Partial Restoration of Activity to *Lactobacillus casei* Thymidylate Synthase following Inactivation by Domain Deletion†

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ABSTRACT: Thymidylate synthase (TS) from *Lactobacillus casei* has a 50 amino acid insert (residues 90–139) in the small domain that is found in only one other TS. A deletion mutant was constructed which lacked the entire insert, thereby reducing the small domain to the size found in *Escherichia coli* TS. This mutant did not catalyze the formation of dTMP. From the crystal structure of *L. casei* TS, we surmised that the loss of activity might have resulted from the exposure of residues of helices C and D, which were previously buried by the insert. To restore the local structure of helices C and D in the deletion mutants, we replaced several residues in this region by the corresponding residues found in *E. coli* TS. The mutant whose sequence most closely resembled that of *E. coli* TS carried six mutations and possessed partially restored TS activity. The mutant which had all those mutations except F87D did not catalyze any dTMP formation. The crucial role of F87D was proven in a deletion mutant which had only this change and showed greatly increased activity. All of the mutants catalyzed the debromination of BrdUMP in the absence of cofactor about as well as wild type TS. The kinetic parameters for dTMP formation of the active mutants show that the deletion has its major effect on k_{cat} and binding of cofactor CH_2H_4folate, with less effect on binding of the substrate dUMP. Removal of residues 90–139 is believed to disorder helices C and D, which in turn decreases cofactor binding and catalysis.

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. TS has been isolated from different sources such as bacteriophages, bacteria, fungi, protozoans, and vertebrates, and comparison of over 20 known sequences shows that TS is among the most highly conserved enzymes (Perry et al., 1990). Three-dimensional structures of TS from several species have been determined (Hardy et al., 1987; Matthews et al., 1990; Montfort et al., 1990; Perry et al., 1990).

TS from *Lactobacillus casei* is a dimer of identical subunits with a molecular weight of about 35 kDa per subunit. Each monomer is composed of a large and a small domain, separated by a cleft. Whereas the large domain comprises highly conserved regions that form important secondary structural elements common in all TS molecules, the small domain is poorly conserved.

The small domains of all TSs consist of two short helices, C (residues 71–78)[2] and D (residues 84–88), followed by an insert of variable length in different species (for aligned sequences of TS species, see Perry et al., 1990). In 11 of the known sequences this insert comprises 13 amino acids, which form a loop on the surface of the protein. In three TSs the insert consists of only one amino acid, connecting the D helix of the small domain with the G helix of the large domain. The largest insert of 40 amino acid residues is found only in *L. casei* TS and in TS from the transposon Tn4003 from *Staphylococcus aureus* (Rouch et al., 1989). The insert comprises about 70% of the small domain from *L. casei* TS and forms a surface loop (residues 89–103), followed by helices D′ (residues 104–109), E (residues 121–130), and F (residues 133–139) (Finer-Moore et al., 1993).

The function of the small domain is not known, but because of its high variability it is not expected to be relevant to fundamental aspects of structure or function. The X-ray crystallographic structure of TS indicated to us that deletion of the entire *L. casei* insert (residues 90–139) would leave the remaining COOH- and NH_2-termini in close enough proximity that a connection might be made without major perturbations in the core structure (Hardy et al., 1987).

In this paper we describe the deletion of the entire 50 amino acid insert (residues 90–139) of *L. casei* TS, reducing the small domain to the size found in *Escherichia coli* TS. This deletion mutant catalyzed partial reactions of TS, but did not catalyze dTMP formation. However, further mutations in the adjacent C and D helices, making residues more "*E. coli*-like", resulted in the partial restoration of TS activity. In particular, a single mutation at position 87 was shown to be essential for proper enzyme function.

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[1] Abbreviations used: TS, thymidylate synthase; 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; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; BrdUMP, 5-bromo-2′-deoxyuridine 5′-monophosphate; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; NO_2dUMP, 5-nitro-2′-deoxyuridine 5′-monophosphate; DTT, dithiothreitol; CB3717, 10-propargyl-5,8-dideazafolate; PABA, *p*-aminobenzoic acid.

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

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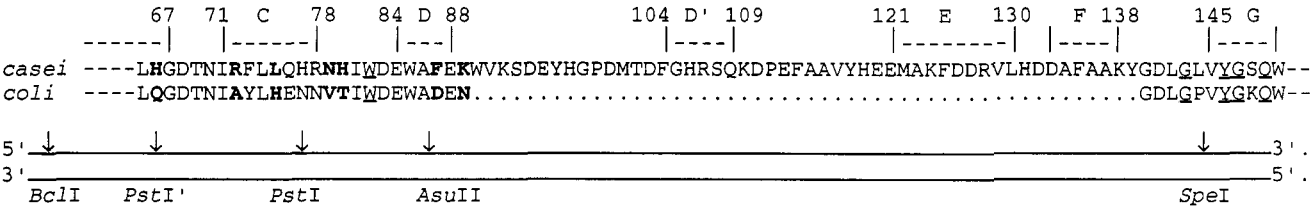

FIGURE 1: Aligned sequences of residues 65–150 of TS from *L. casei* and *E. coli*, comprising the mutated region. Residues that have been replaced by the respective *E. coli* residues are in bold. Residues that are completely conserved among all known TSs are underlined. Aligned below is a part of pSCTS9Δ*Pst*I containing the synthetic TS gene with the restriction sites used for construction of the deletion mutants.

MATERIALS AND METHODS

Materials. The Sequenase DNA sequencing kit was purchased from U. S. Biochemicals. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs and Bethesda Research Laboratories (BRL) and were used according to the manufacturers instructions.

E. coli strains DH5α and Thy⁻ χ2913 (ΔthyA572) were obtained from BRL and R. Thompson (University of Glasgow, UK), respectively. Oligonucleotides were synthesized at the University of California, San Francisco Biomolecular Resource Center. In vitro cassette mutagenesis was performed using a synthetic TS gene carried on the plasmid pSCTS9Δ*Pst* I which has a single *Pst*I site that is located in the region being mutagenized (Climie & Santi, 1990). Q-Sepharose (Fast Flow) was obtained from Pharmacia. [5-^{3}H]dUMP (20 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 purchased from Moravek Biochemicals. (6*R*)-CH_2H_4folate was a generous gift from SAPEC S.A. (Lugano, Switzerland). BrdUMP was obtained from Sigma and was purified by DEAE-cellulose chromatography (Wataya & Santi, 1977). All other materials were obtained from commercial sources and used without further purification.

Construction of Deletion Mutants, pTSDel1 and pTSDel2. The deletion of residues 90–139 was accomplished by restriction of pSCTS9Δ*Pst*I with *Asu*II and *Spe*I (Figure 1) and ligation of the cut vector with a synthetic oligonucleotide duplex containing compatible *Asu*II and *Spe*I ends and the coding sequence for residues 89 and 140–143. The oligos used were 5′ CGAAAA(AC)GGTGACCTGGGA 3′ and 5′ CTAGTCCCAGGTCACC(TG)TTTT 3′. The cassette contained a degenerate codon for residue 89 AA(AC) coding for asparagine or lysine at this position in order to obtain the two mutant enzymes, designated TSDel1 and TSDel2.

To reduce the number of wild type clones the ligation mixture was restricted with *Bam*HI which has a unique recognition sequence within the deleted segment but not in the desired recombinant. The restricted ligation mixture was used to transform *E. coli* DH5α, transformants were pooled, and DNA was extracted and used to transform the Thy⁻ strain χ2913. Resulting transformants were analyzed for their ability to complement TS activity by plating on minimal plates containing ampicillin (100 mg/mL) and containing or lacking thymidine (50 μg/mL).

Restriction analysis was used to identify plasmids with the desired deletion, and DNA sequence analysis (Sequenase DNA sequencing kit) was used to confirm the mutation.

Mutagenesis of Residues within the C and D Helices. First, a "stuffer" plasmid (Wells et al., 1986) containing an additional *Pst*I site (designated as *Pst*I′) at residues 65/66 was engineered by restricting plasmid pTSDel1 described above with *Bcl*I and *Pst*I (Figure 1). The *Bcl*I–*Pst*I fragment was replaced by insertion of a DNA cassette containing *Bcl*I and *Pst*I compatible ends, codons for residues 58–65, and a single base change (CAC)H66Q(CAG) for the new *Pst*I′ site, which was followed by a "stuffer" sequence with sites for *Not*I and *Sph*I. Plasmid DNA was prepared, and the H66Q mutation and "stuffer" sequence were verified by restriction analysis. TSDel3 was prepared as follows. A synthetic oligo cassette (5′ GGGTGATACCAATATTGCTTTCCTGCACCAGCACCGCGTTACTATCTGGGATGAGTGGGCTTT 3′, 5′ CGAAAGCCCACTCATCCCAGATAGTAACGCGGTGCTGGTGCAGGAAAGCAATATTGGTATCACCCTGCA 3′) retained the *Pst*I′ site and the H66Q mutation and contained sequence changes to mutant the following residues: R72A, L75H, N79V, and H80T. This oligonucleotide duplex was ligated into *Pst*I′/*Asu*II digested "stuffer" plasmid, and the ligation mixture was cut with *Not* I in order to restrict transformants carrying the parent plasmid. The ligation mixture was used to transform *E. coli* DH5α. Plasmid DNA was prepared, and the desired recombinant was identifed by restriction analysis. The resulting plasmid, designated as pTSDel3, was used for construction of deletion mutant TSDel4, which had an additional F87D mutation. The oligonucleotide duplex (5′ GGGTGATACCAATATTGCTTTCCTGCACCAGCACCGCGTTACTATCTGGGATGAGTGGGCTGA 3′,5′ CGTCAGCCCACTCATCCCAGATAGTAACGCGGTGCTGGTGCAGGAAAGCAATATTGGTATCACCCTGCA 3′), containing the changes at positions 72, 75, 79, 80, and 87, was inserted between the *Pst*I′ and *Asu*II sites of pTSDel3. The F87D mutation requires destruction of the *Asu*II site; therefore, cleavage by that enzyme was used to restrict transformants carrying the parent plasmid. pTSDel5 was obtained by restricting plasmid pTSDel1 with *Pst*I and *Asu*II and ligating the oligonucleotide duplex (5′ GCACCGCAACCACATCTGGGATGAGTGGGCTGA 3′, 5′ CGTCAGCCCACTCATCCCAGATGTGGTTGCGGTGCTGCA 3′) into these restriction sites. Insertion of this cassette coded for a single mutation F87D and also caused destruction of the *Asu*II site. Thus, restriction with *Asu*II was used to restrict parent transformants. Plasmid DNA was extracted, and the F87D mutation was verified using restriction analysis. The correctness of all three deletion mutants was verified by DNA sequencing.

For construction of pTSDel6 the recombinant circle PCR technique was performed as described (Jones & Winistorfer, 1992). In each of the two PCR amplifications, pTSDel5 served as template and was linearized by restriction enzyme digestion outside the region to be amplified. The primer pairs used in the first mixture were 5′ CGGTGATACCAATATTGCTTTCCTGCACCAGCACCGCAACACTATCTGGGATGAGTGGGCTGA 3′ (mutagenesis primer, top); 5′ GTTTTCCCAGTCACGAC 3′ (M13 sequencing primer, –40) and 5′ CGTCAGCCCACTCATCCCAGATAGTGTTGCGGTGCTGGTGCAGGAAAGCAATATTGGTATCACCGTGCA 3′ (mutagenesis primer, bottom); and 5′ CTGATCAAAAGCGAGCTG 3′ (pSCTS9–706 sequencing

primer) in the second mixture. The mutagenesis primers contained the changes at positions 72, 75, 80, and 87. After amplification, equal volume aliquots of crude PCR products were combined, denatured (94 °C × 3 min), annealed (50 °C × 2 h), and transformed into MAX Efficiency DH5α cells (BRL). Plasmid DNA was extracted, and restriction analysis was used to screen for the transformants. The sequence of the mutagenized region was verified by automated DNA sequencing. To avoid additional sequencing, a 274 bp long DNA cassette was cut using *Kpn*I and *Spe*I, gel purified, and moved into *Kpn*I/*Spe*I digested pSCTS9Δ*Pst*I to give the final construct pTSDel6.

Protein Purification. Expression of all six deletion mutants was sufficiently high that purifiction could be monitored by SDS–PAGE. Deletion mutants TSDel1, TSDel2, and TSDel6 were purified by automated sequential chromatography on phosphocellulose and hydroxylapatite as described (Kealey & Santi, 1992).

TSDel3, TSDel4, and TSDel5 were purified using the following procedure. The cell pellets from 4 L of culture (about 24 g) were resuspended in 20 mM Tris, pH 7.4, 0.1 mM EDTA containing 50 mM KCl (buffer A). All manipulations were performed on ice or at 4 °C. Cells were lysed by two passes through a French Presure cell at 18 000 psi. Cell debris was removed by centrifugation at 27000*g* for 30 min. High molecular weight nucleic acid was precipitated by addition of 5% streptomycin sulfate (0.15 mL/mL), and the precipitate was removed by centrifugation. The supernatant was loaded at a flow rate of 1 mL/min directly onto a Q-Sepharose column (60-mL bed volume), which was equilibrated with buffer A. After the column was washed with 250 mL of the same buffer, the protein was eluted with a 400-mL linear gradient from 50 to 400 mM KCl in buffer A. Fractions were collected and analyzed on SDS–PAGE performed on a Phastsystem (Pharmacia). The TS-containing fractions were pooled, and TSDel3 or TSDel4 were each precipitated by addition of $(NH_4)_2SO_4$ to 24% saturation. The precipitated proteins were recovered by centrifugation and dissolved in buffer A. Purification of TSDel5 required an additional column. The Q-Sepharose pool of TSDel5 was adjusted to pH 7.0 and loaded onto a hydroxylapatite column (25-mL bed volume), which was equilibrated with 20 mM KH_2PO_4, pH 7.0, containing 0.1 mM EDTA. The column was washed with 125 mL of the same buffer, and the protein was eluted with a 150-mL linear gradient from 20 to 400 mM phosphate. After analysis by SDS–PAGE, the TS-containing fractions were pooled, and contaminating proteins were precipitated by addition of $(NH_4)_2SO_4$ to 30% saturation. The $(NH_4)_2SO_4$ concentration of the supernatant was increased to 60% saturation to precipitate TSDel5, which was recovered by centrifugation and dissolved in buffer A. Purified enzymes were stored at −80 °C.

Enzyme Assays. TS activity was monitored spectrophotometrically at 340 nm as described (Pogolotti et al., 1986). The standard assay buffer contained 50 mM TES, pH 7.4, 25 mM $MgCl_2$, 6.5 mM formaldehyde, 1 mM EDTA, and 75 mM β-mercaptoethanol. Both dUMP and (6*R*)-CH_2H_4folate were present at a concentration of 100 μM.

TS-catalyzed exchange of the 5-hydrogen of dUMP for solvent protons was monitored at 20 °C by the decrease in $^3H/^{14}C$ of [2-^{14}C,5-^{3}H]dUMP during the course of reaction (Pogolotti et al., 1979). Reaction mixtures contained 4 μM enzyme, 200 μM (6*R*)-CH_2H_4folate, and 200 μM [2-^{14}C,5-^{3}H]dUMP (22 mCi ^{3}H/mmol, 8 mCi ^{14}C/mmol) in standard assay buffer. Aliquots (50 μL) were withdrawn and analyzed as described (Carreras et al., 1992). For determination of k_{cat} and K_m of CH_2H_4folate, TS concentrations were 2 (TSDel4), 0.25 (TSDel5), or 0.1 μM (TSDel6), the concentration of dUMP was fixed at 200 μM, and CH_2H_4folate was varied between 0.2 and 8 × K_m. For determination of K_m of dUMP, (6*R*)-CH_2H_4folate was present at a concentration of 15 (TSDel4) or 3 mM (TSDel5, TSDel6), and dUMP was varied between 2.5 and 300 μM. Steady-state kinetic parameters were obtained by a nonlinear least-squares fit of the data to the Michaelis–Menten equation using the program Kaleidagraph (Abelbeck Software, 1989) run on a Macintosh II computer. Because of the high K_m of CH_2H_4folate exhibited by deletion mutant TSDel4, saturation concentration was not feasible when measuring the kinetic parameters for dUMP. Therefore, k_{cat} was corrected using the equation for a sequential ordered mechanism, $V_{max} = V_{app}(1 + K_{mB}/[B])$ (Segel, 1975).

TS-catalyzed dehalogenation of BrdUMP was performed as described (Garrett et al., 1979). Reaction mixtures (0.75 mL) contained 1.5 μM enzyme, and BrdUMP was varied between 3 and 200 μM. Kinetic constants were determined as above.

Enzyme concentrations were measured spectrophotometrically. The extinction coefficient of TSDel4 (ϵ_{278} = 94 760 M^{-1} cm^{-1}) was determined by the method of (Edelhoch, 1967), and it was assumed to be the same for all six deletion mutants.

The numbers of active sites of TSDel4, TSDel5, and TSDel6 were measured by difference spectral titration with NO_2dUMP as described (Wataya et al., 1979). NO_2dUMP was added in increments to a sample cuvette containing 6.7 μM enzyme in 50 mM TES, pH 7.4, 25 mM $MgCl_2$, 6.5 mM formaldehyde, 1 mM EDTA, and 5 mM DTT and to a reference cuvette lacking enzyme. Spectra were taken and corrected for light scattering (SCATWAV program, Hewlett-Packard), reference spectra were subtracted from the sample spectra, and the change in absorbance at 345 nm was recorded. $\Delta\epsilon_{345}$ values for enzyme–NO_2dUMP complexes were obtained by addition of equal amounts of the appropriate mutant enzyme to a cuvette containing 6 μM NO_2dUMP in the same buffer. This titration was continued until no further absorbance increase at 345 nm was observed.

SDS–PAGE of Ternary Complexes. TS-[6-^{3}H]FdUMP–CH_2H_4folate complexes were formed and analyzed by SDS–PAGE as described (Sirawaraporn et al., 1990). The reaction mixture contained 2.5 μM enzyme, 0.5 μM [6-^{3}H]FdUMP, and 137 μM CH_2H_4folate in the standard assay buffer. The samples were incubated 2 h; control reactions lacked CH_2H_4-folate.

HPLC Analysis of dTMP Formation. HPLC was performed as described (Carreras et al., 1992). Reaction mixtures contained 100 μM [2-^{14}C]dUMP, 100 μM CH_2H_4folate, and 4 μM enzyme in standard TS assay buffer and were incubated for 2 h at room temperature. Prior to HPLC analysis, samples were deproteinized using centricon 10 ultrafiltration devices.

RESULTS

Deletion of Residues 90–139. Two TS deletion mutants lacking the entire insert from position 90 to position 139 were prepared by cassette mutagenesis using the synthetic *L. casei* gene pSCTS9Δ*Pst*I (Climie & Santi, 1990) and a degenerate oligonucleotide coding for both Lys (found in *L. casei*) and Asn (found in *E. coli*) at position 89 (Figure 1). These mutants were designated as TSDel1 and TSDel2 (see Table 1 for our naming convention). Neither of these mutants was able to complement growth of Thy$^-$ *E. coli* strain χ2913 on plates in

Table 1: Nomenclature of Deletion Mutants from *L. casei* TS

name by convention	abbreviation
TSΔ90–139	TSDel1
TSΔ90–139,K89N	TSDel2
TSΔ90–139,H66Q,R72A,L75H,N79V,H80T	TSDel3
TSΔ90–139,H66Q,R72A,L75H,N79V,H80T,F87D	TSDel4
TSΔ90–139,F87D	TSDel5
TSΔ90–139,R72A,L75H,H80T,F87D	TSDel6

the absence of thymidine, indicating a specific activity of less than 2×10^{-3} unit/mg in the crude extract (Climie et al., 1990).

Mutations within the C and D Helices. Three additional deletion mutants lacking the insert and containing different combinations of *E. coli*-like residues in the adjacent amino acid sequence were prepared by cassette mutagenesis. First, we constructed a "stuffer" vector which contains an additional *Pst*I′ site in the plasmid pTSDel1 (see Figure 1), as well as noncoding sequence containing an unique *Not*I site to be used for restriction selection. Introduction of the *Pst*I′ site caused the replacement of His66 to Gln, which is the amino acid found at this position in *E. coli*. This vector allowed simple insertion of a DNA cassette followed by restriction purification to give the deletion mutant, TSDel3, carrying the following mutations: H66Q, R72A, L75H, N79V, and H80T. pTSDel3 served as parent plasmid for construction of the most *E. coli*-like TS mutant, TSDel4, which in addition contains F87D. pTSDel5, carrying mutation F87D alone, was made from plasmid pTSDel1 by cassette mutagenesis. We used the recombinant circle PCR method for construction of plasmid pTSDel6, which had the mutations R72A, L75H, H80T, and F87D. The mutant enzymes TSDel3 through TSDel6 did not complement Thy⁻ deficient χ2913 cells in medium lacking thymine.

Protein Purification. All five mutant proteins were expressed to about 15% of soluble cell protein as estimated by analysis of the crude extract on SDS–PAGE. An automated purification procedure using sequential phosphocellulose/hydroxylapatite chromatography (Kealey & Santi, 1992) was successfully used to purify TSDel1, TSDel2, and TSDel6. The preparations yielded 20, 15, and 30 mg of pure mutant enzyme, respectively, from 1-L cultures. TSDel3, TSDel4, and TSDel5 did not bind to phosphocellulose. These mutant enzymes were purified by chromatography on Q-Sepharose followed by ammonium sulfate precipitation. In the case of TSDel5 further purification on hydroxylapatite was necessary to obtain homogeneous protein. Four liters of cell culture yielded 105 mg of TSDel3, 75 mg of TSDel4, and 60 mg of TSDel5. After purification, single bands on SDS–PAGE migrating with an apparent molecular mass of 30 kDa were observed, and purity was estimated to be higher than 90% for all mutant enzymes.

Synthesis of dTMP. None of the deletion mutants TSDel1–4 was able to complement Thy⁻ *E. coli* cells. Therefore, more sensitive assays were performed to ascertain whether there was any conversion of dUMP to dTMP. Using high enzyme concentrations and long incubation times, a sensitivity of 10^{-3} unit/mg can be obtained in the spectrophotometric assay. TSDel1–4 were tested at 5 μM enzyme for up to 5 h, but no TS activity was detectable. A more sensitive TS assay measures release of tritium as tritrated water from [5-^{3}H]dUMP when dTMP is formed. A mixture of [5-^{3}H,2-^{14}C]dUMP was used, and ^{3}H release was monitored as a decrease in the ^{3}H/^{14}C ratio (Pogolotti et al., 1979). The mutant enzymes TSDel1–TSDel4, were incubated with the

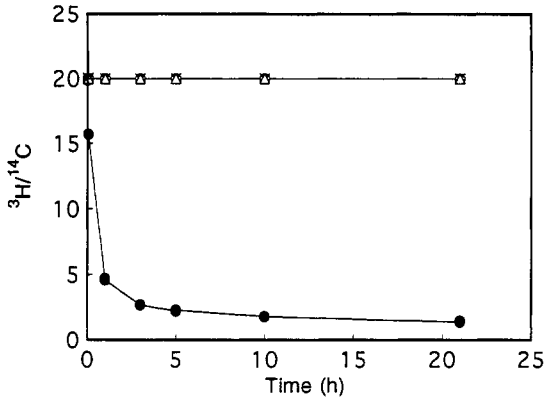

FIGURE 2: Tritium release assay to screen for catalytic activity of deletion mutants from *L. casei* thymidylate synthase: (▲) TSDel1, (○) TSDel2, (×) TSDel3, (●) TSDel4.

labeled mixture in the presence of cofactor. No release of tritium (31 000 cpm ^{3}H, 10 nmol of dUMP) was detected within 24 h for TSDel1, TSDel2, and TSDel3, (Figure 2), indicating that each enzyme has a specific activity of less than 3×10^{-6} unit/mg (<300 cpm ^{3}H, <0.096 nmol of dUMP). However, TSDel4 was able to catalyze the release of tritium from [5-^{3}H]dUMP (Figure 2). In order to determine whether the observed tritium release was accompanied by thymidylate formation, [2-^{14}C]dUMP and CH_2H_4folate were incubated with the enzyme under the conditions described above, and the reaction mixture was separated by HPLC. A new ^{14}C-containing peak was detected, which coeluted with dTMP added as a internal standard.

TSDel5 and TSDel6, designed in an attempt to further increase TS activity were still unable to complement Thy⁻ *E. coli* cells. However, they had activity sufficiently high to be detected by the spectrophotometric method. Steady-state kinetic parameters of TSDel4, TSDel5, and TSDel6 were determined using the tritium release assay (Table 2). Comparing TSDel4 to wild type TS, the K_m values for dUMP and CH_2H_4 folate were increased almost 10- and 300-fold, respectively, and k_{cat} was reduced 500-fold. With TSDel5, K_m values of dUMP and CH_2H_4folate were increased about 3- and 75-fold, and k_{cat} was 37-fold lower. TSDel6 had a slightly higher k_{cat} value than TSDel5, as well as an almost 2-fold decreased K_m for CH_2H_4folate, but K_m for dUMP was found to be about 2-fold higher.

The number of active sites of TSDel4 and TSDel5 were determined by difference spectral titration with NO_2dUMP (Wataya et al., 1979). Values of 1.7 mol of NO_2dUMP/mol of dimer of TSDel4 and 1.5 mol of NO_2dUMP/mol of dimer of TSDel5 were obtained. The values found for TSDel6 and wild type TS were 2 mol of NO_2dUMP/mol of dimer, indicating two active sites per dimer in both enzymes.

Debromination of BrdUMP. It has been shown that wild type TS catalyzes the thiol-dependent dehalogenation of BrdUMP to dUMP in the absence of cofactor (Garrett et al., 1979). Each of the six deletion mutants catalyzed debromination of BrdUMP as determined by UV spectral analysis. The k_{cat} and K_m values for this reaction are very similar to the kinetic parameters of wild type TS (Table 2).

Covalent Complex Formation with FdUMP and CH_2H_4-folate. Wild type TS forms a covalent ternary complex with FdUMP and CH_2H_4 folate (Santi et al., 1974). The deletion mutants were incubated with [6-^{3}H]FdUMP in the presence of cofactor, and covalent ternary complexes were analyzed by SDS–PAGE followed by autoradiography. Five of the deletion mutants, TSDel1, TSDel2, and TSDel4–TSDel6, formed

Table 2: Steady-State Kinetic Constants for Deletion Mutants of *L. casei* TS[a]

	dTMP formation			BrdUMP dehalogenation	
		K_m (μM)			
Enzyme	k_{cat} (s^{-1})	CH_2H_4folate	dUMP	$k_{cat} \times 10^{-3}$ (s^{-1})	K_m (μM)
wild type TS[b]	5.5	14	2.9	13 ± 1	12.0 ± 4.0
TSDel1	nd[c]	nd	nd	11 ± 1	5.1 ± 0.5
TSDel2	nd	nd	nd	7 ± 1	18.0 ± 2.0
TSDel3	nd	nd	nd	7 ± 1	11.4 ± 1.5
TSDel4	0.011 ± 0.001	3090 ± 580	45.0 ± 8.0	16 ± 2	4.1 ± 0.4
TSDel5	0.146 ± 0.004	772 ± 82	8.9 ± 0.8	4 ± 1	17.0 ± 1.6
TSDel6	0.200 ± 0.005	481 ± 85	18.1 ± 1.8	4 ± 1	17.1 ± 2.3

[a] Values given include standard errors from nonlinear least-squares fit of experimental data to kinetic models. [b] Values for wild type TS were taken from Climie et al. (1992). [c] Not detectable.

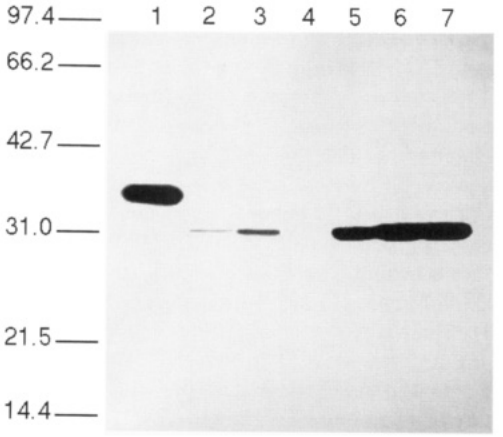

FIGURE 3: Autoradiogram of SDS–PAGE showing the covalent ternary complex between deletion mutants, [6-^{3}H]FdUMP and CH_2H_4folate. Lane 1; wild type TS; lane2, TSDel1; lane3, TSDel2; lane4, TSDel3; lane5, TSDel4; lane6, TSDel5; lane7, TSDel6. Molecular weights ($\times 10^{-3}$) are given.

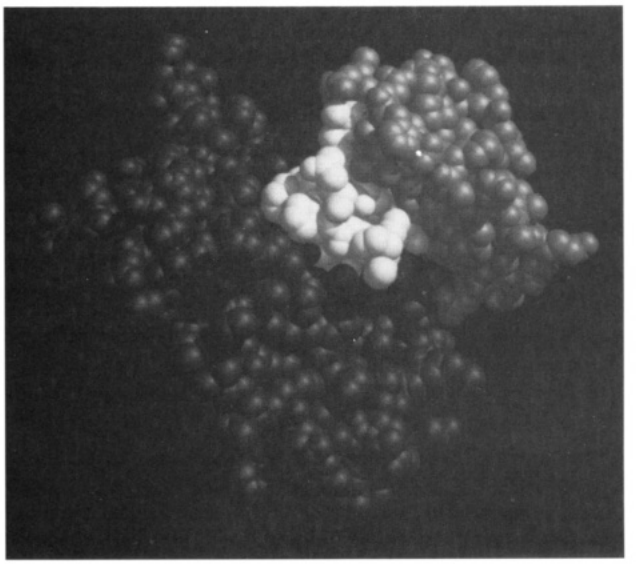

FIGURE 4: Space-filling model of *L. casei* TS. Black, large domain; white, residues 71–88, including helices C and D; gray, insert, comprising residues 90–139 and covering helices C and D.

complexes with FdUMP and CH_2H_4folate, whereas no complex was detected with TSDel3 (Figure 3). The intensity of the bands of TSDel1 and TSDel2 was low compared with that of wild type enzyme. A slightly lower intensity was also observed for the complex formed with TSDel4, whereas bands as strong as for the wild type control were observed with TSDel5 and TSDel6.

DISCUSSION

In the present work we have investigated the importance of a 50 amino acid insert (residues 90–139) which forms a surface loop plus three short helices D′, E, and F within the small domain of *L. casei* TS. In TSs from different species, the small domain varies in length and sequence whereas the large domain contains all of the conserved structural elements which make TS one of the most highly conserved enzymes. Thus, the small domain was not expected to be essential for enzyme function.

It had been suggested (Hardy et al., 1987) that the *L. casei* insert might be deleted without destruction of the core structural elements since the COOH- and NH_2-termini of residues 89 and 140 are in close proximity. Using cassette mutagenesis, we constructed mutant TSDel1 and the corresponding K89N mutant, TSDel2, which lack the entire insert. No dTMP synthesis could be detected for these mutants. Thus, simple deletion of the 50 amino acid insert of *L. casei* TS did not lead to an active enzyme.

In an attempt to understand what effects the deletion might have, we compared the structures of *L. casei* TS to *E. coli* TS. It was our intent to identify residues which might be detrimental in the deletion and to modify them in the hope of restoring function. In all positions mutated we took our lead from nature, making the substitutions that occur in the *E. coli* enzyme (Figure 1).

In the *L. casei* TS crystal structure the insert 90–139 interacts most closely with the C and D helices (residues 71–78, 84–88) (Figure 4). Two hydrophobic side chains of the C and D helices, Leu75 and Phe87, and buried by the insert (Figure 5) and become exposed to water in the deletion mutant, possibly destabilizing helices C and D. The mutations L75H and F87D converted these residues to the hydrophilic residues found in *E. coli* TS. Deleting the insert also removes a specific polar interaction between residue Arg72 of the C helix and Asp133 of the insert. The remaining positive charge was removed by the R72A mutation. In addition to these three residues we targeted Asn79, His80, and His66 for mutagenesis. The N79V mutation converts a buried polar residue to a nonpolar residue of similar volume, possibly increasing the local hydrophobic stability; the H80T mutation converts a completely buried His80 to a residue found in the *E. coli* structure; H66Q converts the flanking residue of the C helix to one found in *E. coli* and was convenient for mutagenesis. Three more residues of the C helix, Gln76, His77, and Arg78, are different in *L. casei* TS from the respective residues in *E. coli* TS (Figure 2). In the crystal structure of *L. casei* TS, none of these residues make contact with the 50 amino acid insert; instead all of them are buried within the large domain of the enzyme. For this reason, no changes at positions 76–78 were made.

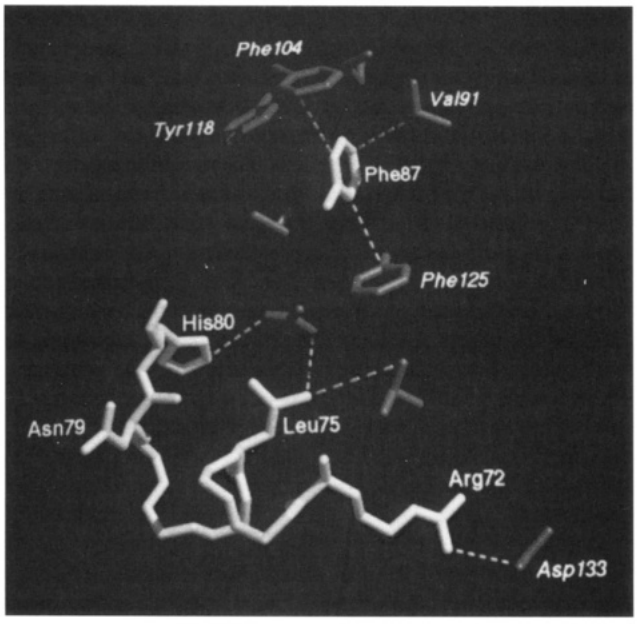

FIGURE 5: Interactions between residues of the C and D helixes (shown in white) and the insert of *L. casei* TS (residues 90–139, gray).

The first set of deletion mutants constructed carried five (TSDel3) and six (TSDel4) of the above changes. Whereas TSDel3 had undetectable TS activity, TSDel4 had a k_{cat} of 0.01 s^{-1}, at least 10^3-fold higher than TSDel3. Also, TSDel3 did not form a ternary TS–FdUMP–CH_2H_4folate complex, whereas TSDel4 readily formed the complex. Since TSDel3 and TSDel4 differ only by the F87D mutation present in TSDel4, we surmised that residue 87 plays a crucial role for enzyme activity and ternary complex formation of the deletion mutants. To prove this we constructed the deletion mutant TSDel5 carrying only the single F87D mutation. We observed a 15-fold increased k_{cat} value compared to TSDel4, as well as lowered K_m values for both dUMP and CH_2H_4folate (Table 2). Further, the F87D mutant TSDel5 formed a TS–FdUMP–CH_2H_4folate complex similar to wild type TS. These experiments clearly demonstrate the detrimental effect of Phe87 when exposed to solvent in the deletion mutants and the ability of a charged group to reverse the effect.

The fact that TSDel5, containing a single F87D mutation, is considerably more active than TSDel4, which contains an additional five *E. coli*-like mutations, suggested that one or more of the other five mutations in TSDel4 was detrimental. Studies of the TS–FdUMP–CH_2H_4folate complex also suggested that not all of these mutations contributed to its formation. Whereas the parent deletion mutant TSDel1 retained some ability to form the covalent complex, addition of five *E. coli*-like residues to give TSDel3 gave an enzyme incapable of forming the complex. In an attempt to further improve TS activity, we made TSDel6, a mutant having four mutations including F87D. Otherwise identical to TSDel4, TSDel6 lacks mutations H66Q and N79V. Compared to TSDel4, TSDel6 had a 20-fold higher k_{cat}, as well as lowered K_m values for dUMP and cofactor. Thus, the *E. coli*-like mutations H66Q and/or N79V seem to be detrimental to activity. TSDel6 showed kinetic properties similar to TSDel5, indicating that the combined contribution of the mutations R72A, L75H, and H80T to activity of the deletion mutants is small.

Concomitant with cofactor binding to TS, and prior to catalytic steps, the C-terminus undergoes a conformational change to cover the active site which is important for folate binding and catalysis (Climie et al., 1992; Perry et al., 1990; Montfort et al., 1990, Carreras et al., 1992). The following evidence suggests that a major effect of the deletion mutants is on their interaction with CH_2H_4folate or steps subsequent to its binding. First, the K_m values of all three dTMP-forming deletion mutants are greatly increased compared to wild type TS, indicating that binding of cofactor is diminished. Second, all of the deletion mutants (even those incompetent at dTMP formation) catalyze the debromination of BrdUMP with kinetic constants similar to wild type enzyme. This reaction does not require the cofactor and reflects only the initial stages of the reaction (i.e., the formation of the Michael adduct), which are apparently not impaired by the deletion. Finally, the capability to form the ternary complex with FdUMP and CH_2H_4folate is decreased in deletion mutants TSDel1–TSDel4, probably due to the loss in binding of cofactor. In TSDel5 and TSDel6, tighter binding of cofactor, also expressed as lower K_m values, restores the competence to form the covalent complex as in the wild type control.

The effect of the deletion mutants on folate binding and catalysis may be due to local structural perturbations of residues of the C and D helices. In the crystal structure of the ternary *E. coli* TS–dUMP–CB3717 complex several residues from helices C and D are directly involved in binding of the cofactor analog (Finer-Moore et al., 1990; Montfort et al., 1990). Ile81 is part of the hydrophobic binding site of the PABA ring, and residues Trp82 and Trp85 make hydrophobic contacts with the quinazoline ring of CB3717. Trp85 also makes a hydrogen bond with the C-terminal carboxyl group upon transition from the open to the closed conformation of the protein. Deletion of the *L. casei* TS insert could destabilize the C and D helices, thereby impairing cofactor binding and the interaction between the C-terminus and Trp85. We suggest that structural destabilization is caused by exposure of hydrophobic protein surface area or lost interactions with the deleted part of *L. casei* TS. Indeed, site-directed mutagenesis of residues in the C and D helices enabled us to restore partial TS activity in the deletion mutants.

Determination of crystal structures of these mutants will test whether our hypothesis is correct. Furthermore, random mutagenesis and selection of active TS mutants by genetic complementation will show whether the structures of helices C and D can be further optimized for activity.

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