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The structural roles of conserved Pro196, Pro197 and His199 in the mechanism of thymidylate synthase

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We generated replacement sets for three highly conserved residues, Pro196, Pro197 and His199, that flank the catalytic nucleophile, Cys198. Pro196 and Pro197 have restricted mobility that could be important for the structural transitions known to be essential for activity. To test this hypothesis we obtained and characterized 13 amino acid substitutions for Pro196, 14 for Pro197 and 14 for His199. All of the Pro196 and Pro197 variants, except P197R, and four of the His199 variants complemented TS-deficient *Escherichia coli* cells, indicating they had at least 1% of wild-type activity. For all His199 mutations, k_{cat}/K_m for substrate and cofactor decreased more than 40-fold, suggesting that the conserved hydrogen bond network coordinated by His199 is important for catalysis. Pro196 can be substituted with small hydrophilic residues with little loss in k_{cat}, but 15- to 23-fold increases in K_m^{dUMP}. Small hydrophobic substitutions for Pro197 were most active, and the most conservative mutant, P197A, had only a 5-fold lower k_{cat}/K_m^{dUMP} than wild-type TS. Several Pro196 and Pro197 variants were temperature sensitive. The small effects of Pro196 or Pro197 mutations on enzyme kinetics suggest that the conformational restrictions encoded by the Pro–Pro sequence are largely maintained when either member of the pair is mutated.

Keywords: hydrogen bond/modeling/protein dynamics/residue specificity/saturation mutagenesis

Introduction

Thymidylate synthase (TS) catalyzes the conversion of 2′-deoxyuridine-5′-monophosphate (dUMP) and 5,10-methylene-5,6,7,8-tetrahydrofolate (mTHF) to 2′-deoxythymidine-5′-monophosphate (dTMP) and 7,8-dihydrofolate. Because of its central role in maintaining adequate levels of deoxythymidylate for DNA synthesis, TS is a major chemotherapeutic target and several potent drugs that act as inhibitors of the enzyme have been developed. Crystal structures of TS from several species, including *Lactobacillus casei* (Hardy *et al*., 1987), *Escherichia coli* (Matthews *et al*., 1990; Montfort *et al*., 1990; Perry *et al*., 1990), T4 phage (Finer-Moore *et al*., 1994), human (Schiffer *et al*., 1995; Almog *et al*., 2001; Phan *et al*., 2001; Sayre *et al*., 2001), *Leishmania major* (Knighton *et al*., 1994), mouse (Sotelo-Mundo *et al*., 1999), *Bacillus subtilis* (Stout *et al*., 1998; Fox *et al*., 1999) and *Pneumocistis carinii* (Anderson *et al*., 2000), show that TS is a dimer of identical subunits of ~35 kDa, and reveal a set of 25 conserved residues that define the cavernous active site cavity and may be involved in catalysis. Roles of many of these residues in the reaction mechanism have been defined by mutagenesis and structure (Carreras and Santi, 1995; Finer-Moore *et al*., 2003).

TS is among the most highly conserved of all enzymes and undergoes a cycle of conserved structural changes to reorient the reactants through several reaction steps (Stroud and Finer-Moore, 2003). The reactants bind in an ordered, sequential fashion, with dUMP binding first to the open conformation of the enzyme. The conserved Asn229 carboxamide group forms hydrogen bonds with N-3 and O-4 on the base, while two other conserved residues, His259 and Tyr261, hydrogen bond to O-3′ on the ribose ring. The backbone amide of Asp221 and carboxamide of Gln217 donate hydrogen bonds to O-2 on the base. Four arginines, Arg23 and Arg218 from one protomer and Arg178′ and Arg179′ from the second protomer, each donate two hydrogen bonds to the dUMP phosphate moiety. Except for Arg23, which is disordered in the apoenzyme, all dUMP contact residues are pre-oriented for forming ideal hydrogen bonds to dUMP, and these hydrogen bonds are maintained during subsequent conformational changes of the enzyme.

The cofactor binds on the surface of dUMP and induces a major conformational change that closes the active site cavity. Enzyme closure brings several hydrophobic residues into van der Waals contact with the cofactor, most notably, Ile81, Trp82, Trp85, Leu195, Leu224 and Phe228. The C-terminus swings into the active site and coordinates a hydrogen bond network with the pterin ring of the cofactor, Arg23, Trp85 and ordered water. Concomitant with this conformational change, the catalytic nucleophile forms a covalent bond to C-6 of dUMP. The essential nucleophile, the sulfhydryl group of Cys-198, lies in a highly conserved sequence, Pro196–Pro197–Cys198–His199. With its restrictive geometry, proline often plays a structural role. To probe the effects and roles of Pro196, Pro197 and His199 in the dynamic structural mechanism, we elaborated a set of replacements at each of the residues using a synthetic gene approach (Climie *et al*., 1990).

Materials and methods

Materials

The *E.coli* strain TB-1 from T.O.Baldwin (Texas A&M) was used as the host strain for plasmid-mediated transformations during construction of the mutants, and a TS-deficient (Thy^-) strain of *E.coli*, χ2913 (ΔthyA572) from R.Thompson (University of Glasgow, UK) was used to test plasmids for

TS activity by complementation and to produce mutant protein. Media used for cell growth were Lurie broth (LB) plus 100 μg/ml ampicillin (LBA), or LBA containing 50 μg/ml thymidine (LBAT) for growing Thy⁻ cells. The Sequenase DNA sequencing kit was purchased from US Biochemical Corp. Restriction endonucleases and T4 ligase were purchased from New England Biolabs and Bethesda Research Laboratories, respectively, and used as recommended.

Molecular biology

Plasmid DNA was sequenced (both strands) by the dideoxy chain-termination method using a modified T7 polymerase (Tabor and Richardson, 1987) and ^{35}S-ATP. Oligonucleotides were synthesized at the UCSF Biomolecular Resource Center using an Applied Biosystems model 380 B DNA synthesizer.

Construction and characterization of mutants

Mutant proteins were obtained *in vitro* by cassette mutagenesis as described using a synthetic *L.casei* TS gene contained within plasmid pSCTS9 (Climie *et al*., 1992). Substitutions that led to >~1% of wild-type activity were identified by screening for the ability of Thy⁻ cells harboring the mutant plasmids to grow on a minimal agar plate lacking thymidine (Climie *et al*., 1992).

For temperature-dependence studies, Thy⁻ cells harboring different mutant plasmids were grown overnight to 10^9 cells/ml in LBAT. Cells were harvested, and resuspended in an equal volume of phosphate-buffered saline (PBS); 1 μl of the suspension was spotted on minimal agar plates and incubated at 30, 37 and 42°C for 18 h.

To test for reversion of inactive mutants to catalytically active enzyme, mutants that were inactive by the complementation screen were grown overnight in 5 ml of LBAT to 10^9 cells/ml. Cells were harvested, washed with PBS and resuspended in 1 ml of PBS. A 100 μl aliquot was serially diluted and plated on LBA plates to evaluate the cell count. Five 100 μl aliquots ($2–10\times10^{-9}$ cells) were plated on minimal plates plus ampicillin (100 mg/ml) and incubated at 37°C for 48 h. Colonies were transferred to LBAT plates and plasmid DNA was used to transform χ2913 and the resulting transformants were checked again for TS complementation by patching cells on minimal agar plates in the absence of thymidine. Plasmid DNA from cells that scored positive was sequenced.

Protein analysis

Mutant TS was purified by sequential chromatography on phosphocellulose and hydroxyapatite (Kealey and Santi, 1992). Protein was quantitated by the method of Read and Northcote (Read and Northcote, 1981).

TS activity was determined at 25°C by monitoring dTMP formation spectrophotometrically at 340 nm (Pogolotti *et al*., 1986) for both crude extracts and homogeneous protein. (One unit of activity is the amount of TS that catalyzes the formation of 1 μmol of product per minute in a 1-ml reaction mixture.) For determining K_m^{dUMP}s, initial velocities were measured as the concentration of dUMP was varied in the presence of excess (300 μM) mTHF. For determining K_m^{mTHF}s, initial velocities were measured as the concentration of mTHF was varied in the presence of excess (125 μM) dUMP. Kinetic constants were determined by a least-squares fit of these data to the appropriate equations.

Dehalogenation of BrdUMP

Kinetic assays for TS-catalyzed dehalogenation of 5-bromo-2′-deoxyuridine-5′-monophosphate (BrdUMP) (Garret *et al*., 1979) were performed by monitoring the decrease in absorbance at 285 nm, using ε_{285} = 5320 (Kawase *et al*., 2000). (One unit of TS activity catalyzes the dehalogenation of 1 μM of BrdUMP per minute in 1 ml of reaction mixture.) For the determination of kinetic parameters, the concentration of TS was fixed at 5–10 μM and the concentration of BrdUMP was varied up to 160 μM. The dehalogenation of BrdUMP occurs in the absence of cofactor and kinetic parameters for this reaction indicate how well the enzyme orients dUMP in a binary complex for covalent addition to the active site nucleophile (Finer-Moore *et al*., 1998).

Computer modeling

For modeling of Pro196 and Pro197 variants, side chains in the LcTS-dUMP binary complex crystal structure (Finer-Moore *et al*., 1993) were replaced manually using the computer graphics program CHAIN (Sack, 1988). The conformation chosen for the replaced side chain was that member of a subset of likely rotomers that had the fewest steric collisions with the rest of the structure. Structures were refined in CNS (Brunger *et al*., 1998) by several cycles of simulated annealing molecular dynamics without the X-ray term, using a 'slowcool' procedure in which the temperature was initially set to 2500 K, then reduced in increments of 25 K per cycle until reaching a final temperature of 0 K (Rice and Brunger, 1994). Portions of the structure outside the 15 Å radius of the mutation site were harmonically restrained using a harmonic restraint constant of ten, since proteins typically adapt to mutations with local changes in structure (Perry *et al*., 1990).

When substrate-bound TS binds to its cofactor to form a covalent ternary complex, the enzyme undergoes extensive conformational shifts that close the active site cavity (Perry *et al*., 1990). The impact of a mutation on catalysis will depend in part on whether the substituted residue experiences different structural environments in the open and closed enzyme states (Finer-Moore *et al*., 2003). Therefore, we also modeled the Pro196 and Pro197 mutations into a pair of open and closed TS structures: a binary complex of *E.coli* TS (EcTS) with dUMP, and the ternary complex of EcTS with dUMP and a cofactor analog, 10-propargyl-5,8-dideazafolate (CB3717), respectively.

Results

Mutagenesis

Mutants were prepared by cassette mutagenesis of pSCTS9 (st *Nco*I–*Sna*BI) using synthetic oligonucleotides that contained a mixture of 32 codons NN(G+C) in place of the target codon. Following initial transformation, colonies were pooled, and the mutagenized plasmid DNA was recovered and used to transform Thy⁻ χ2913 *E.coli* strain. Individual plasmids were passed through several rounds of transformation to insure segregation and/or repair of the heteroduplex DNA molecules, and mutants were identified by DNA sequencing. For a mixture of 32 plasmids encoding 20 amino acids, a sampling of 160 random clones are necessary to achieve a 95% probability of obtaining all amino acid substitutions (Climie *et al*., 1990). All transformations exceeded that number, so that all mutants were probably represented in the final libraries. However, only 20–

30 isolates of a replacement set were sequenced to give 10–15 different mutants of a given residue.

Using this procedure, 14 mutants of His199 were isolated of which five were fully active and one showed marginal activity in the complementation assay. Likewise, we obtained 13 mutants of Pro196 and the parental CCG Pro codon after sequencing 32 clones. For the Pro197 mutants, 13 amino acid substitutions were identified and one of them, the variant in which Pro197 was replaced with Arg (P197R), was inactive.

Temperature dependence of mutants

We screened for temperature sensitivity by comparing the ability of Thy⁻ cells containing plasmids encoding the different active mutant proteins to grow on minimal agar plates at 30, 37 and 42°C. Cells containing wild-type protein and most mutants grow well at these temperatures. Among the Pro196 mutants, P196I grew poorly at 37°C, but did not grow at 42°C; P196C and P196F grew well at 37°C, but grew poorly at 42°C. Among the Pro197 mutants, P197Q complemented Thy⁻ cells at 37°C, but did not grow at 42°C; P197H, P197F and P197Y grew poorly at 37°C, but did not grow at 42°C.

Spontaneous revertants

We used a complementation screen to detect reversion of catalytically inactive Cys198 mutants to active ones. About $0.2–3\times10^{10}$ Thy⁻ cells harboring plasmids coding for inactive TS mutants were plated on minimal media and incubated overnight. Plasmid DNA was isolated from colonies that grew over Thy-selective media and used to retransform Thy⁻ cells. The retransformed cells were grown under selective conditions, and sequenced.

None of the Cys198 mutants complement TS-deficient *E.coli* (Climie *et al*., 1990). We screened C198A(GCC), C198G(GGC), C198S(TCC) and C198T(ACC) for reversions and obtained one revertant from C198G and seven from C198S. When sequenced, all gave the TGC Cys codon, which represented a single base change. Reversions of C198S and C198G occurred at frequencies of $\sim1\times10^{-9}$ cells. The C198A and C198T mutants gave no revertants. More than one base change in the A and T mutants would be required to provide Cys198, and a second site revertant not containing the essential Cys198 is unlikely to be active. Thus, although revertants are easily screened by this method, we were only successful in recovering wild-type TS, demonstrating that Cys198 is essential for >1% enzyme activity.

Purification and characterization of mutants

His199. Four active His199 mutants, H199A, H199S, H199T and H199V, were purified to homogeneity and steady-state kinetic parameters were determined (Table I). For these mutants, K_ms for dUMP and mTHF were within a factor of four of the K_ms for wild-type TS, except for K_m^{dUMP} for H199A, which was ~15-fold higher than the wild-type K_m^{dUMP}. The k_{cat} values were 37– 400-fold lower than the wild-type k_{cat}.

Pro196. Steady-state parameters were measured for 12 Pro196 mutants (Table II). These data show that small polar side chains at this site maintain k_{cat}, while hydrophobic side chains best maintain K_m values. Proline is presumably a best compromise, although the geometric restraints of proline are not essential for good activity.

K_m values for dUMP were either similar to the wild-type value or moderately (<23-fold) higher for all of these mutants. K_m for dUMP was most impaired for the small polar substituents P196D, P196H, P196N and P196T, which had K_m^{dUMP} values 15–23-fold higher than that of the wild-type enzyme. K_m values for dehalogenation of BrdUMP were affected in a similar fashion, further showing that substrate binding was diminished in these mutants. In models of these mutants, the substituted side chains formed new hydrogen bonds to two of the conserved phosphate-binding arginines, Arg218 or Arg179, at the expense of ideal hydrogen bond geometry between the arginines and phosphate moiety of dUMP. Thus, alteration of the structural and electrostatic

Table I. Kinetic constants for *L.casei* TS His-199 mutants

Residue	K_m (μM)		k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	
	dUMP	mTHF		dUMP	mTHF
His (wt)	2.7	10	7.8	2.9	0.39
Ala (A)	42	20	0.035	8.38×10^{-4}	1.77×10^{-3}
Ser (S)	6.4	8.9	0.0196	3.07×10^{-3}	2.19×10^{-3}
Thr (T)	10	31	0.0626	6.00×10^{-3}	1.99×10^{-3}
Val (V)	3.6	22	0.2126	5.86×10^{-2}	9.47×10^{-3}

Table II. Kinetic constants for *L.casei* TS Pro-196 mutants

Residue	dTMP formation						BrdUMP dehalogenation	
	K_m (μM)		K_m^{dUMP}/K_m^{mTHF}	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\mu M^{-1}$)		K_m (μM)	k_{cat} (s^{-1})
	dUMP	mTHF			dUMP	mTHF		
Pro (wt)	2.7	10	0.27	7.8	2.9	0.39	9	0.01
Cys (C)	10	37	0.27	0.86	0.08	0.02		
Asp (D)	61	62	0.99	3.7	0.06	0.06	55	
Phe (F)	3.1	11	0.27	0.57	0.18	0.05	6.4	0.01
Gly (G)	3.2	7	0.45	0.12	0.04	0.02		
His (H)	46	32	1.4	0.60	0.01	0.02		
Ile (I)	3.2	68	0.05	0.28	0.09	0.0041		
Lys (K)	4.0	16	0.25	0.17	0.04	0.01		
Asn (N)	41	44	0.93	1.8	0.04	0.04	43	0.0014
Arg (R)	18	23	0.77	0.58	0.03	0.03		
Thr (T)	47	83	0.57	1.6	0.03	0.02		
Tyr (Y)	11	37	0.30	0.4	0.04	0.01		
Val (V)	19	63	0.29	0.64	0.03	0.01		

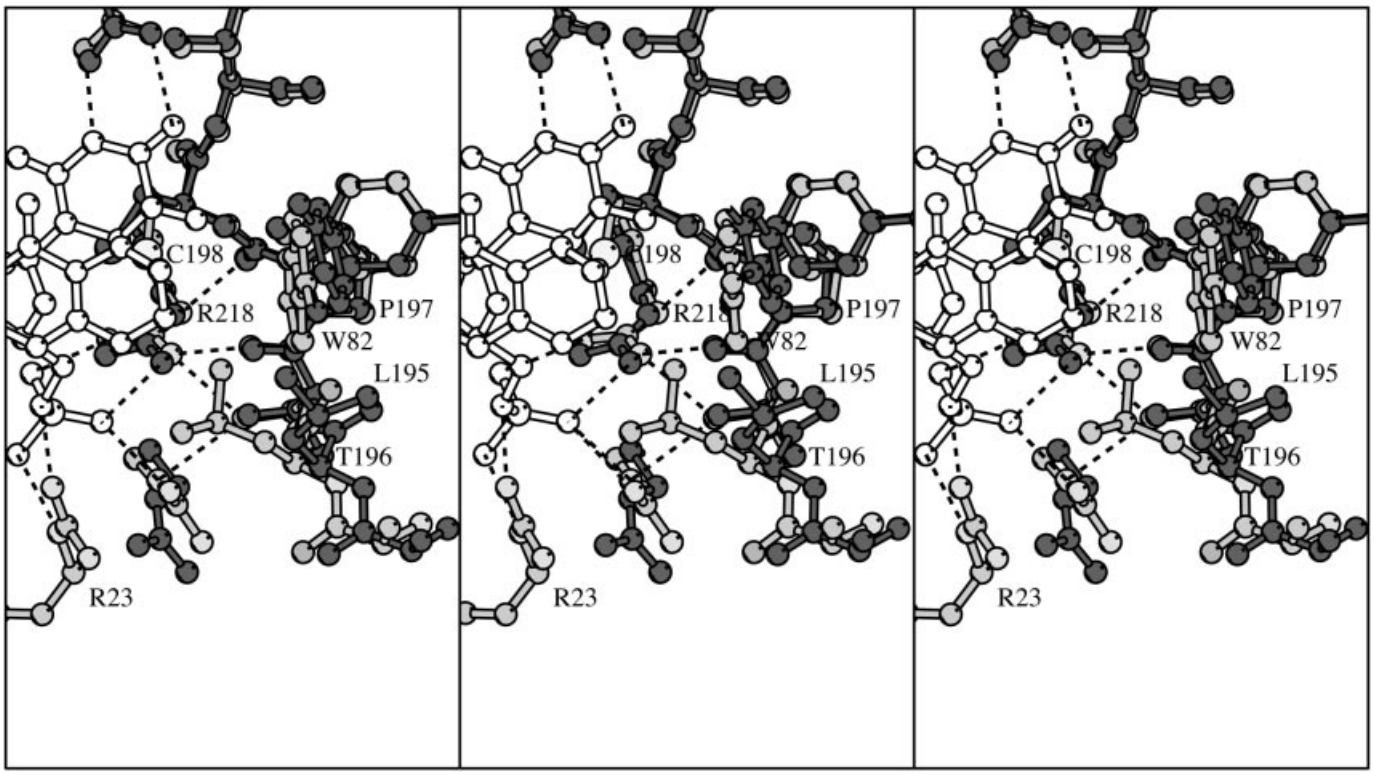

Fig. 1. Stereo plot (Kraulis, 1991) illustrating the conserved conformational change in the vicinity of Pro-196 that occurs when the ternary complex forms in TS. Apo-TS from *B.subtilis* (dark gray) (Stout *et al*., 1998) is overlapped with the ternary complex of *B.subtilis* TS with 5-fluoro-dUMP and mTHF (light gray protein, white ligands) (Fox *et al*., 1999). The picture shows how Leu-195(158) moves to make contact with mTHF in the ternary complex. Hydrogen bonds are shown with dashed lines. The figure is labeled using the LcTS numbering scheme. In this and subsequent stereo diagrams, the left two panels constitute a divergent-eyes view while the right two panels are the cross-eyed view.

Table III. Kinetic constants for *L.casei* TS Pro-197 mutants

Residue	K_m (μM)		K_m^{dUMP}/K_m^{mTHF}	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	
	dUMP	mTHF			dUMP	mTHF
Pro (wt)	2.7	10	0.27	7.8	2.9	0.39
Ala (A)	3.8	9.3	0.41	2.33	0.6	0.25
Cys (C)	4.1	11	0.36	0.8	0.19	0.07
Phe (F)	21	87	0.24	0.031	0.0014	0.00035
His (H)	68	96	0.71	0.4	0.01	0.0042
Ile (I)	12	19	0.67	0.75	0.06	0.04
Leu (L)	6.1	188	0.03	0.141	0.02	0.00078
Met (M)	12	29	0.39	0.31	0.03	0.01
Gln (Q)	250	34	7.4	3.31	0.01	0.10
Ser (S)	38	50	0.75	4.12	0.11	0.08
Thr (T)	16	41	0.39	0.693	0.04	0.02
Trp (W)	64	25	2.5	0.22	0.0035	0.0087
Tyr (Y)	5.8	68	0.08	0.148	0.03	0.0022

environment at the binding site for the dUMP phosphate moiety may increase K_m^{dUMP}s.

K_ms for mTHF among the 12 mutants varied by less than 12-fold. The K_m^{mTHF} for P196T was the most affected (8-fold) followed by those for P196I, P196V, P196D and P169N, while the K_m^{mTHF}s for P196G, P196K and P196F were approximately the same as the wild-type TS value. The small changes in K_m^{mTHF} for the Pro196 mutants indicate first that the substituted side chains do not impinge on the cofactor-binding site, a result that is verified by modeling. Secondly, the mutations do not prevent the closure of the enzyme active site that is triggered by, and is essential for, tight cofactor binding (Stroud and Finer-Moore, 2003) (Figure 1). However, for the three mutants with the highest K_m^{mTHF}s, which are all branched at their β-carbons, the cofactor-triggered conformational change may be perturbed due to steric crowding in the closed enzyme.

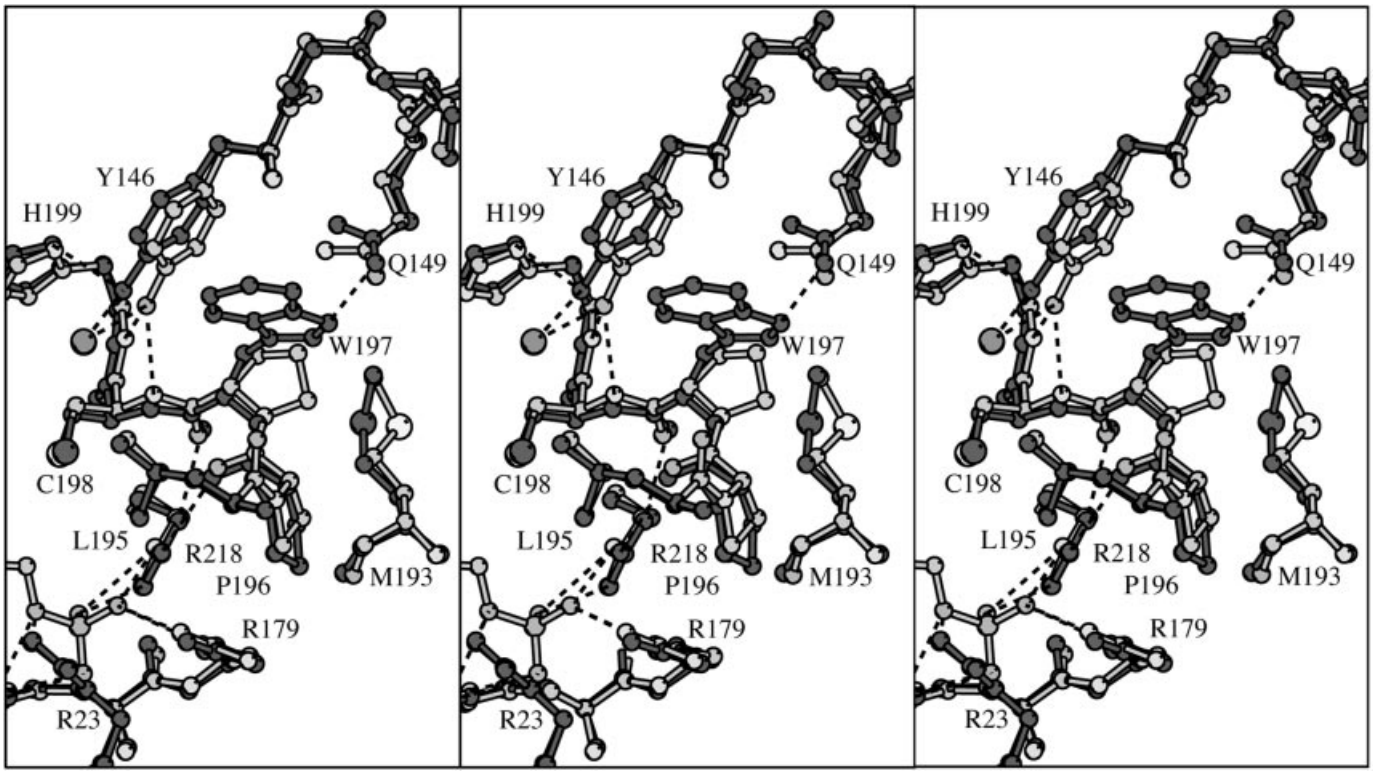

Fig. 2. Stereo plot (Kraulis, 1991) of the X-ray crystal structure of LcTS-dUMP, in light gray, overlapped with a model of LcTS P197W-dUMP after simulated annealing refinement, in dark gray. Hydrogen bonds are shown with dashed lines. In the model, Tyr-146 makes a new hydrogen bond to His-199 Nδ1, Trp-197 Nε1 donates a hydrogen bond to Gln-149 Oε1, and Cys-198 has shifted ~0.5 Å into the active site.

For example, the Ile side chain is 3.3 Å from the guanidinium group of Arg179′(127′) in the model of P196I based on the EcTS·dUMP·CB3717 structure. The rigid structure of proline might be expected to facilitate the switch to a closed enzyme conformation. However, the fact that three of the mutants had K_m^{mTHF}s approximately equal to the wild-type value suggests that the geometric constraints of the Pro196 side chain are incidental to this conformational change.

The K_m^{dUMP}/K_m^{mTHF} ratio for a mutant, when compared with the wild-type ratio, indicates whether the mutation affects the binding of one ligand more than the other. Several Pro196 mutants (Arg, Gly, Asn, Asp, His, Thr) had a greater impact on dUMP binding than on mTHF binding; only P196I showed an increased effect on mTHF binding. This is not surprising since Pro196 forms part of the substrate-binding cavity and does not directly contact the cofactor. Interestingly, for six of the mutants, K_m values of both substrates increased by about the same amount, reflecting the interrelation between dUMP and cofactor binding in TS. dUMP binds before mTHF and makes up part of its binding surface, thus perturbation of dUMP binding, indicated by an elevated K_m^{dUMP}, frequently propagates to cofactor binding, leading to an increase in K_m^{mTHF} (Finer-Moore *et al*., 1998).

For all 12 mutants, k_{cat} decreased with respect to wild-type TS and k_{cat} values span a range of ~30-fold. Paradoxically, P196D, P196N and P196T, which show some of the largest increases in K_m^{dUMP}, have the highest k_{cat}s, while the three mutants with the lowest values of k_{cat}, P196G, P196I, and P196K, have values of K_m^{dUMP} close to that of wild-type TS. Seven mutants have almost the same k_{cat}/K_m^{dUMP} values (in the 0.03–0.04 range) even though their K_ms and k_{cat}s vary widely (by up to 15-fold). Thus, in general, the sensitivities of substrate binding and reaction rates, respectively, to Pro196 mutations are inversely related.

Pro197. Small hydrophobic side chains at this site support wild-type K_m values, but introduce small changes to the protein that are reflected in reductions of more than 10-fold in k_{cat}. Kinetic parameters were measured for 12 mutants of Pro197 that complemented Thy⁻ *E.coli* (Table III). P197A, P197C and P197S had the highest k_{cat}/K_m values, indicating a preference for small, hydrophobic side chains at position 197 in the sequence.

Two of the mutants, P197A and P197C, had K_ms for dUMP and mTHF that were not significantly different from the wild-type values. This is consistent with the fact that the Pro197 side chain does not contact dUMP or mTHF in TS complexes and further shows that the proline geometry is not essential for orienting ligand-binding residues. In several instances, substitutions of hydrogen-bonding residues for Pro197 significantly increased K_m^{dUMP}. These residues may have introduced hydrogen-bond rearrangements or side chain reorientations at the dUMP-binding site via Tyr146. Tyr146 forms an interface between residue 197 and a hydrogen bond network with the pyrimidine ring of dUMP.

Moderately large, even hydrophilic, residues are tolerated at the Pro197 site and can be fit into the site by minor adjustments to surrounding side chains. P197Q had the second highest k_{cat}/K_m^{mTHF} next to P197A, although its K_m^{dUMP} was nearly 100-fold higher than the wild-type value. Therefore, even though the Gln side chain must alter the structure of the binding cavity in a way that interferes with dUMP binding, the productive

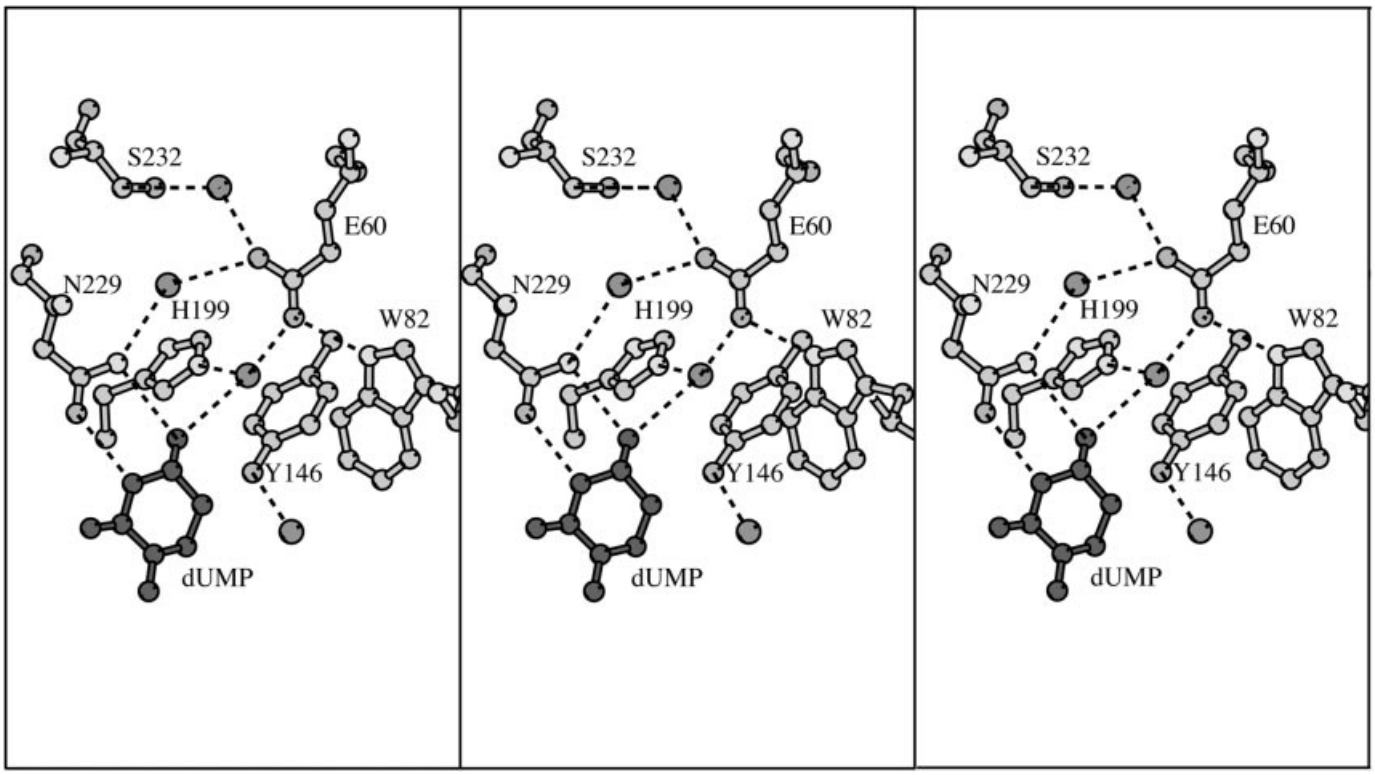

Fig. 3. Stereo plot of the water-mediated hydrogen bond network above the pyrimidine ring of dUMP (shown in dark gray) in the *E.coli* ternary complex with dUMP and the cofactor analog CB3717. Hydrogen bonds are shown with dashed lines.

active site structure is apparently restored once both substrate and cofactor are bound.

The bulkiest residues (Arg, Tyr, Phe, Trp and Leu) led to the largest reductions in k_{cat} and the largest increases in K_m^{mTHF}. This result is not unexpected since Pro197 is an internal residue whose side chain forms part of the protein core. Arginine does not complement Thy$^-$ *E.coli*, while the other mutants had k_{cat}/K_m^{mTHF} values that were 177–1114-fold less than that of the wild-type enzyme. Bulky residues such as Trp could be modeled into both binary and ternary complex structures of TS without major rearrangement of the protein backbone or change in ligand orientation (Figure 2). However, side chains in the neighborhood of the substitution were very close-packed, suggesting an increase in free energy of the productive binary or ternary complex.

Discussion

We have found that the highly conserved residues flanking the catalytic nucleophile do not have essential chemical roles in the TS reaction and do not contribute to K_ms for substrate or cofactor. At the same time, all mutants of these residues that we have characterized have shown more than a 10-fold decrease in k_{cat}/K_m^{dUMP} except for the most conservative mutant, P197A. This suggests that each of the residues contributes to the structural mechanism of TS, in which conformational dynamics is used to orient substrate and cofactor at each reaction step. By using a saturation mutagenesis approach, we have identified the properties of these residues that optimize their role in the structural mechanism.

Hydrogen bonding by the His199 side chain contributes to catalysis

His199 is conserved in all reported TS sequences except for the enzymes from bacteriophage ϕ3T and *B.subtilis*, where the corresponding residue is Val. His199 lies in the active site cavity just above the dUMP binding site. In *L.casei* TS binary complexes, the weakly acidic Cε1 methylene of His199 forms a weak hydrogen bond to dUMP O-4 (Finer-Moore *et al*., 1993). However, in covalent ternary complexes of TS from several species, the space above the dUMP base has closed down via ~0.5 Å shifts of the peptide chain lining the cavity and His199 is in a different conformation where it participates in a hydrogen bond network involving several conserved side chains and water molecules (Matthews *et al*., 1990; Montfort *et al*., 1990; Knighton *et al*., 1994; Anderson *et al*., 2001; Phan *et al*., 2001; Sayre *et al*., 2001) (Figure 3). This hydrogen bond network connects the important catalytic residue Glu60 with dUMP (Sage *et al*., 1996).

Our results show that several His199 mutants with K_ms nearly identical to wild-type values show significant reductions in k_{cat} (Table I), demonstrating a clear though non-essential role for His199 in catalysis. Dev *et al*. observed similar results for the mutants of *E.coli* TS, and further showed that mutation of His199 specifically slowed the rate of ternary complex formation, k_{on} (Dev *et al*., 1989). Effects of the H199G mutation on k_{cat} and k_{on} were more pronounced at a higher pH, leading Dev *et al*. to postulate that His199 altered the pK_a of a catalytically important residue (Dev *et al*., 1989). LaPat-Polasko *et al*. (LaPat-Polasko *et al*., 1990) mutated the same conserved histidine in bacteriophage T4 TS to a valine, and

also saw that while K_ms for dUMP and mTHF were very similar to wild-type values, k_{cat} was only 23% that of the wild-type enzyme.

TS-A from *B.subtilis*, which has a valine at the position analogous to His199, is even more active than either *E.coli* or *L.casei* TS. However, in this enzyme, covariant changes in neighboring residues preserve the main features of the His199–coordinated hydrogen bond network seen in other TS species (Fox *et al*., 1999). Thus, it is the hydrogen bond network that is important for enzyme activity and His199 contributes to catalysis through its structural role in maintaining this network.

The hydrogen bond network could enhance k_{on} in at least two ways. First, the water-mediated hydrogen bonds dUMP O-4 makes with Glu60 Oε2 and His199 Nε2 may facilitate hydrogen transfer to O-4 of dUMP during the Michael addition of Cys198 Sγ to C-6 of dUMP (Huang and Santi, 1997). Secondly, the hydrogen bond network could help stabilize the closed form of the enzyme seen in analogs of the covalent ternary complex intermediate (Figure 3). The closed enzyme conformation appears to be required for the Michael addition step to occur. The second mechanism seems more likely, since neither mutations of His199 nor of Glu60 impair dehalogenation of 5-bromo-dUMP (Huang and Santi, 1997). The dehalogenation reaction, which is cofactor independent, mimics early steps in catalysis that involve proton transfer to O-4 of dUMP.

Pro196 and Pro197

Pro196 and Pro197 are highly conserved but not invariant residues that immediately precede the catalytic cysteine, which lies at the N-terminus of β-strand IV of the central β-sheet. Pro197 is conserved in almost all reported TS sequences, but is replaced with Phe in the highly divergent TS from *Methanococcus jannaschii* (Aurora and Rose, 1998). Pro196 is replaced with Ala in *E.coli* and *B.subtilis* B TS, with Thr in ϕ3T and *B.subtilis* A TS, and with Leu in *L.lactis* TS.

The Pro–Pro sequence is rare in proteins and its backbone conformation angles are confined to $\phi \approx -60°$ and $\psi \approx 95–190°$ (Schimmel and Flory, 1967). The Pro196, Pro197 sequence is in a conformationally sensitive region of the active site cavity. It is preceded by an important cofactor binding residue, Leu-195, and followed by the catalytic cysteine. The allowed (ϕ, ψ) angles of the Pro–Pro sequence orient the proline carbonyl groups to accept hydrogen bonds from Arg218 Nη1 and Nε, respectively. These hydrogen bonds, in turn, orient the Arg218 guanidinium group to make hydrogen bonds of ideal geometry to the dUMP phosphate group. Arg218 not only binds to the phosphate moiety of dUMP, but also is postulated to activate the catalytic nucleophile, Cys198 (Hardy *et al*., 1987), and all mutants of Arg218 are inactive (Kawase *et al*., 2000). Thus, Pro196 and Pro197 may have an important structural role in shaping the substrate-binding cavity and orienting three critical ligand-binding residues.

Besides restricting the geometry of catalytic residues, the Pro–Pro sequence may facilitate the conserved conformational changes used by TS to orient its reactants during the reaction. The rigid conformation of the Pro–Pro sequence reduces the conformational degrees of freedom of the protein, which may help guide the protein along conserved paths between conformational states. The conformational transition between the open (apo-enzyme) conformation and the closed (ternary complex) conformation is a key part of the cofactor-binding mechanism. The segment containing Pro196 and Pro197 shifts only a small amount (~0.3 Å) during this transition but this shift is significant since it allows Leu195 to make hydrophobic contacts with the cofactor (Figure 1). However, the fact that K_m^{mTHF} values are close to wild-type values for most of the Pro196 and Pro197 mutants argues that the rigid geometry of proline is incidental to the conformational change. A more sensitive test of the importance of proline geometry for the conformational change would be the changes in rates of ternary complex formation, k_{on}, in the mutants, which we did not determine.

The kinetic parameters for the most conservative mutations of Pro196 and Pro197 indicate the degrees to which the prolines at these two sites uniquely contribute to substrate binding and catalysis in TS. These contributions are surprisingly small, considering the conserved nature of these residues. The most active mutant, P197A, showed only a 5-fold decrease in k_{cat}/K_m^{dUMP} and a 1.5-fold decrease in k_{cat}/K_m^{mTHF}. We did not isolate P196A, but P196F, the most active of the Pro196 variants, showed a 16-fold decrease in k_{cat}/K_m^{dUMP} and a 7.8-fold decrease in k_{cat}/K_m^{mTHF}. Part of the reason why the kinetics of TS are relatively insensitive to mutations of the individual prolines may be that the prolines work together to optimally restrict the geometry of the chain, and when one proline is mutated, the other still maintains the correct backbone conformation. For example, the most favorable (ϕ, ψ) angles for any residue preceding a proline are similar to the allowed (ϕ, ψ) angles for a Pro–Pro dipeptide (Schimmel and Flory, 1968), so mutation of Pro196 alone does not alter the conformational restraints very much. The effect on enzyme kinetics of making a (Pro196, Pro197) double mutant would likely be greater than the sum of the effects of mutating each proline by itself.

The rigid geometry of Pro may play a larger role in catalysis at higher temperatures. We identified several temperature-sensitive Pro196 and Pro197 mutants, most involving substitutions of bulky residues. LaPat-Polasko *et al*. found that even the conservative substitution of the smaller residue Ala for Pro197(155) in bacteriophage T4 TS, while not altering k_{cat}, made the enzyme dramatically less stable to heat and resulted in a blue shift of the fluorescent spectrum, which indicated a structural change in the vicinity of a Trp (LaPat-Polasko *et al*., 1990).

As expected for residues with a structural role, the most active substitutions for Pro196 and Pro197 were residues that were sterically compatible with the sites. For the internal residue, Pro197, k_{cat}/K_ms were inversely correlated with size of the substituted side chain. However, substitutions of even bulky amino acids such as Trp could be modeled at Pro197 without major structural rearrangements. The side chain packing in models of these mutants was highly constrained, with several more van der Waals contacts to the substituted side chain than in wild-type TS. Thus there is an entropic cost to maintaining the productive fold of the enzyme when a bulky residue replaces Pro196 or Pro197, and several of these mutants were temperature sensitive. In an enzyme such as TS that is highly dependent on its structure and dynamics to align its substrate and cofactor for catalysis, such changes to the energetic landscape of the folded protein could affect enzyme specificity and be responsible for some of the drop in k_{cat}/K_m^{dUMP} seen for these mutants.

Even minor perturbations to the orientations of ligand binding or catalytic residues in the vicinity of each site, particularly to the catalytic nucleophile, Cys198, may also

decrease k_{cat}/K_m^{dUMP} for the proline mutants. Catalytic activity may be especially sensitive to changes in the position or hydrogen bonding partners of Tyr146 (such as those seen in Figure 2) since this residue is essential for abstracting a proton from a steady-state intermediate in the enzyme reaction and also appears to have some, perhaps indirect, role in cofactor binding (Liu *et al*., 1999). Besides their small size, another important attribute of Pro196 and Pro197 is that they are chemically neutral. Introducing hydrogen-bonding capability at either of these positions in many cases reduced enzyme specificity by increasing K_m^{dUMP}. Presumably the hydrogen-bonding side chains altered the detailed structure or electrostatics of the dUMP-binding site.

Conclusion

Kinetic analysis of His199 mutants strengthens the argument that the water-mediated hydrogen bonds dUMP O4 makes with Glu60 and His199 contribute to catalysis. They may assist hydrogen transfer to O-4 of dUMP during the Michael addition of C198 Sγ to C-6 of dUMP, or they may help stabilize the closed form of the enzyme in covalent ternary complexes.

Pro196 and Pro197 mutants show a general reduction of specificity, k_{cat}/K_m, probably due either to structural perturbations of the closest neighboring residues, such as Cys198, Tyr146 and Arg218, which are directly involved in substrate binding and catalysis, or to destabilization of the conformations of reaction intermediates, which are required for productive alignment of substrate and cofactor. The Pro side chain is unique in being able to constrain the protein backbone of the loop containing Cys198 in an active conformation without interfering sterically or through hydrogen bonding with the adjacent residues that have direct roles in binding or catalysis. The unique role of proline in constraining the geometry of the active site loop makes a minor contribution to catalysis at room temperature, but becomes more important at higher temperatures. Since each proline can partially compensate for mutation of the other, it is likely that double mutants would have a much greater impact on function than mutations of each proline individually.

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