud, unpublished results). In contrast, K_m values of CH_2H_4folate varied about 60-fold among mutants and were correlated ($r = 0.71$–0.75) with several different hydrophobicity scales. The correlation suggests that hydrophobic interactions of the C-terminal side chain are involved in the initial interaction of the cofactor with the TS–dUMP complex to form the Michaelis complex.

There is an inverse correlation ($r = -0.67$) between log k_{cat} and log K_m of the cofactor, which reflects the dependence of both cofactor binding and catalysis on hydrophobicity of the C-terminal side chain. In general, mutants with hydrophobic side chains at residue 316 show lower K_m values and higher k_{cat} values. Thus, hydrophobicity may be loosely correlated with both tighter binding of cofactor and more facile catalysis. Although k_{cat}/K_m values show apparent correlations with K_m, the correlations are not interpreted since they seem to be computational artifacts that are manifestations of the changes in k_{cat}.

We have also examined the kinetic properties of several Val 316 mutants using the dehalogenation of BrdUMP. As an alternate substrate, BrdUMP undergoes a partial reaction which involves nucleophilic attack by Cys 198 of TS, followed by exogenous thiol-mediated dehalogenation to give dUMP (Garrett et al., 1979); CH_2H_4folate is not involved in the dehalogenation. In dTMP formation, the k_{cat} values of wild-type TS and the mutants examined span a range of about 180-fold, but the K_m values for dUMP are essentially unchanged. The K_m values for BrdUMP are not significantly different in the mutants examined. This agrees with and supports the previous conclusion that binding of nucleotide is not significantly affected by mutations at position 316 of TS. Unlike dTMP formation, k_{cat} values for dehalogenation are essentially the same with these mutants; there is no correlation of k_{cat} with hydrophobicity of the 316 side chain. This agrees with the hypothesis that binding of the cofactor is required to trigger the conformational change associated with closure of the carboxy-terminal "lid" over the active site.

It has been shown that isolated, noninteracting mutations on enzymes often have additive effects on catalytic properties (Wells, 1990). It would be interesting to identify additional sites of TS which show a linear free energy relationship of k_{cat} versus properties of the side chains within a replacement set. With this information, it may be possible to predict the properties of all permutations of multiple mutations within the two replacement sets.

We speculate that the C-terminal residue of TS has evolved toward hydrophobic amino acids to optimize activity, cofactor binding, and the conformational change. In 14 of the 18 TS sequences known, valine is the C-terminal residue, and it is more active than any mutant at that position for *L. casei* TS. Ile, Leu, and Ala substitutions at position 316 were among the most active mutants in the replacement set; these residues are at the C-termini of TSs from *E. coli* (Ile), *Plasmodium falciparum* (Ala), *Plasmodium carinii* (Leu), and *Varicella zoster* (Leu). However, in *L. casei* TS, all of these mutations result in significant increases in K_m for folate and decreases in k_{cat} compared to wild-type TS, so it is likely there are covariant residues in these TSs important in the functions of the C-terminal residue. We are attempting to identify what those residues are.

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Registry No. TS, 9031-61-2; CH_2H_4folate, 3432-99-3; Val, 72-18-4; dUMP, 964-26-1; Ile, 73-32-5; Leu, 61-90-5; Phe, 63-91-2; Thr, 72-19-5; Cys, 52-90-4; Ala, 56-41-7; Met, 63-68-3; Pro, 147-85-3; His, 71-00-1; Ser, 56-45-1; Asn, 70-47-3; Gln, 56-85-9; Tyr, 60-18-4; Glu, 56-86-0; Lys, 56-87-1; Trp, 73-22-3; Arg, 74-79-3; Gly, 56-40-6.

REFERENCES

Aull, J. L., Loeble, R. B., & Dunlap, R. B. (1974) *J. Biol. Chem. 249*, 1167–1172.

Birnboim, H. C., & Doly, J. (1979) *Nucleic Acids Res. 7*, 1513–1523.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Bruice, T. W., & Santi, D. V. (1982) *Biochemistry 21*, 6703–6709.

Carreras, C. W., Climie, S. C., & Santi, D. V. (1992) *Biochemistry* (following paper in this issue).

Chen, E. J., & Seeburg, P. H. (1985) *DNA 4*, 165–170.

Chothia, C. (1984) *Annu. Rev. Biochem. 53*, 537-572.
Climie, S., & Santi, D. V. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 633-637.
Climie, S. C., Ruiz-Perez, L., Gonzales-Pacanowska, D., Prapunwattana, P., Cho, S. W., Stroud, R. M., & Santi, D. V. (1990) *J. Biol. Chem. 265*, 18776-18779.
Cornette, J. L., Cease, K. B., Margalit, H., Spouge, J. L., Berzofsky, J. A., & DeLisi, C. (1987) *J. Mol. Biol. 195*, 659-685.
Danenberg, P. V., & Danenberg, K. D. (1978) *Biochemistry 17*, 4018-4024.
Daron, H. H., & Aull, J. L. (1978) *J. Biol. Chem. 253*, 940-945.
Estell, D. A. (1987) *Protein Eng. 1*, 441-447.
Finer-Moore, J. S., Montfort, W. R., & Stroud, R. M. (1990) *Biochemistry 29*, 6977-6986.
Galivan, J., Maley, F., & Baugh, C. M. (1977) *Arch. Biochem. Biophys. 184*, 346-354.
Garrett, C., Wataya, Y., & Santi, D. V. (1979) *Biochemistry 18*, 2798-2804.
Hardy, L. M., Finer-Moore, J. S., Montfort, W. R., Jones, M. O., Santi, D. V., & Stroud, R. M. (1987) *Science 235*, 448-455.
Laemmli, U. K. (1970) *Nature 227*, 680-685.
Matthews, D. A., Appelt, K., Oatley, S. J., & Xuong, N. H. (1990) *J. Mol. Biol. 214*, 923-936.
Michaels, M. L., Kim, C. W., Matthews, D. A., & Miller, J. H. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3957-3961.
Miller, J. H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Montfort, W. R., Perry, K. M., Fauman, E. B., Finer, M. J. S., Maley, G. F., Hardy, L., Maley, F., & Stroud, R. M. (1990) *Biochemistry 29*, 6964-6977.
Perry, K. M., Fauman, E. B., Finer-Moore, J. S., Montfort, W. R., Maley, G. M., Maley, F., & Stroud, R. M. (1990) *Proteins 8*, 315-333.
Pogolotti, A. L. J., Danenberg, P. V., & Santi, D. V. (1986) *J. Med. Chem. 29*, 478-482.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning—A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Santi, D. V., & Danenberg, P. V. (1984) in *Folates and Pterins* (Blakley, R. L., & Benkovic, S. J., Eds.) pp 345-398, John Wiley & Sons, Inc., New York.
Santi, D. V., McHenry, C. S., & Sommer, H. (1974) *Biochemistry 13*, 471-480.
Santi, D. V., McHenry, C. S., Raines, R. T., & Ivanetich, K. M. (1987) *Biochemistry 26*, 8606-8613.
Segel, I. H. (1975) *Enzyme Kinetics: Behavior and Analysis of Steady-State and Rapid Equilibrium Enzyme Systems*, Wiley-Interscience, New York.
Tabor, S., & Richardson, C. C. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4767-4771.
Wells, J. A. (1990) *Biochemistry 29*, 8509-8517.
Wells, J. A., Vasser, M., & Powers, D. (1985) *Gene 34*, 315-323.

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

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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-*

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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.

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