A C-Terminal Conformational Equilibrium in Thymidylate Synthase Observed by Electron Paramagnetic Resonance Spectroscopy†

Christopher W. Carreras,‡ Nariman Naber,§ Roger Cooke,§ and Daniel V. Santi*,‡,§

Department of Pharmaceutical Chemistry and Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, California 94143-0448

Received September 29, 1993; Revised Manuscript Received December 2, 1993®

ABSTRACT: A spin-label was attached to the C-terminal side chain of *Lactobacillus casei* thymidylate synthase (TS, EC 2.1.1.45), and EPR spectroscopy was used to study the change in conformational equilibrium that occurs when the enzyme binds nucleotides or the methylenetetrahydrofolate analog CB3717. The C244T/V316C mutant TS has only two cysteines, the active site Cys-198 and an engineered cysteine which replaces valine as the C-terminal residue. dUMP was used to block the active-site cysteine while the C-terminus was reacted with the spin-label 4-maleimido-2,2,6,6-tetramethylpiperidinyl-1-oxy. Exclusive attachment of the label to the C-terminal cysteine was verified by a study of the labeled enzyme's reaction with 5,5′-dithiobis(2-nitrobenzoic acid). EPR spectra of the labeled enzyme and its complexes were composed of two components corresponding to populations of both flexible and more immobilized forms of the C-terminus (τ_C = 1 and 9.7 ns, respectively). Ligand binding increased the population of the more immobilized form of the C-terminus with the following series: free enzyme < E·dUMP ≈ dTMP ≈ E·FdUMP < E·CB3717 < E·dUMP·CB3717. Ligand-induced perturbation of the conformational equilibrium was titratable and indicated approximate K_d values of 3 and 13 μM for formation of the E·dUMP and E·CB3717 binary complexes, respectively, and 7 μM for the binding of CB3717 to the E·dUMP complex. Immobilization of the spin-label correlated well with crystallographic *B*-factors of the C-terminal residue in corresponding TS crystal structures. These results show that TS has two major conformations which are in equilibrium, and the position of the equilibrium changes in the presence of ligands.

Thymidylate synthase (TS,[1] EC 2.1.1.45) undergoes a major C-terminal conformational change during its catalysis of the reductive methylation of dUMP by CH_2H_4folate to produce dTMP and H_2folate. The mechanism of TS has been extensively characterized [for a review, see Santi and Danenberg (1984)], and crystal structures of free and bound forms of the enzyme have been determined (Finer-Moore et al., 1993; Kamb et al., 1992; Matthews et al., 1990; Montfort et al., 1990; Fauman et al., 1994). These studies indicate that after ordered binding of dUMP and CH_2H_4folate, the C-terminus moves more than 5 Å to form a "lid" which covers bound reactants. The C-terminal carboxylate is especially important, and coordinates an extensive hydrogen bond network that links active-site residues with both the substrate and cofactor. In crystal structures of the unliganded enzyme, the C-terminus is in the "open" conformation, and the C-terminal tetrapeptide is relatively disordered, with crystallographic *B*-factors between 40 and 60 Å^2. In ternary complexes, however, C-terminal residues cover the active site in a "closed" conformation, and have *B*-factors near 16 Å^2, close to the average for the remainder of the enzyme (Montfort et al., 1990). Since crystallographic studies indicate a dramatic immobilization of the C-terminus upon completion of the dUMP/CB3717-induced conformational change, we reasoned that it might be possible to monitor subtle aspects of the conformational change by attaching a spin-label to the C-terminal residue.

Here we describe the specific attachment of a nitroxide–maleimide spin-label (Griffith & McConnell, 1966) to the C-terminus of *Lactobacillus casei* TS, and demonstrate the ability of the label to report changes in the relative populations of flexible and immobilized forms of the C-terminus in the presence of molecules which are known to bind to TS. The results demonstrate the enzyme's dynamic role in ligand binding, and are consistent with a model where the free enzyme exists in two conformational states which are in an equilibrium that is perturbed by ligand binding [cf. Alber et al. (1983) and Monod et al. (1965)].

MATERIALS AND METHODS

Materials. The plasmid pSCTS9, which overexpresses wild-type *L. casei* TS in the Thy$^-$ *Escherichia coli* host χ2913 (ΔthyA572), has been described (Climie & Santi, 1990). The plasmids pSCTS9-C244T and pSCTS9-C244T/V316C were constructed from pSCTS9 by Dr. V. N. S. K. Francis using cassette mutagenesis as described (Climie et al., 1990). Mutant and wild-type *L. casei* thymidylate synthases were purified from plasmid-containing χ2913 *E. coli* using the automated FPLC method described by Kealey and Santi (1992). [6-^{3}H]FdUMP (20 Ci/mmol) was from Moravek Biochemicals. dU and nucleotides were obtained from Sigma

† This work was supported by U.S. Public Health Service Grants CA-14394 (D.V.S.) and AR-30868 (R.C.) from the National Institutes of Health. C.W.C. received a supplement to Grant CA-14394 for Minority Graduate Research Assistants.

* Correspondence should be addressed to this author at the Department of Pharmaceutical Chemistry, University of California, San Francisco.

‡ Department of Pharmaceutical Chemistry.

§ Department of Biochemistry and Biophysics.

® Abstract published in *Advance ACS Abstracts,* February 1, 1994.

[1] Abbreviations: TS, thymidylate synthase; MSL, the "maleimido spin-label" 4-maleimido-2,2,6,6-tetramethylpiperidinyl-1-oxy; MSL-TS, C244T/V316C TS labeled with MSL on the C-terminal cysteine; 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; dU, 2′-deoxyuridine; TES, *N*-[tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); βME, 2-mercaptoethanol.

0006-2960/94/0433-2071$04.50/0 © 1994 American Chemical Society

and analyzed by thin-layer chromatography on silica gel (Baker-flex) developed with 1-propanol/NH_4OH/water (6:3:1) with 0.5 g/L EDTA. (6*R*,6*S*)-H_4folate was obtained from Sigma and condensed with formaldehyde to produce (6*R*,6*S*)-CH_2H_4folate. Concentrations of CH_2H_4folate given refer to the concentration of (6*R*)-CH_2H_4folate competent for the TS reaction. CB3717 was a gift from Dr. Hilary Calvert. MSL was obtained from Aldrich and stored at −20 °C as a 10 mM solution in ethanol. DTNB was obtained from Sigma and recrystallized from ethanol (Riddles et al., 1983). All other materials were obtained from commercial sources and used without purification.

Specific Labeling of C244T/V316C TS with MSL. Enzymes (40–120 μM) were reduced by incubation with a 100-fold excess (βME/enzyme thiols) of βME for 1 h at 0 °C, followed by removal of excess thiol by Sephadex G-25 chromatography (1.5 × 4.9 cm, NAP-25; Pharmacia) in 50 mM TES, pH 7.0, and 0.5 mM EDTA. Protein-containing fractions were identified using Bradford reagent (Bradford, 1976); TS concentration was determined using ϵ_{278} = 125 600 M^{-1} cm^{-1}, and free thiols were assayed using DTNB (see below). Specific spin-labeling of C244T/V316C TS was accomplished using dUMP to block the active-site thiol of Cys-198 (Figure 1). Labeling reactions (0.5–0.75 mL) contained 55 μM C244T/V316C TS dimer, 50 mM TES, pH 7.0, 0.5 mM EDTA, 3–4 mM dUMP, and 130 μM MSL at room temperature. Parallel control reactions omitted either MSL or dUMP, or substituted C244T TS for C244T/V316C TS. The concentration of unreacted thiols in the labeling reaction was determined over 1 h by reaction with DTNB under denaturing conditions (see below). Preparative labeling reactions were quenched after 15 min by addition of βME to ~5 mM, and MSL-TS was purified from excess dUMP and unreacted MSL by Sephadex G-25 chromatography as above.

Reaction of Enzyme Thiols with DTNB. The progress of MSL-labeling reactions was followed by quantitating enzyme thiols during the reaction. Total enzyme thiol concentrations were measured by mixing 100-μL aliquots (4–5 nmol of TS dimer) of labeling reactions with 1 mL of denaturing DTNB reagent (6 M urea, 75 mM KH_2PO_4, pH 8.0, and 2 mM DTNB) and measuring the increase in absorbance at 412 nm (ϵ_{412} = 13 600 M^{-1} cm^{-1}; Gething & Davidson, 1972). The kinetics of the reaction of DTNB with βME, C244T TS, C244T/V316C TS, and MSL–TS were also measured under nondenaturing conditions in the presence and absence of dUMP. Reactions (1 mL) containing 50 mM TES, pH 7.0, 0.5 mM EDTA, and 10–15 μM TS dimer or βME were initiated by addition of DTNB to 6.25 mM. When dUMP was included, its concentration was 3.2 mM. UV/vis spectra were recorded at 25 °C using a Hewlett-Packard 8452A diode-array spectrophotometer, and rate constants were obtained from a fit of the first-order rate equation to the data (Jencks, 1969).

Enzyme Assays. TS activity was monitored at 25 °C spectrophotometrically at 340 nm in the standard TES assay buffer (50 mM TES, pH 7.4, 25 mM $MgCl_2$, 6.5 mM HCHO, and 1 mM EDTA) containing 75 mM βME and 1.7 μM MSL-TS dimer (Pogolotti et al., 1986). K_m and k_{cat} values were determined as previously described (Climie et al., 1992). The specific activity of MSL-TS for the dehalogenation of BrdUMP was measured in the presence of 90 μM BrdUMP as previously described (Carreras et al., 1992; Garret et al., 1979). Covalent TS·FdUMP·CH_2H_4folate complexes were formed by incubating a mixture containing 2.7 μM MSL-TS dimer, 5.4 μM [6-^{3}H]FdUMP (13.4 Ci/mmol), and 170 mM CH_2H_4folate in standard TES buffer containing 10 mM DTT at room temperature for 1 h. Reaction mixtures were analyzed by 12% SDS–PAGE as previously described (Carreras et al., 1992).

EPR Spectroscopy of MSL-TS Complexes. EPR spectra were acquired between 22 and 25 °C using a Bruker ER/200D spectrometer (Billerica, MA) equipped with a TE_{102} cavity. First-derivative, X-band absorption EPR spectra were obtained with the following instrument settings: microwave power, 25 mW; modulation, 0.2 mT at 100 kHz. Samples (30 μL) were prepared in 50-μL capillaries and contained either 8.5 μM or 24 μM MSL-TS dimer in 50 mM TES, pH 7.0, and 0.5 mM EDTA. When dTMP, FdUMP, or dU were included, their concentration was 280 μM. For determination of the K_d of dUMP, dUMP was varied between 3.5 and 280 μM. K_d values for CB3717 were determined by varying the concentration of CB3717 between 3.8 and 375 μM both in the presence and in the absence of 280 μM dUMP. K_d values were obtained from a nonlinear least-squares fit of EPR spectral changes to an equilibrium binding equation which corrects for ligand depletion by enzyme [see p 74 of Segal (1975)].

Rotational correlation times, τ_c, for the two spectral components were determined from the shape of the spectra. $2T'_\parallel$ was measured as the separation of the outer hyperfine extrema of each component; the rigid limit for this quantity, $2T_\parallel$, was determined from a spectrum of MSL-TS frozen in liquid nitrogen. The correlation time of the more immobilized component was calculated using $\tau_c = a(1 - 2T'_\parallel/2T_\parallel)^b$, where $a = 5.4 \times 10^{-10}$ s and $b = -1.36$ (Goldman et al., 1972). The correlation time of the more freely moving population of spin-label was determined from the relative heights of the peaks in the first-derivative spectrum using the method of Stone et al. (1965).

RESULTS

Preparation and Characterization of MSL-TS. The C244T and C244T/V316C mutants were expressed in *E. coli* χ2913 as approximately 30% of the total soluble protein, and were purified to >95% homogeneity as estimated by SDS–PAGE. The C244T/V316C TS dimer has four cysteine residues which are available for reaction with thiol reagents. Two of these are the active-site Cys-198 residues of each subunit which are essential for enzyme activity, and the remaining two are engineered to replace the C-terminal residue, Val-316. dUMP was used to protect Cys-198 during the labeling reaction (Figure 1), and exclusive attachment of the spin-label to Cys-316 was verified by three methods. First, we followed the reaction of MSL with enzyme thiols by measuring unreacted thiols with DTNB under denaturing conditions. The reaction of MSL with C244T/V316C TS was rapid (Figure 2). When MSL is added to C244T/V316C TS in the absence of dUMP, all four protein thiols reacted within 10 min. However, when dUMP was included in the reaction at ~1000 times its K_d, only two of the enzyme's thiols reacted with MSL even after incubation for 1 h.

Next, we compared the reactivity of thiols in MSl-TS to the reactivities of unmodified Cys-198 and Cys-316 residues by reacting C244T TS, C244T/V316C TS, and MSL-TS with DTNB in the presence and absence of dUMP. In the presence of dUMP, C244T TS and MSL-TS each had less than 10% of their cysteine residues available for reaction with DTNB. In the absence of dUMP, MSL-TS reacted with DTNB with a pseudo-first-order rate constant similar to C244T TS which has Cys-198 as its only cysteine (k_{obs} = 0.012 and 0.018 s^{-1},

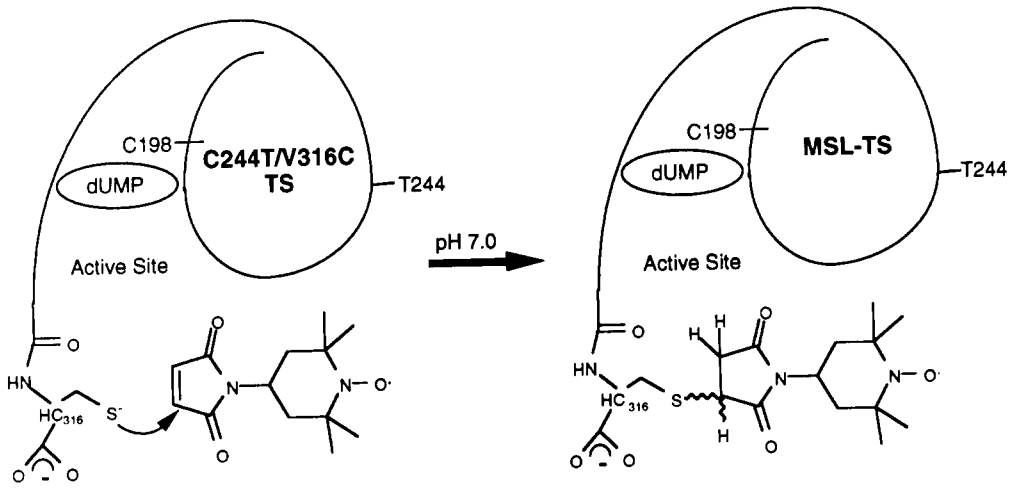

FIGURE 1: Schematic representation of the spin-labeling procedure. To ensure specific labeling of the engineered Cys-316, dUMP was used to block the active-site thiol of Cys-198, and the nonessential Cys-244 was mutated to a threonine.

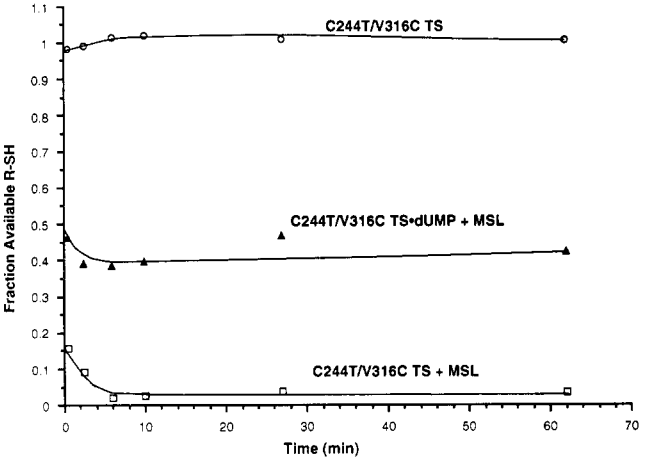

FIGURE 2: Consumption of protein thiols by MSL. Aliquots were removed from reaction mixtures containing 50 μM C244T/V316C TS dimer and assayed with DTNB under denaturing conditions (see Materials and Methods). When dUMP or MSL was included, its concentration was 3 mM or 250 μM, respectively.

respectively). C244T/V316C TS reacted with DTNB both in the presence and in the absence of dUMP. In the absence of dUMP, the first 50% of enzyme thiols reacted before measurements could be made ($k_{obs} > 0.693\ s^{-1}$); however, the remaining cysteines reacted with a pseudo-first-order rate constant of 0.011 s^{-1}, a rate similar to that obtained for the reaction of Cys-198 in C244T TS. In the presence of dUMP, only half of the enzyme thiols reacted, but with a pseudo-first-order rate constant of 0.082 s^{-1}, ~8-fold faster than Cys-198 in C244T TS. βME reacted with DTNB with a pseudo-first-order rate constant of 0.025 s^{-1} in the presence and absence of dUMP. Thus, reactivity toward DTNB increases as the following series: Cys-316 > βME > Cys-198.

Finally, to directly assess the reactivity of Cys-198 toward MSL, we examined the reaction of 250 μM MSL with 50 μM C244T TS dimer for 15 min. Although these conditions completely label Cys-316 of C244T/V316C TS in the presence of dUMP, EPR spectra showed that no labeling of C244T TS occurred in the presence of dUMP, and only weak (< 30%) labeling occurred in the absence of dUMP. These experiments demonstrate the ability of dUMP to protect the active-site Cys-198 from reaction with MSL, and confirm that Cys-198 is less reactive than Cys-316 toward thiol-modifying reagents.

Enzymatic Activities. C244T TS had steady-state kinetic parameters that were unchanged compared to wild-type TS [K_m(dUMP) = 4.8 μM, K_m(CH_2H_4folate) = 28 μM, and k_{cat} = 4.25 s^{-1} (Pinter, Davisson, and Santi, unpublished data)]. The V316C mutant had K_m values for dUMP and CH_2H_4-folate of 1.6 and 77 μM, respectively, and a k_{cat} value of 1.1 s^{-1} (Climie et al., 1992). The steady-state kinetic parameters of the C244T/V316C double mutant were the same as for V316C TS. MSL-TS catalyzed dTMP formation with a k_{cat} of 0.07 s^{-1}, about 16-fold lower than the value obtained with unmodified C244T/V316C TS, and had K_m values for dUMP and CH_2H_4folate of 1.4 and 140 μM. When MSL-TS was incubated with [6-^{3}H]FdUMP and (6*R*)-CH_2H_4folate, tritium-labeled enzyme was detected on SDS–PAGE. C244T/V316C TS and MSL-TS dehalogenated BrdUMP with specific activities of 0.004 and 0.0014 unit/mg, respectively.

EPR of MSL-TS Complexes. Representative EPR spectra of MSL-TS and its complexes are shown in Figure 3. In all cases, two major spectral components were observed, with $2T'_{\parallel}$ values of 3.36 and 6.25 mT. We define the I ("immobilized") peak as the low-field peak of the component with $2T'_{\parallel}$ = 6.25 mT, and the M ("mobile") peak is the low-field peak of the component that has a $2T'_{\parallel}$ = 3.36 mT. The $2T'_{\parallel}$ splittings were normalized to a rigid-limit value of 7.1 mT, measured for MSL-TS in liquid nitrogen, and rotational correlation times, τ_c, of 1 and 9.7 ns were obtained. Consistent with a model corresponding to two populations of the spin-label which have different mobilities, all EPR spectra of unliganded MSL-TS and MSL-TS complexes showed the same $2T'_{\parallel}$ values, and differed only in the relative amount of each component. Several isosbestic points are apparent, indicating the absence of intermediates between the two populations of spin-label that are in equilibrium.

The presence of only two components was further demonstrated by deconvolution of the two spectral components (Figure 4). Spectra of pure immobilized and pure mobile spin-label were constructed by scaling the M peaks of the spectra of unliganded MSL-TS and MSL-TS•dUMP•CB3717, and constructing a difference spectrum which represented only the relatively immobilized conformation of the spin-label. The I peak from this spectrum was then scaled to the I peak of the unliganded MSL-TS spectrum and subtracted from it to yield a difference spectrum that represents only the less immobilized population of spins. These difference spectra were normalized so that their integrated areas were equal, derivatives were taken, and the resulting spectra (Figure 4A,B) were used in weighted summations to fit the experimentally observed EPR spectra of MSL-TS and MSL-TS complexes

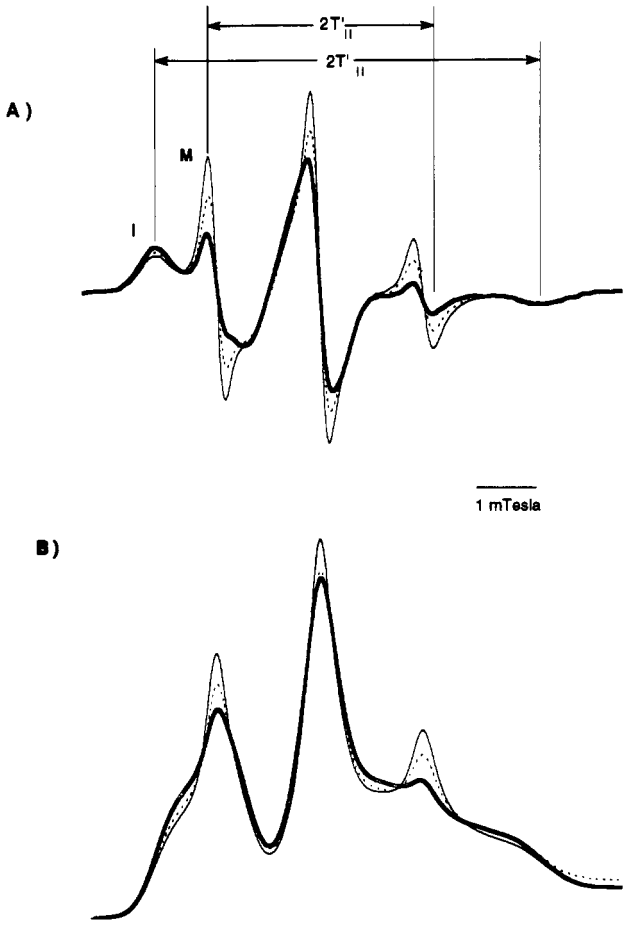

FIGURE 3: (A) First-derivative and (B) absorption EPR spectra of unliganded MSL-TS (thin solid curve), the MSL-TS·dUMP complex (dashed curve), and the MSL-TS·dUMP·CB3717 complex (thick solid curve). Each spectrum was obtained from a sample containing 24 μM MSL-TS dimer. When dUMP or CB3717 was included, its concentration was 280 or 375 μM, respectively.

(Figure 4C,D). The weighting factors represent the relative abundance of each form of the spin-label. Results of this simulation procedure indicate that free MSL-TS, the MSL-TS·dUMP complex, and the MSL-TS·dUMP·CB3717 ternary complex have 65%, 74%, and 90% of their spin-label in the more immobilized conformation, respectively.

M/I peak-height ratios were also used to quantitate changes in EPR spectra. Unliganded MSL-TS had a M/I peak ratio of 3.5 (Table 1). Formation of binary complexes between MSL-TS and dUMP or dTMP resulted in a 23% reduction in this ratio, corresponding to increased population of the immobilized conformation of the C-terminus in the presence of substrate and product nucleotides. dUMP-induced spectral changes were titrated and showed an approximate K_d value of 3 μM (Figure 5A), similar to the K_m value observed for wild-type TS.

Incubation of either the free enzyme or the MSL-TS·dUMP complex with CB3717 also resulted in increased populations of immobilized spin-label to yield M/I peak-height ratios of 1.9 and 1.4, respectively. In all cases, spectral changes were complete by the time of measurement (~3–5 min) and were stable for more than an hour. Spectral changes induced by the binding of CB3717 both to free enzyme and to the MSL-TS·dUMP complex were titrated and indicated approximate K_d values of 7 and 13 μM, respectively (Figure 5B,C). Since the concentrations of MSL-TS needed for EPR are similar to the K_d values, these K_d values are approximate. Addition of CH_2H_4folate to the MSL-TS·FdUMP complex resulted in

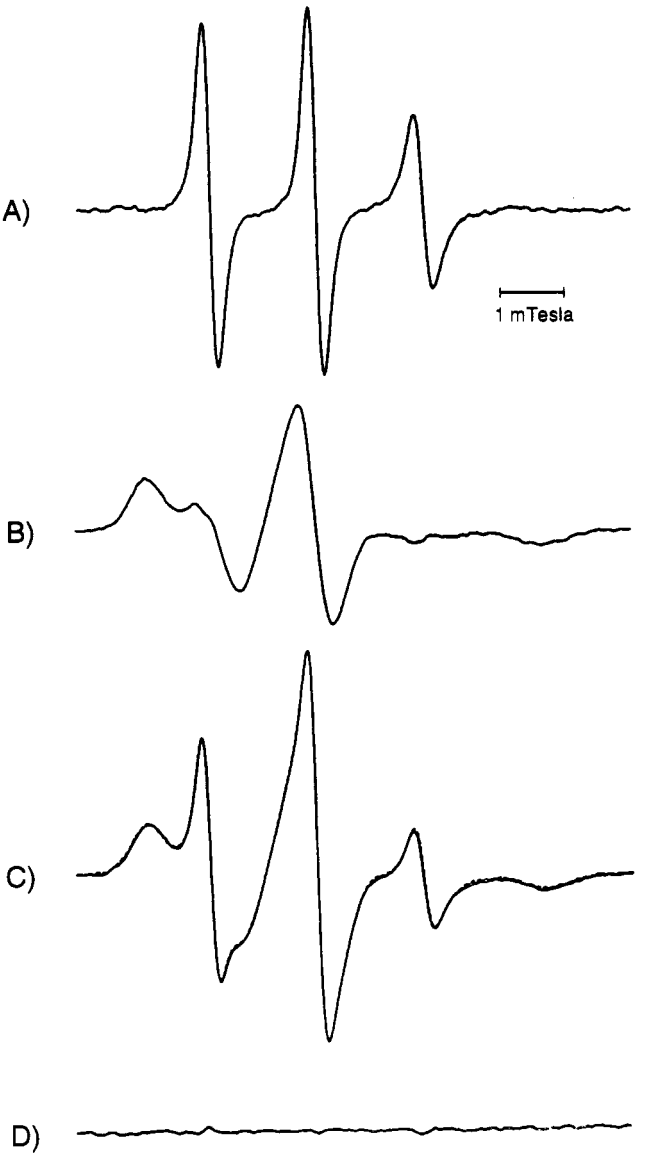

FIGURE 4: Simulation of the spectrum of the MSL-TS·dUMP complex. (A) The more mobile component of the spectrum of free MSL-TS. (B) The more immobilized component of the spectrum of the MSL-TS·dUMP·CB3717 complex. (C) Superposition of the spectrum of the MSL-TS·dUMP complex onto a weighted summation of the spectra in (A) and (B). (D) Difference between the modeled and observed spectra of the MSL-TS·dUMP complex shown in (C).

Table 1: Summary of EPR Peak Ratios

enzyme form	M/I peak ratio[a]	crystallographic *B*-factor of C-terminal residue (Å²)
free E	3.5 ± 0.1	67[b]
E·dUMP	2.7 ± 0.1	45[b]
E·dTMP	2.7 ± 0.1	nd[c]
E·FdUMP	2.9 ± 0.1	nd[c]
E·CB3717	1.9 ± 0.1	97[d]
E·dUMP·CB3717	1.4 ± 0.1	16[e]

[a] Ratio of the low-field peaks of spectral components with $2T'_{\parallel}$ = 3.36 and 6.25 mT (τ_c = 9.7 and 1 ns, respectively). [b] Finer-Moore et al. (1993). The structure of the free enzyme was at 2.36-Å resolution, while the TS·dUMP structure was at 2.55-Å resolution. [c] Structure not determined. [d] Kamb et al. (1992). This structure was at 3-Å resolution, and has a Wilson *B*-factor of 65 Å². [e] Montfort et al. (1990). 1.97-Å resolution.

a spectrum similar to that obtained for the MSL-TS·dUMP·CB3717 complex (data not shown); however, the EPR signal rapidly faded, suggesting that reduction of the spin-label was occurring. When either 100 mM DTT or 70

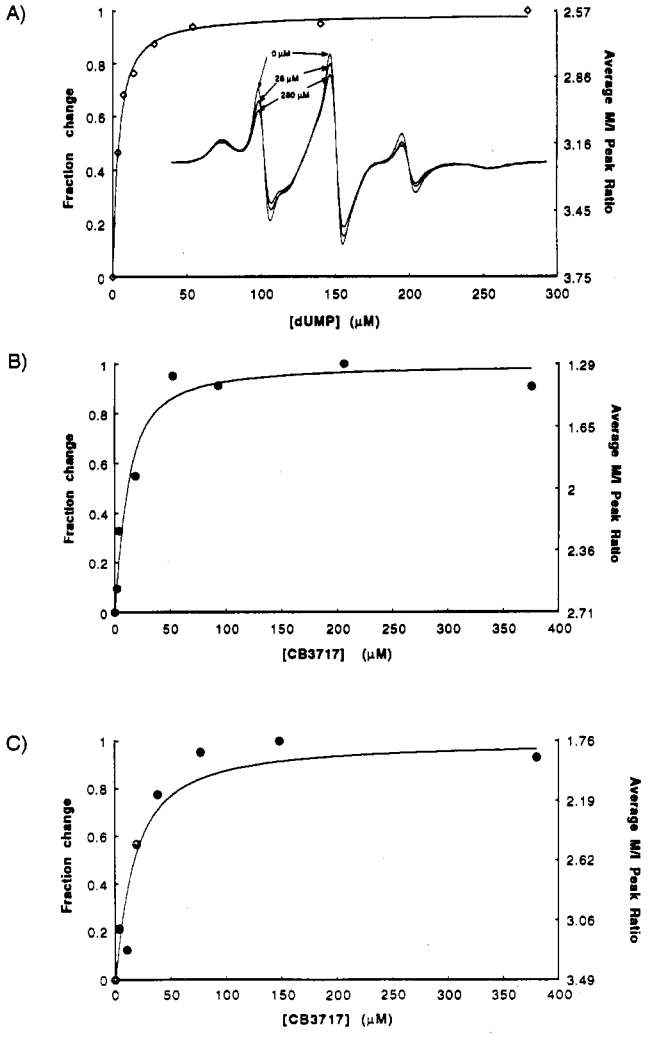

FIGURE 5: (A) EPR titration of 24 μM MSL-TS dimer with dUMP to yield an approximate K_d of 3 μM. The inset shows changes in the EPR spectrum of MSL-TS that occur as dUMP is added. (B) EPR titration of 8.5 μM MSL-TS dimer with CB3717 in the presence of 280 μM dUMP to yield an approximate K_d of 7 μM. (C) EPR titration of 8.5 μM MSL-TS dimer with CB3717 in the absence of dUMP to yield an approximate K_d of 13 μM. In the above plots, points are experimental, and lines are a nonlinear least-squares fit to equation II-54 from Segel (1975, p 174).

μM CH_2H_4folate was mixed with free MSL, a 200-fold reduction in the EPR signal was observed, demonstrating that CH_2H_4folate can reduce the nitroxide spin-label even more effectively than thiol reagents. No changes in the spectrum of free MSL were observed in the presence of either dUMP or CB3717.

DISCUSSION

Wild-type *L. casei* TS has two cysteine residues on each subunit: Cys-244, which is not essential for enzyme activity, and Cys-198, which is the active-site nucleophile. In order to specifically introduce a spin-label at the C-terminus, it was necessary to replace the unreactive Val-316 with a cysteine residue and to prevent reaction of the labeling reagent with Cys-244 and Cys-198. Residue 244 was made unreactive toward the labeling reagent by a cysteine to threonine mutation which did not affect the enzyme's steady-state kinetic parameters. To prevent reaction of the spin-labeling reagent with Cys-198, a binary complex was formed between the enzyme and dUMP. The ability of the enzyme's substrate, dUMP, to protect Cys-198 from reaction with thiol-modifying reagents has been previously demonstrated (Danenberg et al., 1974; Galivan et al., 1976; Leary et al., 1975).

We unambiguously confirmed that the conditions used to label C244T/V316C TS modified Cys-316 but not Cys-198. First, it was shown that all four thiol groups on the dimeric enzyme can react with MSL; however, when dUMP is included in the incubation, two of the four enzyme thiol groups are protected from MSL-labeling (Figure 2). Second, we measured the rate constant for the reaction of DTNB with thiol groups of MSL-TS and found that it was the same as the rate constant for reaction of DTNB with Cys-198 residues of C244T TS. Third, using an EPR assay, we found that C244T TS is not spin-labeled under the conditions used to label the C-terminus of C244T/V316C TS.

Preparations of MSL-TS showed a k_{cat} value that was reduced relative to unmodified C244T/V316C TS. MSL-TS had the same K_m value for dUMP as the unmodified enzyme; however, the K_m value for CH_2H_4folate was about 2-fold higher. In accordance with what has been observed for other modifications of the C-terminus of TS, MSL modification of the C-terminus has caused a decrease of TS activity and an increase in the CH_2H_4folate K_m without affecting the K_m for dUMP (Climie et al., 1992).

MSL-TS shares other important similarities with wild-type TS and C-terminal mutants. MSL-TS catalyzes the cofactor-independent dehalogenation of BrdUMP at a rate that approaches that of the wild-type enzyme, thus demonstrating its ability to bind to and form Michael adducts with pyrimidine nucleotide substrates (Garrett et al., 1979). The observation of a covalent ternary complex between MSL-TS, FdUMP, and CH_2H_4folate shows that the spin-labeled enzyme is capable of catalyzing steps of the TS reaction at least up to formation of a covalent ternary complex (Santi et al., 1987).

EPR spectra of MSL-TS are composed of two components and are consistent with a two-state model in which the motion of the spin-label is different in each state. The only difference between spectra of MSL-TS and spectra of MSL-TS complexes was the relative amount of each of the two components. On the basis of our ability to detect changes in $2T'_{\parallel}$ of 0.1 mT and the unlikelihood that dramatically different conformations will result in the exact same $2T'_{\parallel}$, we conclude that each spectral component represents a single major conformation; i.e., the active site either is closed by an immobilized C-terminus or is open and the C-terminus is relatively freely moving. The EPR spectrum of unliganded MSL-TS (Figure 3) indicated that approximately 45% of the label was relatively freely moving while the remaining 65% was significantly immobilized. Upon additon of dUMP, changes in the EPR spectrum of MSL-TS indicated that about 74% of the spin-label was in the immobilized conformation. Titration of the EPR spectral change with dUMP indicated a K_d value similar to the dUMP K_m values obtained for wild-type TS and unmodified C244T/V316C TS. Addition of the cofactor analog CB3717 to the MSL-TS·dUMP complex caused further changes in the EPR spectrum. Titration of these changes with CB3717 showed a K_d value of 7 μM and indicated that approximately 90% of the spin-labeled can be converted to the more immobilized conformation.

We also observed binding of CB3717 to unliganded MSL-TS, with an approximate K_d of 14 μM. Although a crystal structure of *E. coli* TS bound to CB3717 in the absence of dUMP was recently reported (Kamb et al., 1992), to our knowledge this is the first time a complex between TS and a monoglutamyl folate analog has been observed in solution in the absence of nucleotide. However, neither this work nor

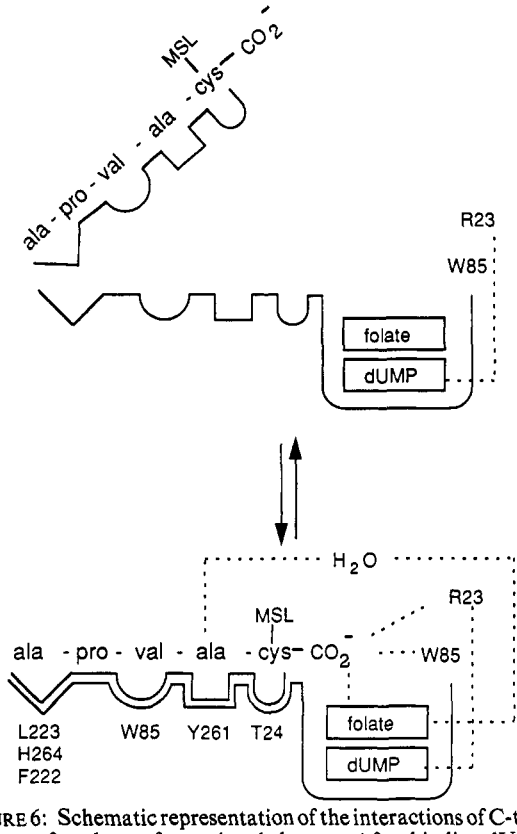

FIGURE 6: Schematic representation of the interactions of C-terminal residues after the conformational change. After binding dUMP and cofactor, the C-terminus moves to form a lid over the active site which is anchored by side-chain interactions and an extensive hydrogen-bond network (dotted lines).

the TS·CB3717 crystal structure has demonstrated that a binary TS·CH_2H_4folate complex is on a productive reaction pathway.

The crystal structures of free TS and the binary TS–dUMP complex are very similar. Here, the active sites are in an open conformation, and high *B*-factors (40–60 Å^2 for residues of the C-terminal tetrapeptide in the structure of the free enzyme) suggest that C-terminal residues are flexible (Hardy et al., 1987). Upon formation of the ternary TS·dUMP·CB3717 complex, a large conformational change occurs in which the C-terminus moves about 5 Å to form a lid over the bound substrate and cofactor to "close" the active site. 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 (Montfort et al., 1990). In the ternary complex, the carboxylate of the C-terminal residue forms a hydrogen-bond network with several conserved residues, including Arg-23, which has moved from its position in the structure of the unliganded enzyme 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 experienced new interactions (Figure 6).

When MSL-TS binds dUMP and CB3717, changes in its EPR spectrum occur which closely parallel changes in the atomic *B*-factors of C-terminal residues in the corresponding crystal structures (Figure 7). We interpret the higher *B*-factors observed for the C-terminus of the unliganded enzyme as indicating that this structure corresponds to the solution conformation that gives rise to the more freely-moving population of spin-label. Low *B*-factors for the C-terminus in TS·dUMP·CB3717 ternary complexes suggest that this structure corresponds to the solution conformation responsible

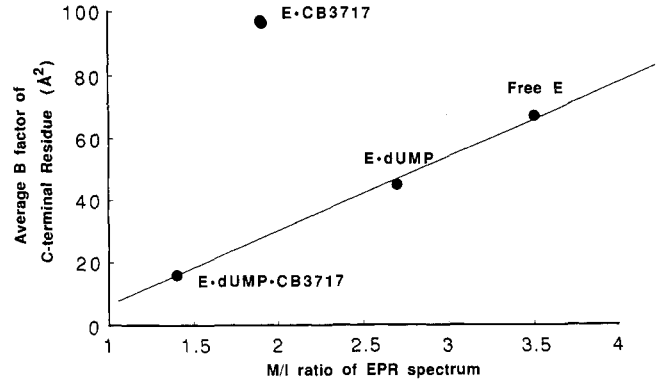

FIGURE 7: Correlation of crystallographic *B*-factors with EPR measurements. *B*-factors are the average of atomic *B*-factors for atoms of the C-terminal residue. M/I ratio is the ratio of the M ("mobile") peak to the I ("immobilized") peak, which is defined in Figure 3. Structures used are referenced in Table 1. Note that the entire E·CB3717 structure was of relatively low resolution (3 Å) and was highly disordered (Wilson *B*-factor = 65 Å^2).

for the more immobilized population of spin-label. If the two conformations are not compatible with the same crystal lattice, crystallization must select and stabilize one conformation. Crystal-packing forces probably stabilize the open form of the wild-type free enzyme and the closed form of ternary complexes. In accordance with the idea that both the open and closed conformations are present in solution, crystallization drops which yield crystals of ternary complexes usually also contain crystals which have the same morphology as crystals of the unliganded enzyme (R. M. Stroud, unpublished observations).

Our studies of MSL-TS are consistent with a model in which the enzyme is in equilibrium between two conformational states: a flexible, open conformation observed in the crystal structures of the free enzyme and binary complexes, and a rigid, closed structure observed for the TS·dUMP·CB3717 complex (Figure 6). Binding of ligands changes the energy of one or both conformations to perturb the conformational equilibrium. Consistent with this model is the reactivity of the engineered Cys-316, which is reduced more than 8-fold by the presence of dUMP. We attribute this difference in reactivity to a dUMP-induced change in the conformational equilibrium which makes the C-terminal Cys-316 less accessible to thiol-modifying reagents. We recognize that MSL-TS is highly modified relative to wild-type TS, and avoid speculation of conformational equilibrium constants for wild-type TS based on those obtained for MSL-TS.

A similar model of ligand-induced perturbation of conformational equilibria has been discussed for triosephosphate isomerase (Alber et al., 1983; Ringe & Petsko, 1986). Since the enzyme is in equilibrium between two conformations, it can sense that a ligand is present and change the conformational equilibrium to take advantage of favorable interactions with the ligand. This model also provides for disruption of favorable protein–ligand interactions that must occur if products are to be released. Since the enzyme samples the open conformation even in the presence of bound substrates, these interactions are routinely broken. During this time, the products have an opportunity to escape so that a new set of substrates can bind and continue the catalytic cycle.

Finally, we note that since TS exists in two conformations which interconvert during catalysis, effective stabilization of either conformation could provide a novel method of inhibiting the enzyme.

ACKNOWLEDGMENT

We thank Dr. V. N. S. K. Francis for providing plasmids which express C244T and C244T/V316C thymidylate synthases. We are grateful to David Yee, Pat Greene, Jim Kealey, Jens Eckstein, and Janet Finer-Moore for helpful discussions.

REFERENCES

Alber, T., Gilbert, W. A., Ringe, D., & Petsko, G. A. (1983) in *Mobility and Function in Proteins and Nucleic Acids*, Pitman, London.

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

Carreras, C. W., Climie, S. C., & Santi, D. V. (1992) *Biochemistry 31*, 6038–6044.

Climie, S., & Santi, D. V. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 633–637.

Climie, S., Ruiz-Perez, L., Gonzalez, P. D., Prapunwattana, P., Cho, S. W., Stroud, R., & Santi, D. V. (1990) *J. Biol. Chem. 265*, 18776–18779.

Climie, S. C., Carreras, C. W., & Santi, D. V. (1992) *Biochemistry 31*, 6032–6038.

Danenberg, P. V., Langenbach, R. J., & Heidelberger, C. (1974) *Biochemistry 13*, 926–933.

Fauman, E. B., Rutenber, E. E., Maley, G., Maley, F., & Stroud, R. M. (1993) *Biochemistry* (in press).

Finer-Moore, J. S., Fauman, E. B., Foster, P. C., Perry, K. M., Santi, D. V., & Stroud, R. M. (1993) *J. Mol. Biol. 232*, 1101–1116.

Galivan, J. H., Maley, F., & Baugh, C. M. (1976) *Biochem. Biophys. Res. Commun. 71*, 527–534.

Garrett, C., Wataya, Y., & Santi, D. V. (1979) *Biochemistry 18*, 2798–2804.

Gething, M. J., & Davidson, B. E. (1972) *Eur. J. Biochem. 30*, 352–353.

Goldman, S. A., Bruno, G. V., & Freed, J. H. (1972) *J. Phys. Chem. 76*, 1858–1860.

Griffith, O. H., & McConnell, H. M. (1966) *Proc. Natl. Acad. Sci. U.S.A. 55*, 8–11.

Hardy, L. M., Finer-Moore, J. S., Montfort, W. R., Jones, M. O., Santi, D. V., & Stroud, R. M. (1987) *Science 235*, 448–455.

Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, McGraw-Hill Book Co., New York.

Kamb, A., Finer-Moore, J. S., & Stroud, R. M. (1992) *Biochemistry 31*, 12876–12884.

Kealey, J. T., & Santi, D. V. (1992) *Protein Expression Purif. 3*, 380–385.

Leary, R. P., Beaudette, N., & Kisliuk, R. L. (1975) *J. Biol. Chem. 250*, 4864–4868.

Matthews, D. A., Appelt, K., Oatley, S. J., & Xuong, N. H. (1990) *J. Mol. Biol. 214*, 923–936.

Monod, J., Wyman, J., & Changeux, J. (1965) *J. Mol. Biol. 12*, 88–119.

Montfort, W. R., Perry, K. M., Fauman, E. B., Finer-Moore, J. S., Maley, G. F., Hardy, L., Maley, F., & Stroud, R. M. (1990) *Biochemistry 29*, 6964–6977.

Pogolotti, A. L., Danenberg, P. V., & Santi, D. V. (1986) *J. Med. Chem. 29*, 478–482.

Riddles, P. W., Blakeley, R. L., & Zerner, B. (1983) *Methods Enzymol. 91*, 49–60.

Ringe, D., & Petsko, G. A. (1986) *Methods Enzymol. 131*, 389–433.

Santi, D. V., & Danenberg, P. V. (1984) in *Folates and Pterins*, pp 345–398, John Wiley & Sons, Inc., New York.

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 Rapid-Equilibrium and Steady-State Enzyme Systems*, pp 1–957, Wiley-Interscience, New York.

Stone, T. J., Buckman, T., Nordio, P. L., & McConnell, H. M. (1965) *Proc. Natl. Acad. Sci. U.S.A. 54*, 1010–1017.