Structures of Thymidylate Synthase with a C-terminal Deletion: Role of the C-terminus in Alignment of 2′-Deoxyuridine 5′-Monophosphate and 5,10-Methylenetetrahydrofolate[†,‡]

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ABSTRACT: Thymidylate synthase undergoes a major conformational change upon ligand binding, where the carboxyl terminus displays the largest movement (~4 Å). This movement from an "open" unliganded state to the "closed" complexed conformation plays a crucial role in the correct orientation of substrates and in product formation. The mutant lacking the C-terminal valine (V316Am) of the enzyme is inactive. X-ray crystal structures of V316Am and its complexes with dUMP, FdUMP, and both FdUMP and CH_2H_4-folate are described. The structures show that ligands are bound within the active site, but in different modes than those in analogous, wild-type thymidylate synthase structures. The 2.7-Å binary complex structures of V316Am with FdUMP and dUMP show that the pyrimidine and ribose moieties of the nucleotides are pivoted ~20° around the 3′-hydroxyl compared to dUMP in the wild-type enzyme. The 2.7-Å crystal structure of V316Am complexed with cofactor, CH_2H_4folate, and the substrate analog, FdUMP, shows these ligands bound in an open conformation similar to that of the unliganded enzyme. In this ternary complex, the imidazolidine ring of the cofactor is open and has reacted with water to form 5-$HOCH_2H_4$-folate. 5-$HOCH_2H_4$folate is structural evidence for the 5-iminium ion intermediate, which is the proposed reactive form of CH_2H_4folate. The altered ligand binding modes observed in the three V316Am complex structures open new venues for the design of novel TS inhibitors.

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. A critical aspect of this reaction is a large conformational change, where segments of protein move from an "open" conformation to form a "closed" active site cavity. Comparison of the ternary complex structure of *Escherichia coli* TS containing the substrate, dUMP, and the folate analog, 10-propargyl-5,8-dideazafolate (CB3717) with the structures of the unliganded *E. coli* or *Lactobacillus casei* TS and the *E. coli* or *L. casei* TS·dUMP binary complex reveals that a major part of the conformational change is induced by cofactor binding (Matthews et al., 1990; Montfort et al., 1990; Kamb et al., 1992). In this process, ligands are sequestered from solvent and the cofactor is oriented to facilitate one carbon unit transfer to C5 of dUMP (Finer-Moore et al., 1990). The C-terminal residue, Val 316, undergoes the largest movement (~4 Å) upon binding of folates. The C-terminal carboxyl participates in an extensive hydrogen bond network which includes interactions with CB3717, Arg 23, and the ring nitrogen of Trp 85 on the opposite side of the binding pocket. In this closed conformation, the penultimate carbonyl oxygen of Ala 315 forms one water-mediated hydrogen bond to the N1 hydrogen and one direct hydrogen bond to the exocyclic NH_2 of CB3717 (Figure 1). In the *E. coli* WT·dUMP·CB3717 structure, C-terminal residues are well localized in the electron density, with thermal factors near the average for the rest of the protein (16 Å^2). In contrast, the structure of unliganded *L. casei* TS completely lacks the aforementioned hydrogen bond network and has a relatively disordered C-terminal tetrapeptide, with an average thermal factor of 49.5 Å^2. Aull et al. (1974) found that enzymatic removal of the C-terminal valine of *L. casei* TS inactivates the enzyme. Using mutagenesis, Carreras and co-workers (1992) show that a single amino acid deletion at the C-terminus also abolishes product formation. Due to the key role of the C-terminus in closure of the TS active site cavity and product formation, we selected this mutant to study the role of this residue at the level of molecular structure.

Although V316Am does not catalyze dTMP formation, it does catalyze two partial reactions: (1) a CH_2H_4folate-dependent exchange of the 5-hydrogen of dUMP for protons in water and (2) a thiol-dependent dehalogenation of BrdUMP or IdUMP (Carreras et al., 1992). These reactions proceed with kinetic constants similar to those of the wild-type (WT) enzyme and indicate that the C-terminal deletion does not abolish the enzyme's ability to bind and form Michael adducts with nucleotides. V316Am is also capable of forming a covalent ternary complex with FdUMP and CH_2H_4folate; however, the equilibrium between the noncovalent and covalent ternary complexes is dramatically altered from 1:17 000 for WT to 1:7 for the mutant (Carreras, et al., 1992).

We solved the crystal structures of V316Am and its complexes with dUMP, FdUMP, and FdUMP and CH_2H_4-

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[‡] Protein Data Bank file names: V316Am unbound = 1TDA; V316Am·FdUMP = 1TDB; V316Am·dUMP = 1TDC; V316Am·FdUMP·5-$HOCH_2H_4$folate = 2TDD.

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[1] Abbreviations: TS, thymidylate synthase; V316Am, *Lactobacillus casei* TS lacking C-terminal valine; dUMP, 2′-deoxyuridine 5′-monophosphate; CH_2H_4folate, 5,10-methylenetetrahydrorfolate; dTMP, thymidine 5′-monophosphate; H_2folate, 7,8-dihydrofolate; CB3717, 10-propargyl-5,8-dideazafolate; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; WT, wild type; DTT, dithiothreitol; PABA, *p*-aminobenzoic acid; 5-$HOCH_2H_4$folate, 5-(hydroxymethyl)tetrahydrofolate; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

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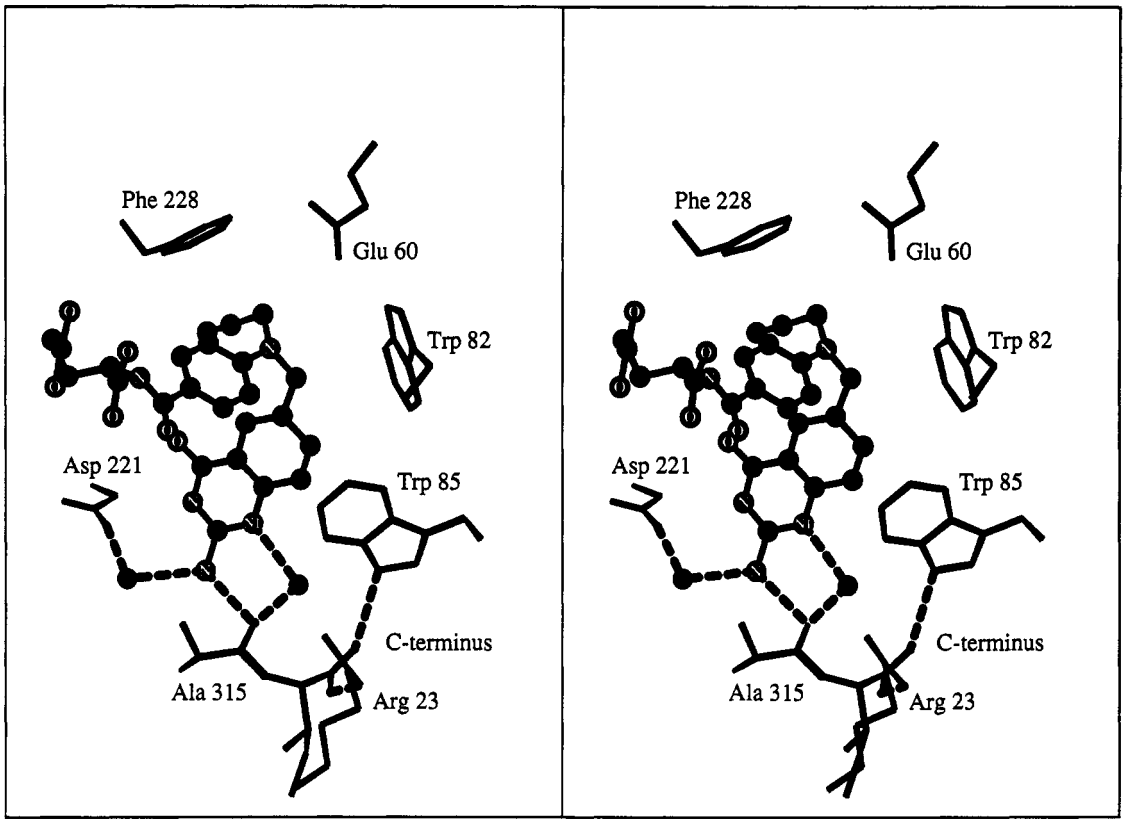

FIGURE 1: Cross-eyed stereoview depicting interactions of the C-terminus with the cofactor analog CB3717 from the *E. coli* WT·dUMP·CB3717 ternary complex. The CB3717 molecule is represented with balls and sticks.

folate. These structures show that, in the absence of the C-terminal valine, both substrate and cofactor bind in orientations that differ from those found for the WT enzyme (Finer-Moore et al., 1990; Matthews et al., 1990; Montfort et al., 1990). The ternary complex of V316Am with FdUMP and CH_2H_4folate provides structural evidence for formation of the highly reactive iminium ion form of the cofactor, which is the proposed electrophile for nucleophilic attack by C5 of dUMP during dTMP formation by the WT enzyme (Santi & Danenberg, 1984). This V316Am ternary complex is in the open conformation, demonstrating that a closed active site is not required for iminium ion formation in the mutant.

MATERIALS AND METHODS

Materials. *E. coli* strain χ2913 (ΔthyA572) was provided by Russell Thompson, University of Glasgow. The plasmid pSCTS-V316Am has been described (Climie et al., 1990). FdUMP was obtained from Sigma and was used without further purification. CH_2H_4folate was prepared from (6*RS*)-H_4folate obtained from Sigma (Wataya et al., 1980). All other reagents were obtained from commercial sources and used without purification.

Protein Purification. Purification of the *L. casei* TS mutant V316Am from χ2913 *E. coli* containing the vector pSCTS-V316Am was as described by Carreras et al. (1992). Purified enzyme was stored in 20 mM potassium phosphate, pH 7.4, and 0.1 mM EDTA at −80 °C. Before use, protein was either dialyzed against 1 L of 20 mM potassium phosphate, 0.1 mM EDTA, and 1.0 mM DTT, pH 7.4 (phosphate buffer), or concentrated in a Centricon-30 (Amicon) three times to 40 μL with 1 mL of 50 mM sodium citrate, 1.0 mM EDTA, 2.0 mM $MgSO_4$, and 10 mM DTT (citrate buffer) added after each spin to exchange the buffer.

Crystallization. Crystals of unliganded V316Am and binary complexes were grown by vapor diffusion in 10-μL hanging drops containing phosphate buffer with 5 mg/mL (70 μM) V316Am and ca. 1% saturated ammonium sulfate. The drop was suspended over a well containing 1 mL of phosphate buffer lacking ammonium sulfate. For binary complexes, 500 μM FdUMP or dUMP was included in the drop. Ternary complex crystals were obtained by soaking FdUMP and CH_2H_4folate into unbound crystals of the mutant, which were grown via controlled pH (Tykarska et al., 1986). By this method, 8 μL of 18.5 mg/mL protein in citrate buffer at pH 6.2 was mixed with 12 μL of citrate buffer at pH 4.2 and sealed in a depression well. Crystals, which grew in 1–3 days, were then soaked with 5 μL of a mixture containing 800 μM CH_2H_4folate, 1.5 mM FdUMP in 50 mM TES, 6.5 mM formaldehyde, 25 mM $MgCl_2$, and 5 mM DTT at pH 7.4. After 4.5 h, an additional 5-μL aliquot of this mixture was added. The crystals were incubated for an additional 2 h but not for longer than 1 day prior to data collection.

Data Collection, Reduction, and Refinement. All data from single crystals were collected using a three-circle goniostat equipped with a Siemens area detector. CuKα X-rays were generated on a Rigaku rotating anode with either a 200-μM focal spot source and graphite monochromator or a 100-μM focal spot with Franks focusing optics (Franks, 1955). Data were processed using either the XENGEN (Howard et al., 1985) or the BUDDHA (Blum, 1987) data reduction package.

All mutant structures are solved by difference Fourier techniques (Chambers & Stroud, 1977) using α_{calc} values from the highly refined WT *L. casei* TS structure (Hardy et al., 1987; Finer-Moore, 1992). Difference density ($\geq 3\sigma$) in $F_o - F_c$ maps showed the general location of ligands, and either free atoms or fragments of the ligands were built into the map. Successive rounds of positional or molecular dynamics refinement using XPLOR (Brunger et al., 1987), manual

Table I: Statistics on Crystallographic Data for V316Am TS in Unbound and Complexed Forms

ligand(s)	max res.	no. of indep. reflections collected	% of possible reflections	redundancy	av, $I/\sigma(I)$	R_{sym}[a]	accum. % R-factor[b]
unbound	∞ – 5.61	1471	93	9.0	29.0	10.1	19.0
	5.61 – 4.45	1481	100	9.5	23.5	11.4	16.7
	4.45 – 3.89	1423	100	6.7	16.4	12.8	15.7
	3.89 – 3.53	1412	99	6.4	10.7	16.6	15.5
	3.53 – 3.28	1336	96	5.7	6.6	21.4	15.9
	3.28 – 3.09	1029	73	4.1	3.4	27.3	16.0
	∞ – 3.09	8152	94	7.1	15.7	13.3	16.0
FdUMP	∞ – 4.84	2206	90	5.6	37.9	6.6	18.3
	4.84 – 3.84	2172	95	6.0	33.3	9.0	18.2
	3.84 – 3.36	2137	95	5.6	21.3	11.6	18.7
	3.36 – 3.05	2132	97	4.6	15.1	14.3	19.9
	3.05 – 2.83	2047	93	3.5	13.1	15.1	20.3
	2.83 – 2.67	1467	67	2.8	12.2	15.0	20.7
	∞ – 2.67	12 161	89	4.8	22.9	10.8	20.7
dUMP	∞ – 4.53	2858	97	6.0	34.1	5.2	18.7
	4.53 – 3.60	2734	100	7.8	25.5	7.7	19.1
	3.60 – 3.14	2702	100	5.0	7.3	15.6	19.6
	3.14 – 2.85	2587	97	4.0	2.8	28.1	20.8
	2.85 – 2.65	2329	88	3.3	1.5	42.2	21.3
	∞ – 2.65	13 210	96	5.3	13.6	8.8	21.3
FdUMP and CH_2H_4folate	∞ – 4.91	1951	84	10.8	37.7	6.6	15.2
	4.91 – 3.90	1984	92	11.7	29.3	8.5	15.9
	3.90 – 3.41	1905	90	7.0	12.0	13.8	16.5
	3.41 – 3.09	1843	87	5.6	5.1	26.8	18.4
	3.09 – 2.87	1719	83	4.5	2.6	38.6	18.4
	2.87 – 2.70	1270	62	3.0	1.5	46.9	19.1
	∞ – 2.70	10 672	83	7.4	15.9	9.7	19.1

[a] $R_{sym} = \left\{ \sum_{h,k,l}^{\sigma} \sum_{i=1}^{N} (I_{avg} - I_i)^2 \Big/ \sum_{h,k,l} \sum_{i=1}^{N} (I_i)^2 \right\}^{1/2}$ where $I_{avg} = 1/N \sum_{i=1}^{N} I_i$. [b] R-factor $= \sum |(|F_o| - |F_c|)|/|F_c|$.

Table II: Crystallographic Refinement Statistics

ligand(s)	total no. of atoms	rms deviations bonds (Å)	rms deviations angles (deg)
unbound	2588	0.031	4.4
FdUMP	2604	0.024	4.4
dUMP	2603	0.024	4.3
FdUMP and CH_2H_4folate	2635	0.025	3.8

rebuilding with the graphics program FRODO (Jones, 1985), and calculation of new maps led to unambiguous locations for the ligand atoms. "Omit", $F_o - F_c$ maps calculated with the refined phases from the complex structures without the ligand atoms confirmed the ligand positions. Individual thermal factors were refined for all protein and ligand atoms. Statistics for data collection and refinement are shown in Tables I and II.

Structural Comparisons. Overlapping of structures was accomplished as described in Perry et al., (1990) using the program Newdome to select a "core" of Cα's whose positions are unchanged relative to each other in the two molecules. Rotational and translational displacements of ligands were determined using the program GEM (Fauman, unpublished results). Differences between protein structures were determined after errors in atomic positions were calculated as a function of temperature factors (Perry et al., 1990).

RESULTS

Crystal Properties. Hexagonal bipyramid crystals of unbound V316Am, binary V316Am·dUMP, and binary V316Am·FdUMP grew to 400 × 700 μM in 1–3 days. The space group is $P6_122$ with unit cell dimensions $a = b = 78.8$ Å and $c = 243.2$ Å. Each unit cell contains one TS monomer. This space group is identical to that of WT *L. casei* TS (Finer-Moore, 1992), and the unit cell dimensions are very similar ($a = b = 78.3$ Å and $c = 243.2$ Å).

Crystals of the V316Am ternary complex with FdUMP and CH_2H_4folate were also hexagonal bipyramids in the space group $P6_122$ with unit cell dimensions $a = b = 78.4$ Å and $c = 242.2$ Å. Most of the V316Am crystals remained intact upon addition of FdUMP and CH_2H_4folate, while crystals of WT TS prepared under identical conditions did not survive the soaking process. Thus, WT TS cannot tolerate intercalation of ligands into the protein without disruption of the crystal lattice, whereas changes in the mutant are less severe. Ternary complex crystals of V316Am were stable for over 1 week in crystallization drops and for at least 2 weeks in the X-ray beam during data collection.

Structure of V316Am without Ligands Is Unchanged Compared to WT. The unbound V316Am protein, solved at 3.09–Å resolution, is in the same open conformation of the enzyme as is observed for unbound WT TS (Figure 2a). Analysis of structural differences between unliganded *L. casei* WT TS and unliganded V316Am based on the correlation of atomic thermal factors with errors in coordinates indicates that these structures are the same within experimental error (Chambers & Stroud, 1979; Perry et al., 1990). Superposition of the WT C-terminus on V316Am shows that residues 313-315 of the WT lie within the $2F_o - F_c$ density calculated for V316Am (Figure 2b). An $F_o - F_c$ map calculated using refined

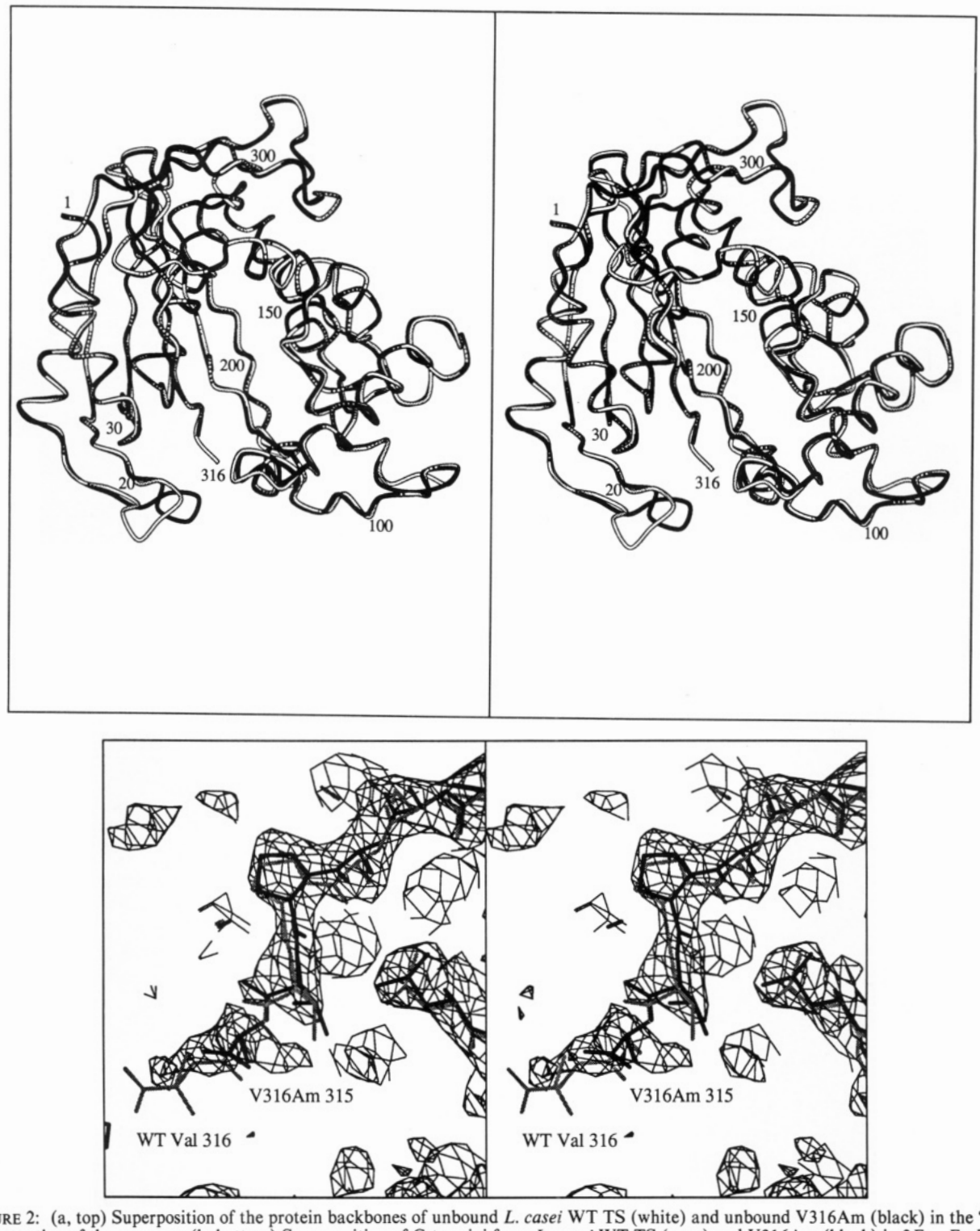

FIGURE 2: (a, top) Superposition of the protein backbones of unbound *L. casei* WT TS (white) and unbound V316Am (black) in the "open" conformation of the enzyme. (b, bottom) Superposition of C-termini from *L. casei* WT TS (gray) and V316Am (black) in $2F_o - F_c$ electron density calculated for the V316Am structure. Cross-eyed stereoviews.

phases from the unbound WT structure and amplitudes measured for the unbound V316Am crystal resulted in negative difference density (>3σ) for Val 316. Thus, no deviation in position is observed between the position of Ala 315 in the mutant and WT.

Binary Complexes of V316Am with FdUMP of dUMP Show an Altered Conformation of the Pyrimidine Ring Compared to dUMP in the WT Structure. The binary complex structure of V316Am with FdUMP, solved at 2.7-Å resolution, shows two major structural differences compared to the *L. casei* WT·dUMP binary complex: (1) the positions of the pyrimidine and ribose rings of bound nucleotides differ from their observed positions in WT·dUMP and (2) a C-terminal carboxyl oxygen forms a hydrogen bond with δNH_2 of Asn 263 in the V316Am binary complex but not in the WT·dUMP complex (Figure 3a). In the V316Am binary complex, FdUMP is rotated 17.3° and translated 0.66 Å with respect to dUMP in the WT·dUMP structure, while some of the same protein–ligand interactions are maintained. Hydrogen bonds between the 3′-OH of the ribose and both His 259 and Tyr 261 are preserved, as is coordination of the phosphate ion between arginines 178′,[2] 179′, and 218 and Ser 219 (Montfort et al., 1990). However, the amide of Asn 229, which interacts as both a hydrogen bond acceptor from the N3 hydrogen and

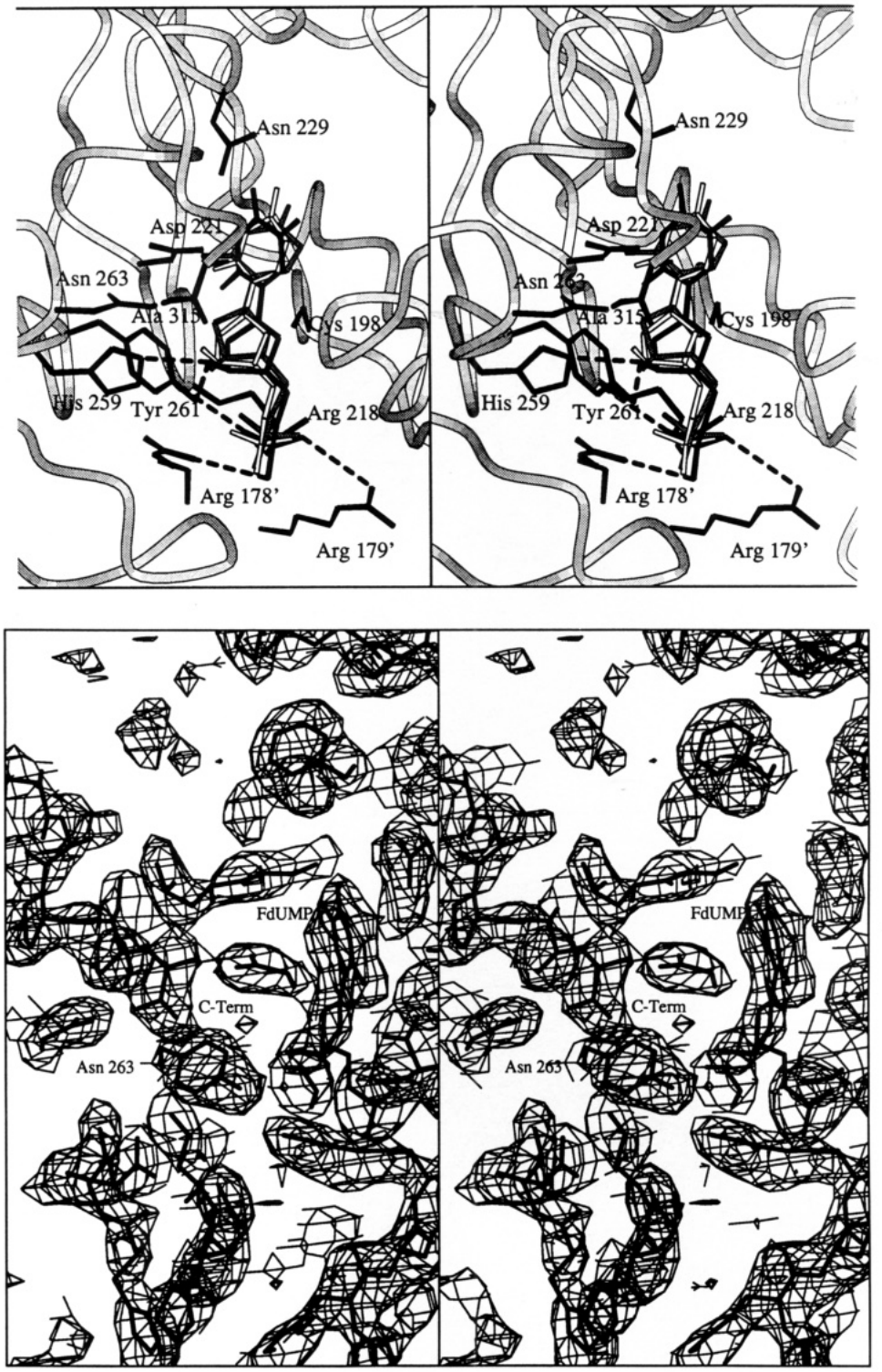

FIGURE 3: (a, top) Superposition of nucleotides from the *L. casei* WT·dUMP (gray), V316Am·dUMP (white), and V316Am·FdUMP (black) binary complexes. The protein backbone and amino acid side chains correspond to the V316Am·FdUMP binary complex. (b, bottom) $2F_o - F_c$ electron density corresponding to the V316Am·FdUMP binary complex depicting the nucleotide, Ala 315, and Asn 263. Cross-eyed stereoviews.

a donor to O4 of the pyrimdine in the WT structure, is 5 Å from these pyrimidine moieties in the V316Am·FdUMP structure. Also, the hydrogen bond between O2 of the pyrimidine and the backbone NH of Asp 221 is not conserved in the V316Am·FdUMP structure. All aforementioned amino acids are absolutely conserved with the exception of Ser 219, which is replaced by a cysteine in *Crithida fasiculata* (Harrap et al., 1989).

In the V316Am·FdUMP complex we see a hydrogen bond between the C-terminal carboxyl and Asn 263. An $F_o - F_c$ difference map calculated using amplitudes corresponding the V316Am·FdUMP crystal and refined phases from the unbound V316Am model showed significant ($\geq 3\sigma$) negative difference density at the position of Ala 315 and corresponding positive difference density close to the side chain of Asn 263. Upon rebuilding and refinement of the V316Am·FdUMP complex, a hydrogen bond interaction between a C-terminal carboxyl

[2] Primes are used to designate amino acids which are donated from the second subunit of the dimer.

oxygen and δNH_2 of Asn 263, which is adjacent to the activesite at the C-terminal end of β strand ii, was apparent in the $2F_o - F_c$ electron density map (Figure 3b).

In order to determine if the structural differences between the V316Am·FdUMP and WT·dUMP were due to the deletion of Val 316 or the presence of a fluorine atom at C5 of FdUMP, we studied the structure of V316Am with dUMP at 2.65-Å resolution. As indicated in Figure 3a, identical changes in the positions of the nucleotide and the C-terminal alanine (i.e., loss of hydrogen bonds with Asn 229 and Asp 221 and relocation of the C-terminus) were observed, confirming that these differences are due to the deletion of Val 316 and not the fluorine substituent. Since the interaction between Ala 315 and Asn 263 is absent in both the WT·dUMP and the unbound V316Am structure, we conclude that this conformation is induced by ligand binding in the V316Am mutant only. An average *B*-factor of 13.7 Å^2 for Ala 315 in both V316Am binary complexes, versus 51.4 Å^2 for WT·dUMP and 32.8 Å^2 for unbound V316Am, is evidence for ligand-induced ordering at this position in the mutant. Hence, even though the mutant binds these substrates in the open conformation, as does the WT enzyme, some local structural differences are observed.

Structure of the V316Am·FdUMP·CH_2H_4folate Ternary Complex. We have solved the structure of V316Am complexed with FdUMP and CH_2H_4folate at 2.7-Å resolution. Comparison of this structure with the *E. coli* WT TS·dUMP·CB3717 complex shows that the mutant active site is not closed (Figure 4a). As stated above, crystals of WT *L. casei* TS crack upon addition of FdUMP and CH_2H_4folate, presumably due to a conformational change. The observation that V316Am crystals are stable to addition of ligands suggested that the conformation of crystalline V316Am·FdUMP·CH_2H_4folate differs from the closed ternary complex structure of the *E. coli* TS enzyme even before the structure was solved. In the *E. coli* WT TS·dUMP·CB3717 complex, the C-terminal tetrapeptide shows the largest displacement relative to the unbound enzyme, mediating a hydrogen bond network between cofactor, water molecules, and protein side chains (Figure 1). Although the C-terminus of V316Am also responds to ligand binding, where a 4-Å shift from its position in the unbound enzyme places a terminal carboxyl oxygen in hydrogen bond contact with Nϵ1 of Gln 109, the active site cavity remains in the open conformation (see Figures 2a and 4).

In this open conformation observed for V316Am·FdUMP·CH_2H_4folate, the position of CH_2H_4folate differs from that of CB3717 in the *E. coli* WT TS·dUMP·CB3717 structure in several respects (Figure 4a). The PABA ring of CH_2H_4folate is rotated 87.3° but is surrounded by the same highly conserved, hydrophobic residues as the PABA of CB3717 (i.e., Ile 81, Leu 224, Phe 228, and Val 314). The pterin of CH_2H_4folate in the V316Am·FdUMP·CH_2H_4folate structure is rotated out of the active site cavity and into the region occupied by Trp 85 in the WT TS·dUMP·CB3717 structure. The pterin ring has moved away from the position of the CB3717 quinazoline, which stacks against the dUMP pyrimidine.

CH_2H_4folate has adopted a "folded" geometry in which the plane of the pterin is roughly perpendicular to the PABA and the five-membered imidazolidine ring is open (Figures 4 and 5). Excess electron density in $2F_o - F_c$ maps and positive electron density in $F_o - F_c$ difference maps indicated chemical modification of the transferable methylene group, C11. Positional and molecular dynamics refinement of this moiety as a hydroxymethyl group satisfied the positive $F_o - F_c$ difference peak at the location of the oxygen and resulted in an improved fit for the entire cofactor molecule in the electron density (Figure 5). Refinement of the structure with the imidazolidine ring in closed conformation resulted in negative $F_o - F_c$ difference density ($>3\sigma$) for atoms of the pterin and imidazolidine ring and a crystallographic *R*-factor of 23.0%, versus 19.1% for the open conformer. $2F_o - F_c$ density maps also show that the transferable methylene group is attached only to N5 and that the N10–C11 bond is broken. Thus, in the V316Am ternary complex, the imidazolidine ring of CH_2H_4folate has opened with hydroxylation of the methylene at N5 and protonation of N10 (see species **IIa**, Figure 6).

The Hydrogen Bond Pattern for FdUMP in V316Am·FdUMP·CH_2H_4folate Is Identical to That Observed in the V316Am·FdUMP and V316Am·dUMP Binary Complexes. As was observed in the V316Am binary complex structures, the hydrogen bonds between the protein and the pyrimidine in V316Am·FdUMP·CH_2H_4folate are not conserved, while those for the 3′-OH of the ribose and the phosphate moiety are maintained, compared to *L. casei* WT·dUMP. The covalent bond between Cys 198 and the pyrmidine, which is observed in the *E. coli* WT TS·dUMP·CB3717 structure and is necessary for catalysis, is not present between V316Am and FdUMP. The plane of the pyrimidine ring is rotated 83.2° with respect to that of dUMP in the WT ternary complex, placing C6 4.2 Å away from the γS of Cys 198 and out of position for nucleophilic attack.

DISCUSSION

Thymidylate synthase undergoes a major structural change upon ligand binding, whereby the active site reconfigures from an open disordered conformation to a closed ordered conformation. The C-terminal tetrapeptide plays a major role in this process. In this study, X-ray crystallographic analysis of a TS mutant lacking the C-terminal valine reveals the influence of this deletion on ligand binding and the conformational change. We find that each structure adopts the open conformation, with a new location for the truncated carboxyl terminus and altered ligand positions compared to analogous WT structures.

Deletion of the C-terminal Valine Affects the Orientation of the Pyrimidine Nucleotide in Binary and *Ternary Complex Structures.* Comparison of the V316Am·dUMP and V316Am·FdUMP structures with the *L. casei* WT·dUMP binary complex shows that interactions of the sugar 3′-hydroxyl with Tyr 261 and His 259 and of the phosphate moiety with Arg 23, 218, 178′, and 179′ are conserved. Rotation of the substrate in V316Am places the pyrimidine out of range for hydrogen bond formation (Figure 3a). Hydrogen bonds from the N3 hydrogen and from O4 and O2 of the pyrimidine to Asn 229 and the backbone NH of Asp 221 are not observed in the mutant structures, and neither compensatory hydrogen bonds nor van der Waals contacts are made to the nucleotide.

The position, flexibility, and length of the carboxyl terminus influences the position of the nucleotide in V316Am binary complexes. In the WT·dUMP structure, thermal factors for the C-terminal tetrapeptide range from 24.0 to 42.6 Å^2, and the closest distance between a C-terminal carboxyl oxygen and dUMP is 10.2 Å. For V316Am binary complexes, the C-terminus is less mobile than in WT·dUMP, evidenced by the hydrogen bond with Asn 263 and thermal factors ranging from 10.5 to 21.0 Å^2 for residues 313–315. FdUMP and dUMP are 5.7 Å from the closest C-terminal oxygen in the mutant structures. These data suggest that, in WT TS, the

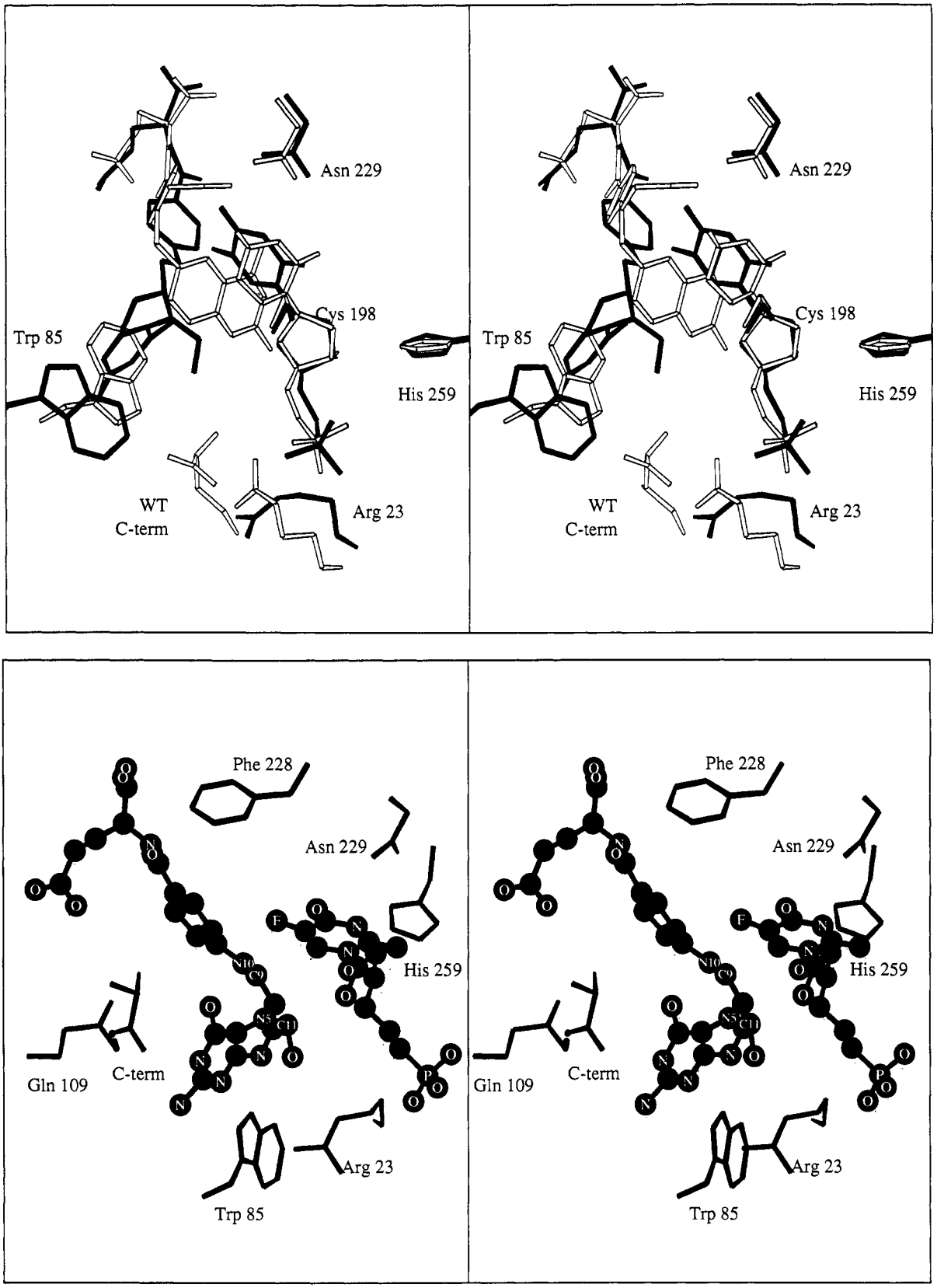

FIGURE 4: (a, top) Superposition of *E. coli* WT·dUMP·CB3717 (white) and V316Am·FdUMP·CH_2H_4folate (black) ternary complexes showing the ligands and surrounding amino acid environments. The noncoincident positions of the WT C-terminus, Arg 23, Trp 85, and Asn 229 illustrates their shift upon ligand binding in the "closed" conformation. The V316Am·FdUMP·CH_2H_4folate C-terminus is out of view. (b, bottom) V316Am·FdUMP·CH_2H_4folate ternary complex depicting the ligand atoms and the hydrogen bond between the C-terminus and Gln 109. Cross-eyed stereoviews.

pyrimidine and ribose rings bind further from the C-terminal carboxyl group to avoid steric clash with the flexible tail. Since thermal factors are high for the unbound V316Am structure, we deduce that the interaction of Asn 263 and Ala 315 is one of many possible locations for the conformationally flexible C-terminus, which is stabilized upon substrate binding. In turn, the nucleotide is no longer constrained in the normal hydrogen-bonding pattern, as is reflected in higher average

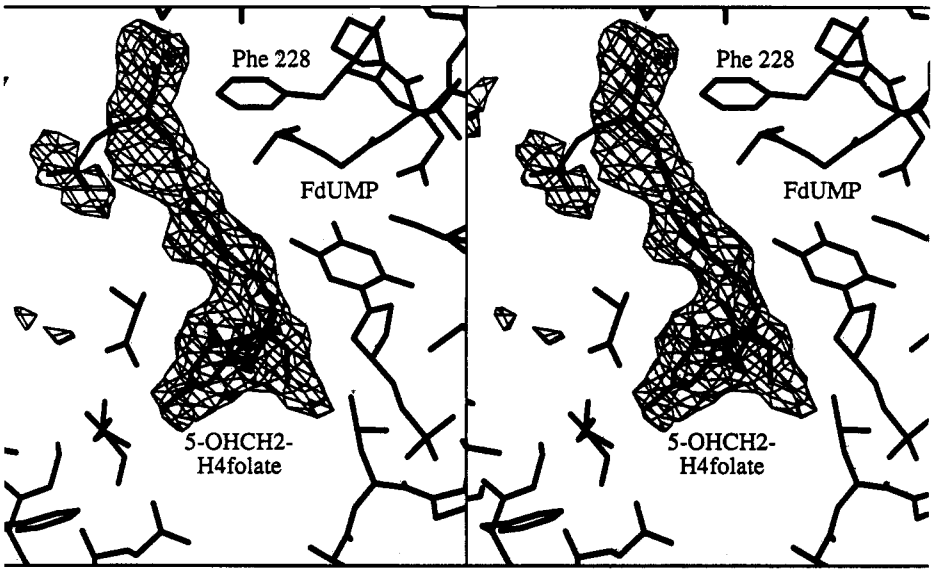

FIGURE 5: "Omit" $F_o - F_c$ map calculated for the V316Am·FdUMP·5-HOCH$_2$H$_4$folate ternary complex using phases from a model which lacked the cofactor atoms. The hydroxymethyl group is attached to N5 of the cofactor. Cross-eyed stereoview.

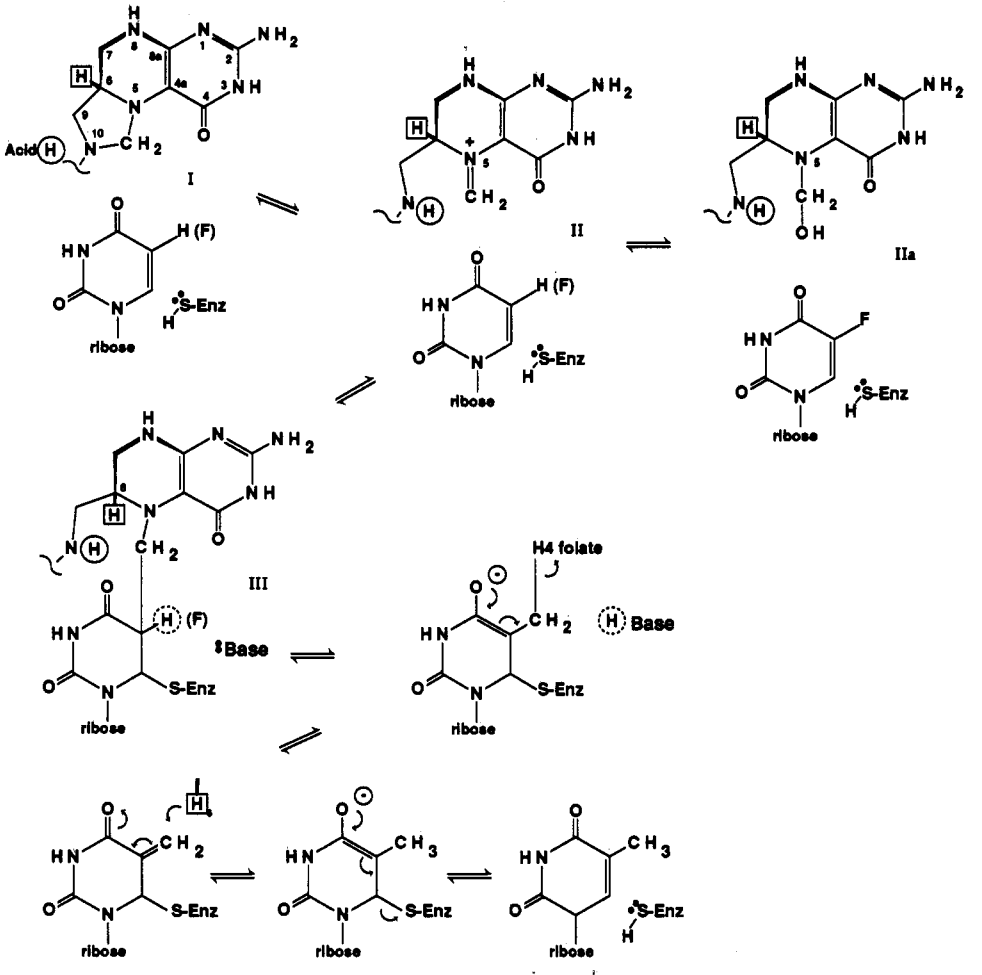

FIGURE 6: TS mechanism showing off-the-pathway formation of 5-HOCH$_2$H$_4$folate (**IIa**).

thermal factors for dUMP (16.5 Å^2) or FdUMP (18.5 Å^2) in V316Am versus 9.2 Å^2 for dUMP in the WT complex. Thus, in WT enzyme the substrate is well-ordered, and the C-terminus is disordered; in V316Am the C-terminus is well-ordered, and the nucleotide is relatively mobile.

Although a covalent bond is observed between C6 of dUMP and the γS of Cys 198 in the WT·dUMP·CB3717 complex, C6 of FdUMP is out of range for formation of a covalent bond with Cys 198 in V316Am·FdUMP·CH$_2$H$_4$folate (Figure 4). The hydrogen bond pattern of FdUMP in V316Am·FdUMP·CH$_2$H$_4$folate is identical to that of FdUMP in V316Am·FdUMP. Even though cofactor is packed against the nucleotide in V316Am, CH$_2$H$_4$folate binding does not cause rotation of the nucleotide into the conformation found in the WT·dUMP and WT·dUMP·CB3717 structures.

The Presence of 5-HOCH$_2$H$_4$folate in V316Am·FdUMP·

CH_2H_4folate is Structural Evidence for the 5-Iminium Ion Intermediate in the TS Reaction. A schematic of the TS mechanism is given in Figure 6, where binding of the substrate and the closed ring form of the cofactor (**I**) precedes nucleophilic attack by Cys 198 at C6 of dUMP. It is generally accepted that the reactive form of CH_2H_4folate is the 5-iminium ion (**II**), where the five-membered imidazolidine ring is open (Santi & Danenberg, 1984; Finer-Moore et al., 1990). In the WT enzyme, Cys 198 adds to C6 of dUMP, forming a nucleophilic enol/enolate at C5, which reacts with the iminium ion. In our V316Am·FdUMP·CH_2H_4folate structure, we observe 5-$HOCH_2H_4$folate (**IIa**), which is a product of the reaction of the iminium ion (**II**) with water (Kallen & Jencks, 1966). Thus, species **IIa** is structural evidence for the existence and subsequent hydration of the iminium ion intermediate in a reaction in V316Am.

The Open Noncovalent Ternary Complex Is Stabilized in V316Am Crystals. In contrast to our crystal structure, Carreras and co-workers (1992) detect a covalent ternary complex (**III**) for V316Am in the presence of excess FdUMP and CH_2H_4folate. However, the ratio between noncovalent and covalent ternary complexes is 1:7 for the mutant and 1:17 000 for the WT TS, corresponding to differences in free energy of −1.12 and −5.8 kcal mol^{-1}, respectively. The reduction in the magnitude of ΔG for V316Am provides a larger proportion of noncovalent forms than would be present with the WT enzyme. Since the difference in free energy between noncovalent and covalent mutant ternary complexes is small, it is reasonable to suggest that the noncovalent form of the ternary complex is stabilized by crystal packing forces, which maintain the open conformation. Since ΔG for covalent ternary complex formation by the WT enzyme is more negative than for V316Am, we believe that WT TS crystals crack upon addition of FdUMP and CH_2H_4folate due to a change in conformation to the closed, covalent form.

Role of the C-terminus in the Conformational Change from the Open to the Closed State of TS. In contrast to the V316Am binary complexes, where Ala 315 is located along the wall of the active site cavity, the C-terminus in the V316Am·FdUMP·CH_2H_4folate structure has moved toward the cofactor into the center of the binding pocket to form a hydrogen bond with Gln 109 (Figure 4b). This suggests that, as with WT enzyme, the cofactor stimulates a conformational change but the truncated C-terminus does not span the active site to complete the process in the V316Am crystal. In addition, lack of closure of the active site in the V316Am·FdUMP·CH_2H_4folate complex demonstrates that opening of the imidazolidine ring is independent of the protein conformational change in this mutant.

Opening of the Imidazolidine Ring of CH_2H_4folate in V316Am·FdUMP·CH_2H_4folate. Studies of WT TS have suggested that the imidazolidine form of CH_2H_4folate binds to the enzyme, and opening of the five-membered ring to the 5-iminium ion is a candidate for the rate-determining step in the reaction (Benkovic, 1980; Bruice & Santi, 1982). Using molecular graphics modeling, we attempted to reconstruct the imidazolidine form of the cofactor from 5-$HOCH_2H_4$folate in the V316Am·FdUMP·CH_2H_4folate structure by simply rotating the pterin while holding the PABA and N10 atoms in fixed positions. The N10–C11 covalent bond could not be made without steric clash between the pterin of the cofactor and the pyrimidine of FdUMP. Thus, the imidazolidine form of the cofactor must bind with a different orientation of the PABA. Comparison of CB3717 in the ternary complex from *E. coli* with CH_2H_4folate of V316Am·FdUMP·CH_2H_4folate (Figure 4a) shows that the PABA ring can rotate up to 87.3°, and N10 can translate up to 3.3 Å, while remaining in the highly conserved, hydrophobic binding site (Montfort et al., 1990). In V316Am, the PABA of the closed imidazolidine ring form of CH_2H_4folate may bind and then rotate. This is consistent with the proposal that an enzyme-induced perturbation of the PABA moiety may assist in opening the imidazolidine ring either by an electronic effect, which would assist protonation of N10, or by stretching the N10–C11 bond, rendering it more susceptible to cleavage (Santi et al., 1987).

V316Am Structures Provide New Insights for Inhibitor Design against TS. TS is already the focus of several major drug design efforts due to its key role in the final step along the sole *de novo* synthesis of dTMP (Santi & Danenberg, 1984; Perry et al., 1992; Reich et al., 1992; Varney et al., 1992). The lesson that TS can accommodate ligands in a variety of binding modes may provide new clues for drug design in this protein. For example, superposition of V316Am·FdUMP·CH_2H_4folate with *E. coli* WT·dUMP·CB3717 shows that the pterin portion of CH_2H_4folate overlaps the position of Trp 85 from the *E. coli* ternary complex (Figure 4a). Recently, we have observed that the inhibitors sulizobenzone and phenolphthalein, which do not resemble dUMP or CH_2H_4folate, also bind in this location in the open form of the enzyme (Shoichet et al., 1992). Thus, inhibitors can bind in orientations that overlap regions of the site normally occupied by amino acid side chains in other conformational states of the protein. Following this logic, compounds that prevent rotation of the C-terminus into the active site should also be viable inhibitors of the enzyme. In addition, on the basis of the nucleotide binding motifs in this mutant, we predict that molecules which maintain interactions with Tyr 261, His 259, and side chains in the phosphate binding pocket should be inhibitors of the enzyme.

In summary, we find that the C-terminus of TS influences the location of ligands in the active site. In the V316Am ternary complex, we have found 5-$HOCH_2H_4$folate which is a "trapped" form of the reactive 5-iminium ion proposed as an intermediate of the TS reaction. From the alternate binding modes of all of the ligands in these mutant structures, we have located atomic positions and specific protein–ligand interactions which may prove helpful in the design of inhibitors against TS

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