Contributions of Orientation and Hydrogen Bonding to Catalysis in Asn229 Mutants of Thymidylate Synthase

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We have determined structures of binary and ternary complexes of five Asn229 variants of thymidylate synthase (TS) and related their structures to the kinetic constants measured previously. Asn229 forms two hydrogen bonds to the pyrimidine ring of the substrate 2′-deoxyuridine-5′-monophosphate (dUMP). These hydrogen bonds constrain the orientation of dUMP in binary complexes with dUMP, and in ternary complexes with dUMP and the TS cofactor, 5,10-methylene-5,6,7,8-tetrahydrofolate. In N229 mutants, where these hydrogen bonds cannot be made, dUMP binds in a misoriented or more disordered fashion. Most N229 mutants exhibit no activity for the dehalogenation of 5-bromo-dUMP, which requires correct orientation of dUMP against Cys198. Since bound dUMP forms the binding surface against which the pterin ring of cofactor binds, misorientation of dUMP results in higher kcat values for cofactor. At the same time, binding of the cofactor aids in ordering and positioning dUMP for catalysis. Hydrophobic mutants, such as N229I, favor an arrangement of solvent molecules and side-chains around the ligands similar to that in a proposed transition state for ternary complex formation in wild-type TS, and kcat values are similar to the wild-type value. Smaller, more hydrophilic mutants favor arrangements of the solvent and side-chains surrounding the ligands that do not resemble the proposed transition state. These changes correspond to decreases in kcat of up to 2000-fold, with only modest increases in Km or kcat. These results are consistent with the proposal that the hydrogen-bonding network between water, dUMP and side-chains in the active-site cavity contributes to catalysis in TS. Asn229 has the unique ability to maintain this critical network, without sterically interfering with dUMP binding.

 Keywords: 2′-deoxyuridine-5′-monophosphate; 5,10-methylene tetrahydrofolate; X-ray crystallography; methylase; kinetics

Abbreviations used: TS, thymidylate synthase; dUMP, 2′-deoxyuridine 5′-monophosphate; CB3717, 10-propargyl-5,8-dideazafolate; CH₂H₂folate, 5,10-methylene tetrahydrofolate; Hfolate, tetrahydrofolate; BrdUMP, 5-bromo-dUMP; PLP, pyridoxal 5′-phosphate; F₁, measured structure factor amplitude; F₁, calculated structure factor amplitude; (F₁ − F₁)αcalc map, electron density map calculated with coefficients (1F₁ − 1F₁) and phases calculated from the coordinates; (2F₁ − F₁)αcalc map, electron density map calculated with coefficients (2F₁ − 1F₁) and phases calculated from the coordinates; PABA, p-aminobenzoic acid.

Introduction

The dUMP methylase thymidylate synthase (TS) binds dUMP and a cofactor, CH₂H₂folate, orients them within a cavernous active-site, and facilitates multiple chemical steps resulting in transfer of a one-carbon unit from cofactor to the pyrimidine base of dUMP. Asn229 is the only side-chain of the protein that forms hydrogen bonds to the base, yet it does not contribute significantly to the free energy of dUMP binding (Liu & Santi, 1993b). However, Asn229 excludes dCMP from the dUMP binding site (Liu & Santi, 1993b), and is conserved
in all known TS sequences, presumably because of its role in determining substrate specificity (Hardy & Nalivka, 1992; Liu & Santi, 1993b). Asn229 is not essential for enzyme activity: six Asn229 mutants in Lactobacillus casei TS have sufficient activity (greater than 1% of wild-type) to support cell growth on thymine-deficient medium (Climie et al., 1992; Liu & Santi, 1993a). However, all mutations of Asn229 result in at least a 50-fold decrease in $k_{cat}/K_m$, suggesting that Asn229 contributes to catalysis (Liu & Santi, 1993a,b).

One way that Asn229 may contribute to catalysis is through orientation of dUMP. In the TS reaction, dUMP binds first and becomes the binding surface against which the pterin ring of the cofactor binds. Thus it must be positioned for both nucleophilic attack at C-6 by the catalytic cysteine residue, and for optimal alignment of CH$_2$H$_6$folate for transfer of the cofactor methylene group to C-5. For most of the Asn229 mutants, decreases in $k_{cat}$ are qualitatively correlated with instability of the TS-FdUMP-CH$_2$H$_6$folate complex, an analog of the ternary complex intermediate (II in Figure 1) in the reaction (Liu & Santi, 1993a). Most of the N229 mutants also fail to catalyze nucleophile-catalyzed dehalogenation of 5-bromo-dUMP (Liu & Santi, 1993a), a reaction used to assay nucleophilic attack of dUMP C-6 by the catalytic cysteine residue in the absence of cofactor (Carreras & Santi, 1995). These results suggest that catalysis in N229 mutants is partly impaired by misorientation of dUMP prior to cofactor binding. To test this hypothesis, we have determined the structures of five dUMP-bound TS N229 mutants, and related the degree of misorientation or disorder of substrate to $K_m$ (dUMP), $K_m$(CH$_2$H$_6$folate) and $k_{cat}$ values.

A second way in which Asn229 could contribute to catalysis is through hydrogen-bond stabilization of reaction intermediates. Asn229 is one of several side-chains and ordered water molecules surrounding C-5 of dUMP. These side-chains and water molecules form a hydrogen-bond network that has been proposed to assist in general base catalysis during elimination of H$_2$folate from the ternary complex (Carreras & Santi, 1995: Figure 1). As in the elimination of H$_2$folate, nucleophilic attack of C-6 of dUMP by Cys198 produces an enolate intermediate (Figure 1). Thus the hydrogen-bond network may similarly facilitate this first chemical step in the TS reaction.

We sought to establish a role for Asn229 in general base catalysis by determining the crystal structures of ternary complexes of three L. casei TS N229 mutants, N229C, N229S and N229A. The structures represent points on the reaction coordinate immediately preceding nucleophilic attack at C-6 of dUMP, and therefore are appropriate for assessing structural changes that might have an impact on this step. For N229S, the $K_m$ values for cofactor and substrate are nearly identical with wild-type values, suggesting that misorientation of ligands is not a factor in the 300-fold decrease in $k_{cat}$ for this variant. In this case, our structural studies would identify a contribution of Asn229 to general base catalysis independent from its role in orientation of ligands.

### Results

Statistics for refinements of the binary and ternary complexes of the N229 mutants are given in Tables 1 and 2. As is typical of single-site mutants, the structures of the N229 variants differ from the structure of wild-type TS only in the region immediately surrounding (within ~7 Å radius of) the mutated residue (Perry et al., 1990). This region includes Glu60 and His199, both implicated in catalysis (Dev et al., 1989; Hardy et al., 1995; Huang & Sanfi, 1994; Zapf et al., 1993), and several conserved, ordered water molecules, which have been postulated to have specific roles in the mechanism (Fauman et al., 1994; Finer-Moore et al., 1990; Hardy et al., 1995; Huang & Sanfi, 1994; Matthews et al., 1990: Tables 3 and 4). The changes in water and protein structure around residue 229, and changes in ligand orientation for the five mutants N229I, N229C, N229S, N229A and N229G (below) relate directly to their differential effect on $k_{cat}$ and $K_m$, and so separate their roles in binding and different enzyme-catalyzed steps.

We reference the mutant structures to analogs of wild-type reaction intermediates: the wild-type

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**Figure 1.** Proposed mechanism for thymidylate synthase. In this scheme, AH represents a general base, perhaps the ordered water molecule Wat1.
Table 1. Refinement statistics, binary complexes with dUMP

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L. casei TS binary complex with dUMP (Finer-Moore et al., 1993: Figure 2), the non-covalent L. casei TS ternary complex with dUMP and the cofactor analog CB3717 (Birdsall et al., 1996), and the covalent ternary complex between Escherichia coli TS, dUMP and CB3717, where Cys3198 S′ is covalently attached to C-6 of the dUMP base (Montfort et al., 1990). Co-crystallization of E. coli TS with dUMP and CB3717 or other folate analogs generally results in a covalent ternary complex where the protein active-site cavity has closed down to sequester the ligands from bulk solvent. Water molecules remaining in the active-site of E. coli TS ternary complexes are highly conserved and are referred to as shown in Figure 2. During closure of the active-site cavity, the hydrogen bonding to Wat1 and Wat5 change; these water molecules are considered to be conserved in the binary complexes if they have some of the hydrogen bonds seen in the covalent ternary complexes. In some instances, water molecules were seen within 1 Å of their positions in covalent ternary complexes, yet showed completely different hydrogen bonding from that seen in the latter structures. Co-crystallization of L. casei TS covalent ternary complexes has not been achieved. The L. casei TS covalent ternary complex is presumed to be structurally analogous to the E. coli complex because of the almost complete conservation of residues in the active-site cavities of these two species. Diffusion of cofactor analogs into L. casei TS binary complex crystals yields non-covalent ternary complexes where dUMP and the folate analog are bound similarly, but less tightly, to the E. coli ternary complexes, and the active-site is not completely closed down. If CH$_2$H$_4$folate, rather than an inhibitor, is diffused into the TS-dUMP crystals, methylation of dUMP occurs within the crystals (Birdsall et al., 1996). We interpret this to mean that the L. casei TS diffusion ternary complexes represent structures along the reaction coordinate, immediately preceding nucleophilic attack of C-6 of dUMP by Cys198. In crystals of the L. casei binary and ternary com-

Table 2. Refinement statistics, ternary complexes with dUMP and CB3717

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<td>R_{free} (%)</td>
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<tr>
<td>Avg. B, dUMP</td>
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<tr>
<td>Avg. B, CB3717</td>
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plexes, the molecular 2-fold relating protomers of the dimer is coincident with a crystallographic 2-fold axis. Thus, the dimers seen in the \textit{L. casei} TS crystal structures are necessarily symmetric, with identical protomer structures.

**TS N229I-dUMP: disordered dUMP, high $K_n$ for dUMP, but high $k_{cat}$**

The electron density for dUMP in the crystal structure of TS N229I-dUMP is poorly defined in $(2F_o - F_i)^2$ maps, but is continuous between the phosphate and ribose moieties so does not arise merely from inorganic phosphate and partially ordered water molecules. The density becomes increasingly more diffuse between the phosphate and the pyrimidine ring, indicating thermal disorder. The refined $B$-factors for dUMP are, on average, 64 Å$^2$ compared to 27 Å$^2$ for the protein, consistent with some lack of restraint on dUMP position in this mutant. The conformation of dUMP shown (Figure 3) best fits the pyrimidine to patchy density, but is likely one of an ensemble of conformations bound to the enzyme. However, the pyrimidine is clearly positioned further from Cys198 than in wild-type TS due to contact with Ile229.

The Ile229 side-chain is oriented such that its C$^β$ atom is oriented towards the interior of the protein and its ethyl group (C$^2$-C$^3$) is pointed out into the active-site cavity. Ile229 C$^3$ is ~2.8 Å, less than van der Waals distance, from the wild-type position of the dUMP O-4 binding site, and is likely responsible for positioning the pyrimidine of dUMP further from Cys198. The distance between the nucleophile Cys198S$^\gamma$ and dUMP C-6 is 5 Å.

The imidazole ring of His199 is rotated clockwise 10°, as viewed from His199 C$^\beta$, with respect to wild-type TS, and more compared to other N229 mutants. For example, the N229I-dUMP and N229C-dUMP data sets could be directly compared, since cell constants for the two were identical. The $(F_o - F_i)^2$ map clearly shows the difference in the His199 imidazole ring orientation between these two mutants (Figure 3). In TS N229I the His199 imidazole group is approximately parallel with the plane of Tyr246, as it is in \textit{E. coli} TS.
covalent ternary complexes. In the covalent ternary complexes, His199 (147) N61 hydrogen bonds to Tyr233(181) O6. In both the wild-type L. casei TS and the TS N229I binary complexes, a water molecule bridges this hydrogen bond and the distance between His199 N61 and Tyr233 O6 is 3.2 Å.

The conformation of Glu60 is unchanged with respect to the wild-type enzyme and its carboxyl group is hydrogen bonded to three ordered water molecules, two of which correspond to Wat5 and Wat6 in the E. coli TS ternary complex. Wat1 and Wat7 are also present, although Wat1 is not hydrogen bonded to dUMP because of the shift in the dUMP base with respect to wild-type TS.

**TS N229C: N229 mutant with the highest \( k_{cat}/K_m \)**

**TS N229C-dUMP**

There is no perturbation in dUMP binding in TS N229C. Except for the missing hydrogen bonds between Asn229 and the dUMP base, the contacts between dUMP and the protein are identical with those in wild-type TS. The Cys229 S'-atom is rotated into the protein core rather than into the active-site like the carboxamide group of Asn229 (Figure 3). Most of the active-site water molecules surrounding the pyrimidine base seen in wild-type L. casei TS-dUMP are conserved, including Wat1 and Wat7, but Wat5 and Wat6 are absent. The imidazole group of His199 is rotated with respect to its position in wild-type L. casei TS and the more active mutant TS N229I (Figure 3). The distance between His199 N61 and Tyr233 O6 is 4.3 Å, too long for even a weak hydrogen bond.

**TS N229C-dUMP-CB3717: dUMP misoriented, water structure conserved**

Only low concentrations of the cofactor analog CB3717 could be diffused into crystals of TS N229C-dUMP without destroying them. Since \( K_m \) for the cofactor is about tenfold higher for TS N229C than for wild-type TS, CB3717 presumably also binds less well in TS N229C. Therefore, it was difficult to obtain crystals of L. casei TS N229C-dUMP-CB3717 with a fully occupied CB3717 site. In only one of three crystals for which data were collected did difference electron density maps indicate the presence of bound CB3717, and even for this crystal structure the density for the cofactor analog was not continuous (Figure 4). Although the discontinuous CB3717 density was not very convincing, other evidence suggested that the crystal structure was a ternary complex. First, as is typically seen when cofactor analogs bind to TS, the C-terminal four residues had moved into the active-site and the carboxyl terminus was hydrogen bonded to Arg23. Second, the position of dUMP had changed significantly from the binding site in the binary complex of the mutant.

The carboxyl terminus, Arg23 and CB3717 all have high temperature factors (Table 2), thus they could not be fit to density with much precision. However, as best fit and refined to the density, the CB3717 is close to the binding site for CB3717 seen in covalent ternary complexes of E. coli TS and makes the conserved hydrogen bond between 3-NH in the quinazoline ring and Asn221 O2 of. There is no density for the PABA moiety of CB3717, but clear density for the Glu moiety attached to the PABA ring in (2Fo − Fc) and dUMP-CB3717 maps; this is typical of other L. casei TS ternary complex structures determined by diffusing cofactor into binary complex crystals (Birdsall et al., 1996).

dUMP, which in TS N229C-dUMP was bound in the same fashion as in wild-type L. casei TS, lies in a slightly different conformation in the ternary complex, with the pyrimidine base pivoted to point O-4 towards the space occupied by the carboxamide group of Asn229 in wild-type TS. The temperature factors for dUMP are high, about 50 Å2 on average, and the density for the pyrimidine ring is not well resolved (Figure 4), but the tilt of the ring is unambiguous and the positions of O-4 and C-4 are significantly different, 1.9 (5) Å and 1.1(5) Å, respectively, from O-4 and C-4 in wild-type TS (Figure 4). The distance between dUMP C-6 and Cys198 S' is 3.3 Å, slightly longer than in the wild-type L. casei TS ternary complex. Part of the reason dUMP is slightly misoriented may be that the active-site has not completely closed down...
Figure 3. MOLSCRIPT (Kraulis, 1991) plot of TS N229C-dUMP (light bonds) and TS N229I-dUMP (black bonds) superposed on the \((F_{o} - F_{c})\) electron density map, where \(F_{o}\) is an amplitude from the N229C-dUMP data set and \(F_{c}\) is an amplitude from the N229I-dUMP data set. The contour level is ±3σ. Positive contours are drawn with continuous lines and negative contours are drawn with broken lines. The most prominent peaks indicate the difference in side-chain at residue 229, shifts in the dUMP position, and a rotation of the imidazole ring of His199.

Figure 4. MOLSCRIPT (Kraulis, 1991) plot of TS N229C-dUMP-CB3717 superposed on the \((F_{o} - F_{c})\) electron density map for TS N229C-dUMP-CB3717, calculated after refinement of the structure without ligands, and contoured at 2.5σ. Plotted in light bonds is dUMP as bound in wild-type TS-dUMP.
around the ligands. Complete closure of the active-site may induce the dUMP to adopt the conformation seen in the wild-type TS binary and ternary complexes.

The His199 Cα-Cβ-Cγ-Cδ2 torsion angle, which defines the orientation of the imidazole ring, is −43°, closer to the angle of −37° seen in wild-type *L. casei* TS-dUMP than it was in the N229C-dUMP structure. His199 Nδ2 is hydrogen bonded to O-4 of dUMP. Water structure is generally conserved: Wat1, Wat5, Wat6 and Wat7 are all within 1 Å of their positions in the *E. coli* TS ternary complex. Wat1, however, makes no hydrogen bond to O-4 of dUMP, Glu60 or His199, so is conserved only in location, not in its interactions.

**TS N229S: small effect on dUMP or CH$_2$H$_4$folate $K_m$, 300-fold decrease in $k_{cat}$**

**TS N229S-dUMP**

dUMP in TS N229S-dUMP is well ordered and bound in the orientation identical with that in wild-type TS. An $(F_1- \bar{F}_2)_{\text{calc}}$ difference map (Figure 5) between wild-type and TS N229S shows that, first, the pyrimidine base is moved away from the active-site cysteine residue by 0.8 (4) Å. Secondly, changes to the protein are limited to His199, Ser229 and Ser232. The side-chain of Ser229 is in a position analogous to that of Asn229 of wild-type TS, that is, when wild-type and mutant protein structures are overlapped, O' of Ser229 is in the same place as C' of Asn229. In contrast, S' of Cys229 in TS N229C has been rotated out of the active-site into a small cavity inside the protein. The His199 imidazole group has rotated 18° relative to wild-type TS, and His199 and Ser229 have moved slightly closer together to hydrogen bond. The difference in position of His199 from wild-type TS is less than the error in coordinates of the structures, but is detectable by the more sensitive comparison of the $(F_1- \bar{F}_2)_{\text{calc}}$ difference map.

There is no hydrogen bond to 4-O or 3-NH of dUMP, either by protein or ordered water molecules. Ordered water molecules are located within 1 Å of the positions of Wat1, Wat6 and Wat7 of wild-type *L. casei* TS-dUMP, and within 1.5 Å of the position of Wat5, but Wat1 and Wat5 make different hydrogen bonds to the protein. In particular, Wat1, which mediates a hydrogen bond between catalytically important Glu60 and 4-O of dUMP in *E. coli* TS covalent ternary complexes, is ~4 Å from each of these groups in TS N229S.

![Figure 5. MOLSCRIPT (Kraulis, 1991) plot of wild-type TS-dUMP (light bonds) and TS N229S-dUMP (black bonds) superposed on the $(F_1- \bar{F}_2)_{\text{calc}}$ difference map, where $F_1$ is an amplitude from the wild-type TS-dUMP data set and $\bar{F}_2$ is an amplitude from the TS N229S-dUMP data set. The contour level is ±3σ. Positive contours are drawn with continuous lines and negative contours are drawn with broken lines. The most prominent peaks indicate the difference in side-chain at residue 229, a shift in the dUMP pyrimidine away from Cys198 in the mutant, and shifts of Ser229 and His199 toward each other so they can form a hydrogen bond.](image-url)
TS N229S-dUMP-CB3717: well-ordered ligands, but no covalent bond to dUMP

Both (2F_o – F_c)\textsubscript{calc} and (F_o – F_c)\textsubscript{calc} maps for crystals where CB3717 has been diffused into binary complexes of TS N229 S-dUMP show clear density for CB3717 (Figure 6). The quinazoline ring of CB3717 is parallel with, and stacked on top of, the pyrimidine ring of dUMP, as seen in E. coli TS covalent ternary complexes. As in the E. coli TS ternary complexes, the quinazoline ring makes one hydrogen bond directly to the protein: 3-NH–Asp221 O\textsubscript{52} = 2.7 Å. Density for two water molecules that mediate contacts between the quinazoline ring and the protein in the E. coli complex is not visible: these two water molecules normally become ordered participants in ligand hydrogen bonding only after full closure of the active-site. In both E. coli and L. casei TS, cofactor binding is normally accompanied by a \( \sim 5 \) Å movement of the carboxy terminus into the active-site, where it hydrogen bonds to Arg23. The carboxy terminus of TS N229S shows this characteristic conformation but is not hydrogen bonded to the guanidinium group of Arg23.

Presumably because of the influence of cofactor, the dUMP is positioned closer to Cys198 than in TS N229S-dUMP but is translated downward, that is away from Ser229, by \( \sim 1.8 \) (7) Å relative to the N229S binary complex. The distance between 6-C of dUMP and Cys198 S\textsubscript{3} is 3.3 Å.

Wat1, which mediates a hydrogen bond between Glu60 and 4-O of dUMP in covalent ternary complexes, is not present in the TS N229 S ternary complex. 4-O is hydrogen bonded to Wat7, the conserved water molecule that is hydrogen bonded to Tyr146 O\textsubscript{58} in almost all ternary and binary complexes. As in the TS N229S binary complex, the His199 imidazole group is rotated relative to wild-type TS such that His199 N\textsubscript{10} forms an ideal hydrogen bond to Ser229 (2.7 Å) and His199 N\textsubscript{11} is out of hydrogen-bonding range of Tyr233 O\textsubscript{59} (3.6 Å).

TS N229A: no measurable activity

TS N229A-dUMP

In TS N229A-dUMP, dUMP is oriented very much like dUMP in TS N229C-dUMP-CB3717, with the base pivoted to move 4-O 2.0 (5) Å into the space occupied by the Asn229 side-chain in wild-type TS (Figure 7). C-6 has moved 0.9 (5) Å and is 3.7 Å from Cys198 S\textsubscript{3}. 4-O is hydrogen bonded to an ordered water molecule, but this water molecule is not Wat1, which normally hydrogen bonds to dUMP 4-O. Although Wat1 is not in the structure, Wat5, Wat6 and Wat7 are conserved. The His199 imidazole group is rotated \( \sim 15^\circ \) such that N\textsubscript{2} can also hydrogen bond to 4-O, and N\textsubscript{61} is over 4 Å from Tyr233 O\textsubscript{57}.

TS N229A-dUMP-CB3717: misoriented dUMP with no contact to ordered water molecules

When CB3717 is diffused into crystals of TS N229A-dUMP, the CB3717 binds in the same site and nearly the same conformation as in cocrystals of E. coli TS-dUMP-CB3717. Temperature factors for the CB3717 are 54 Å\textsuperscript{2} on average. As a result, electron density is not well defined, but it is unambiguously present in (F_o – F_c)\textsubscript{calc} maps as well as in direct difference maps calculated between the TS N229A binary and ternary complex data sets (Figure 8). The quinazoline ring makes the conserved hydrogen bond between 3-NH and Asp221 O\textsubscript{52}. As usual for L. casei TS “diffusion” ternary complexes, the two ordered water molecules mediating other contacts between the quinazoline ring and the protein in E. coli ternary complexes are not present. The propargyl group, present only on CB3717 and not on the cofactor CH\textsubscript{2}H\textsubscript{4}folate, is in a position different from that in the E. coli complex. It has displaced the three water molecules solvating Glu60 in TS N229A-dUMP,

---

Figure 6. MOLSCRIPT (Kraulis, 1991) plot of TS N229S-dUMP-CB3717 superposed on the (F_o – F_c)\textsubscript{calc} omit map for TS N229S-dUMP-CB3717, calculated after refinement of the structure with ligands removed, and contoured at 2.5\( \sigma \).
including the water molecule hydrogen bonded to 4-O in the binary complex and is itself hydrogen bonded to Glu60.

The C-terminal four residues have moved into the active-site in the fashion characteristic of cofactor binding. However, the average atomic B-factor for these residues is 56 Å² compared to 20 Å² for the entire protein, thus they are not well ordered. As best fit to density, the C terminus hydrogen bonds to Arg23 N³¹, as in the *E. coli* TS ternary complex.

As shown in the direct difference map (Figure 8b), dUMP is better ordered and translated 0.6 (3) Å closer to Cys198 in the ternary complex relative to TS N229A-dUMP. The conformation of dUMP is the same as in the TS N229A-dUMP structure, with 4-O pivoted into the space formerly occupied by the Asn229 carboxamide group. The 6-C to Cys198 distance is only 3.1 Å, but a covalently bound dUMP cannot be fit to the density. In contrast to the TS N229C-dUMP-CB3717 structure, the dUMP is very well ordered, with an average atomic B-factor of 18 Å², compared to the overall average B-factor of 20 Å² for the protein.

His199 lies in the same conformation as in the TS N229A-dUMP structure, with His199 Nε² making a hydrogen bond to dUMP 4-O. This hydrogen bond is the only hydrogen bond to either dUMP 4-O or 3-NH in the structure. An ordered water molecule bridges His199 N6¹ and Tyr233 O⁹, while another water is hydrogen bonded to His199 Cε². The latter water molecule is in the same location as Wat1, although its hydrogen-bonding interactions are completely different from those in the *E. coli* ternary complex.

**TS N229G-dUMP: the only N229 mutant that debrominates 5-bromo-dUMP**

As in the TS N229A complexes and in TS N229C ternary complex, dUMP is oriented such that 4-O impinges on space occupied by the Asn229 carboxamide group in wild-type TS. In addition, a new ordered water molecule binds in the Asn229 side-chain space. This new ordered water molecule hydrogen bonds to 4-O of dUMP, Glu217 O²³, and His199 Nε². The direct difference map between the TS N229A-dUMP and TS N229G-dUMP data sets (Figure 9) shows only a few significant differences between the two structures. First, the major difference is the presence of the new ordered water molecule in place of Ala229 C⁵⁰. Other water molecules in the two active-sites are the same. Second, there
is a 30° rotation of the imidazole ring of His199 in TS N229G with respect to TS N229A. Finally, the dUMP is less well ordered in TS N229G, with an average atomic B-factor of 45 Å² compared to the average B-factor of 23 Å² for the protein, and it is closer to Cys198. The distance between dUMP 6-C and Cys198 S' is 3.2 Å in TS N229G, 0.5(2) Å closer than the distance in TS N229A.

**Discussion**

**Perturbations of dUMP binding and hydrogen bonding in binary complexes explain diminished dehalogenation activity**

Normally, dUMP orientation is not greatly affected by the cofactor, rather it is determined by
numerous hydrogen bonds to the protein, including two hydrogen bonds between dUMP and Asn229 (3-NH to N229 Oβ1 and N229 Nε2 to O4; Finer-Moore et al., 1993). In the Asn229 variants, though, two of three hydrogen bonds to the dUMP pyrimidine are missing, dUMP binding is thus less constrained and potentially more influenced by cofactor binding.

We have seen that the orientation of dUMP is different in ternary complexes of Asn229 variants compared to binary complexes. For example, C-6 of dUMP is 3.7 Å from Cys198 Sγ in TS N229A-dUMP, but moves to 3.1 Å from Cys198 Sγ in TS N229A-dUMP-CB3717. The shift toward the catalytic cysteine residue can be measured from difference density peaks in the $(F_c - F_o)\delta_{\text{calc}}$ map (Figure 8b). Although we did not determine the ternary complex structure for TS N229I, which has a very elevated $K_m$ for CH$_2$H$_4$folate, it is clear that the pyrimidine must move much closer to Cys198 in the ternary complex to be consistent with the high activity of this mutant (Table 5).

The fact that dUMP is less constrained by hydrogen bonding in Asn229 variants, and influenced toward a productive orientation by CH$_2$H$_4$folate, explains why several Asn229 variants are highly active, but do not debrominate BrdUMP (Liu & Santi, 1993a). The mechanism for the debromination reaction requires covalent addition of Cys198 Sγ to C-6 of dUMP, as does the enzyme reaction, and debromination of BrdUMP is used to assay the ability of the enzyme to form the

![Figure 9. MOLSCRIPT (Kraulis, 1991) plot of wild-type TS-dUMP (light gray bonds) and TS N229A-dUMP (dark gray bonds) and TS N229G-dUMP (black bonds) superposed on the $(F_c - F_o)\delta_{\text{calc}}$ difference map, where $F_c$ is an amplitude from the TS N229A-dUMP data set and $F_o$ is an amplitude from the TS N229G-dUMP data set. The contour level is ±3σ. Positive contours are drawn with continuous lines and negative contours are drawn with broken lines. The most prominent peaks indicate the difference in side-chain at residue 229, a new water molecule in TS N229G-dUMP compared to TS N229A-dUMP, and lower occupancy or greater thermal motion of dUMP in TS N229G compared to TS N229A.](image)

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covalent bond to dUMP during the normal reaction (Garret et al., 1979). However, since the assay is done in the absence of folate, it may be expected to be sensitive to variations in dUMP orientation in binary complexes. The most active of the N229X mutants also have elevated $K_m$ values for dUMP. As seen in the crystal structure of TS N229I-dUMP, dUMP in these mutant complexes is very disordered and not positioned for covalent addition to S' of Cys198. In the binary complex structure of another active variant, N229C, dUMP binds in the wild-type binding site, but is more disordered: the wild-type binary complex crystal structure and the crystal structure of N229C-dUMP have equivalent overall B-factors, but the average B-factor of atoms in the pyrimidine ring of dUMP in the latter complex is 56 Å² compared to 24 Å² in the wild-type binary complex.

Fewer hydrogen bond contacts to O-4 of dUMP may also contribute to reduction in deamination activity. Hydrogen bonds from water molecules and Asn229 are proposed to stabilize the developing negative charge at O-4 when Cys198 covalently adds to C-6 of dUMP during dUMP methylation (Carreras & Santi, 1995). Hydrogen bonding to O-4 of BrdUMP may similarly be required during the analogous Michael addition step in the dehalogenation reaction. In TS N229S-dUMP, for example, dUMP is well ordered and bound almost identically with dUMP in the wild-type binary complex, but O-4 has no hydrogen-bond partners, and the mutant does not catalyze deamination of BrdUMP.

Of the 15 catalytically active Asn229 mutants tested, only TS N229G and TS N229Q catalyzed deamination of BrdUMP. We have not determined the structure of TS N229Q, but the structure of TS N229G-dUMP is unique among those variants we have studied in having both the pyrimidine ring of dUMP ideally positioned with respect to Cys198 for covalent addition of the catalytic sulphydryl group, and having O-4 of dUMP extensively hydrogen bonded to water molecules and amino acid side-chains. This result suggests that proximity to the catalytic cysteine residue and hydrogen bonding to dUMP O-4 are probably both important for deamination.

Conserved hydrogen bonds from Asn229 are important for orientation, not binding free energy

Table 5 summarizes binding and kinetic data for the Asn229 mutants whose structures we report here (Liu & Santi, 1993a,b). The small changes in $K_a$ for N229 variants with small or absent side-chains indicates that the hydrogen bonds between dUMP and Asn229 do not contribute substantially to dUMP binding energy: water molecules or other hydrogen-bonding side-chains are able to compensate for the hydrogen bonds formed by Asn229 in the wild-type complexes. $K_a$ values for dUMP are measured using a competitive binding assay that measures displacement of PLP from the enzyme (Santi et al., 1993). $K_a$ values for dUMP are less than fivefold higher for the four out of five mutants for which $K_a$ could be measured. In the case of N229I, no PLP binding to TS could be observed by absorbance spectroscopy, thus $K_a$ for this mutant could not be measured.

$K_m$ values for CH$_3$H$_2$folate are a complicated function of rate constants in the multistep, bi-substrate reaction, but can be viewed as apparent cofactor binding constants (Santi et al., 1990). Evidence that $K_m$ values for CH$_3$H$_2$folate reflect binding is that they correlate with average B-factors for the atoms of CB3717 in the ternary complex structures of N229S and N229C ($K_m$ for N229A could not be determined). In contrast to the ternary complex of L. casei TS V316Am, in which the C-terminal valine residue was removed by mutagenesis and consequently the ligands were misaligned in the active-site (Perry et al., 1993), all variants of N229 whose ternary complex structures we have determined bind CB3717 in approximately the orientation seen in the wild-type enzyme (Birdsall et al., 1996), and form the conserved hydrogen bond between Asp221 and 3-NH of the quinazoline ring. Thus, Asn229 affects CB3717 (and CH$_3$H$_2$folate) only indirectly, by perturbing the orientation of dUMP. In the two ternary complexes that have well-defined CB3717 density (the N229S and N229A complexes), the shift in the position of the quinazoline ring of CB3717 tracks the shift of the pyrimidine base of dUMP, illustrating how small changes in dUMP orientation can lead to an increase in $K_m$ for CH$_3$H$_2$folate.

**Sensitivity of $k_{cat}$ to dUMP orientation**

The reduction in $k_{cat}$ for the Asn229 mutants range from approximately threefold (for N229I) to over 2000-fold (for N229A). In bi-substrate reactions, alignment of substrates with each other and with active-site residues contributes greatly to catalysis. Conformational changes in TS on ligand binding, especially movement of the C terminus into the active-site, assists in orienting the cofactor for catalysis. When the carboxy terminus of L. casei TS is missing, cofactor does not bind in a productive orientation and the enzyme is inactive (Perry et al., 1993). The structures of the ternary complexes of the N229 mutants demonstrate that the carboxy-terminal conformational change occurs as in the wild-type enzyme, and only subtle differences in ligand positions are observed. The subtle differences in dUMP orientation observed in the N229 mutant complexes probably do not by themselves account for the drastic reduction in $k_{cat}/K_m$ seen for some of the mutants, but even small changes can have a significant impact on enzyme activity. For example, in altering the specificity of TS from dUMP to dCMP, a mutation that allowed the dCMP to bind approximately 0.6 Å closer to the optimal nucleotide binding site resulted in a
20-fold increase in $k_{cat}/K_m$ for CH$_2$H$_4$folate (Agarwalla et al., in the press). We assessed the sensitivity of the reaction to misalignment of dUMP by comparing $k_{cat}$ values of the mutants to shifts in the position of the dUMP pyrimidine ring relative to Cys198 S'. and CB3717 C-5 in N229C-dUMP-CB3717, N229 S-dUMP-CB3717 and N229A-dUMP-CB3717. In all three structures, dUMP is shifted to a small degree with respect to the wild-type binding site, and the extent of the shift does not correlate with the reduction in $k_{cat}$. However, the $k_{cat}$ values for these three mutants are correlated with the average atomic B-factors of dUMP in their ternary complexes. The average atomic B-factors for dUMP in the N229C, N229S and N229A ternary complexes were 53 Å$^2$, 22 Å$^2$ and 18 Å$^2$, respectively, correlating with $k_{cat}$ values of 0.2 s$^{-1}$, 0.10 s$^{-1}$ and 0.0015 s$^{-1}$.

A similar trend is observed for the binary complex structures: dUMP is identically oriented but more disordered in N229G-dUMP compared to N229A-dUMP (Figure 9 and Table 1), and the $k_{cat}$ is 15-fold higher for N229G than for N229A. In N229L, the dUMP base is shifted away from the catalytic cysteine residue but is very disordered, and $k_{cat}$ is only threefold lower than for wild-type TS. Thus, the extent to which dUMP misorientation affects catalytic rate may depend on how easily dUMP is induced to bind in its optimum catalytic binding site by cofactor binding and protein conformational changes. For mutants that do not bind dUMP well, such as N229L, the free energy of the ground state and transition states of the Michael addition step in the reaction are both raised equally, and $k_{cat}$ is not affected, although $k_{cat}/K_m$ is much lower because of the increase in $K_m$ for dUMP. For N229A, which preferentially binds dUMP in an orientation that is not optimal for forming covalent bonds to cofactor and substrate, the ground state of the Michael addition step is stabilized relative to the transition state, and $k_{cat}$ is lower.

**Hydrogen bond stabilization as a factor in catalysis**

The fact that N229S shows only small perturbations in dUMP and folate orientation in its binary and ternary complexes, and very small increases in $K_m$ values for substrate and cofactor, indicates that ligand orientation effects alone do not account for the 300-fold decrease in $k_{cat}$ for this mutant. This result highlights the role of Asn229 in hydrogen bond stabilization during catalysis. Hydrogen bonding to O-4 of dUMP is proposed to stabilize developing negative charge at O-4 during the Michael addition of Cys198 to C-6 of dUMP (Carreras & Santi, 1995; Matthews et al., 1990), and subsequently, during elimination of dUMP C5-H during breakdown of the ternary complex intermediate II (Carreras & Santi, 1995). The hydrogen bonds Asn229 makes with dUMP O-4 and NH-3 are not major contributors to the proposed stabilization. We estimate that hydrogen bonds between the Asn229 side-chain and dUMP contribute not more than about a factor of 3 to $k_{cat}$ since some mutants in which these hydrogen bonds are absent (e.g. N229I) have $k_{cat}$ only threefold less than wild-type (Liu & Santi, 1993a). However, it may be that in these mutants, new hydrogen bonds from water or neighboring side-chains, such as His199, may form during the reaction and compensate for the absent Asn229 hydrogen bonds.

Asn229 may be more important for its participation in the hydrogen bond network linking dUMP to the invariant residue Glu60. Mutation of Glu60 decreases $k_{cat}/K_m$ by 10$^4$ for all variants except TS E60D, where $k_{cat}/K_m$ decreases by a factor of 10$^2$. (W. Huang & D.V.S., unpublished results). These results identify Glu60 as one of the most important residues for catalysis in TS. A conserved water molecule (Wat1, Figure 10a) that connects dUMP O-4 and Glu60 via hydrogen bonds in TS ternary complexes, has been proposed to act as a general base that promotes proton abstraction prior to elimination of H$_2$folate. How important this water molecule is for catalysis, and whether other hydrogen-bonding groups in the vicinity of the dUMP base can compensate for its removal, remain to be determined. One of our aims in studying the complexes of the N229 mutants is to test the sensitivity of $k_{cat}$ to conservation of Wat1 and its hydrogen-bonding partners. The results of the crystallographic studies of these mutants support the idea that hydrogen bonding between Glu60, Wat1 and dUMP is important, but not essential to catalysis.

In the binary and non-covalent ternary complexes of TS, the protein is in an open conformation and Wat1 is not directly hydrogen bonded to Glu60 or His199 (Figure 2). Closure of the active-site after binding of CH$_2$H$_4$folate (or CB3717) and just prior to Michael addition expels some ordered water molecules and establishes the hydrogen-bond network between Wat1, Glu60 and His199 (Figure 10a), which may be critical to catalysis. The results of our crystallographic studies show that mutations of Asn229 to small amino acids such as alanine, glycine or serine are accompanied by rearrangements of solvent around Glu60, which may preclude forming this hydrogen-bond network. In N229S, the serine side-chain is involved in a new hydrogen bond to His199 and the conserved water molecule is missing altogether from the ternary complexes (Figure 10b). Thus, in these variants lack of hydrogen-bond stabilization may increase transition state energy for the Michael addition step, and/or other steps in the reaction, decreasing reaction rate.

In the ternary complex of N229C, the variant with the highest $k_{cat}/K_m$, well-ordered water molecules are located within 1 Å of their locations in the E. coli TS covalent ternary complex, and therefore are ideally positioned to form stabilizing hydrogen bonds once the active-site closes down.
For the most active variant in the series whose structures we have studied, N229I, water molecules around Glu60 in the covalent ternary complex are also all conserved. Furthermore, the conformation of His199 more nearly resembles its conformation in the covalent ternary complex than the other binary complexes, with His199 $\text{N}^2$ hydrogen bonded to Wat1 (Figure 10c). Thus the transition between the protein structure in the binary complex and a protein structure that could stabilize enolate formation in ternary complexes appears to be energetically less costly for this variant than for others, offering an explanation for the high activity of N229I.

TS N229A is unique among the L. casei TS N229 mutants, in that it has no measurable activity, yet it forms a very stable complex with $[\text{6-}^{3}\text{H}]\text{FdUMP}$ and CH$_2$H$_2$folate, as visualized by autoradiography following SDS/PAGE (Liu & Santi, 1993a). This result suggests that steps following covalent complex formation are impaired in N229A. We predict that the rearrangement of solvent molecules and the His199 orientation that we see in the non-covalent complex with L. casei TS persists after closure of the active-site and covalent addition of Cys198 to dUMP C-6. These rearrangements would interfere with general base catalysis of the removal of the dUMP C-5 proton, and subsequent elimination of H$_2$folate. Indeed, the structure of the E. coli TS covalent complex with dUMP and a cofactor analog shows a rearrangement of water molecules and movement of His199 (147 in E. coli) that supports this prediction (C. Reyes et al., unpublished results). Other specific roles in catalysis have been proposed for the ordered water molecules around the dUMP pyrimidine ring. Matthews et al. (1990) suggest that Wat1 may accept the C-5 proton during breakdown of the ternary complex and subsequently assist in proton transfer from the cofactor to the exocyclic methylene. Hardy et al. (1995) suggest that an ordered water molecule may mediate abstraction of the C-5 proton via N-5 of CH$_2$H$_2$folate, which acts as the general base in catalysis. In both cases, rearrangement of solvent structure as a result of mutation would be expected to decrease the rate of steps involved in breakdown of the covalent intermediate II.

**Conclusion**

Structures of N229X mutants explain the result that many variants with $K_m$ values similar to wild-type have low $k_{cat}$ values, while other variants with greatly elevated $K_m$ values are very active (Liu & Santi, 1993a). Bulky hydrophobic side-chains disfavor dUMP binding in both ground state and transition state structures, thus $k_m$ values residues, and ordered water molecules in the L. casei TS N229I-dUMP structure.
are high, but $k_{\text{cat}}$ values are unaffected by unfavorable dUMP binding. We find that for hydrophobic substitutions, positions for His199 and active-site water molecules resemble those seen in covalent ternary complexes; we surmise that these arrangements of water molecules and His199 favor ternary complex formation and lead to high $k_{\text{cat}}$ values. Small side-chains allow dUMP to bind tightly, but cause rearrangements in dUMP, His199 and water molecule positions, which may interfere with ternary complex formation and subsequent steps in the TS reaction.

Even though Asn229 is not essential for catalysis and does not contribute substantially to substrate binding energy, it is unique in maintaining both high $k_{\text{cat}}$ and low $K_m$(dUMP). The hydrogen-bonding properties of the carboxamid group complement those of dUMP, and of the ordered water molecules in the active-site. Thus it alone among the 20 amino acids interferes neither with dUMP binding nor with a hydrogen-bonding network involving water, dUMP and Glu60, which seems to be important for catalysis. The $k_{\text{cat}}$ is compromised most by well-ordered, but altered alignment and distance of the attacking $\text{S}^\prime$ to $\text{C}-6$ of dUMP, by a factor of 100 (~0.5 Å shift in N229G) to essentially zero (~1.1 Å shift in N229A). Impairment of $K_m$ is most readily seen as poorly ordered binding (N229I, N229C) where the correct alignment can still be accessed.

**Methods**

**Preparation of the protein and crystallization**

$L. \text{casei}$ TS variants were prepared by cassette mutagenesis of the synthetic TS gene pSCTS9 (Climie & Santì, 1990), expressed in $E. \text{coli}$ p2913 (thy⁻), and purified by sequential chromatography on phosphocellulose and hydroxylapatite (Kealey & Santì, 1992) as described (Liu & Santì, 1992). Hexagonal bipyramidal crystals were grown by vapor diffusion from 6 μl drops containing 3.5 to 6.0 mg/ml protein and 2 to 20 mM dUMP or dCMP in 40 mM potassium phosphate (pH 7.0), 1 mM DTT and 1 to 2% saturated ammonium sulfate. Drops were suspended over just the phosphate buffer, DTT and EDTA. Crystals appeared in one to four days and grew to 400 to 600 μm in length. All crystals were isomorphous with either the large or small unit cell forms of the $L. \text{casei}$ TS apoenzyme (Finer-Moore et al., 1993).

Ternary complexes were formed by adding the folate analog CB3717 to crystals of binary complexes with dUMP. Water dilutions of stock solutions of CB3717 ($K_i$ 1.1 nM: Jones et al., 1981) were made and 1 μl aliquots were tested on 4 μl hanging drops until folate analog concentrations were determined that did not dissolve or degrade crystals. The final concentration of CB3717 was ~20 mM. I$_{\text{bd}}$ data were collected two to seven days after addition of CB3717 to the crystals.

**X-ray data collection**

I$_{\text{bd}}$ data were collected on an R-axis IIIC image plate detector system mounted on a Rigaku 18 kV generator run at 60 kV, 50 mA, collimated to 0.3 mm, using 1.0 to 2.0° wide frames in $\omega$. Data were reduced using RaxisII data reduction programs (Higashi, 1990; Sato et al., 1992). Data statistics are compiled in Table 6.

**Structure solution refinement**

Structures were solved by difference Fourier methods starting with the structure of the $L. \text{casei}$ apoenzyme (Finer-Moore et al., 1993). Ligands were fitted to density in the first ($F_o - F_c$)$\sigma_{\text{calc}}$ or ($2F_o - F_c$)$\sigma_{\text{calc}}$ maps. Water molecules were identified in subsequent difference density maps. Structures were refined by fitting to ($2F_o - F_c$)$\sigma_{\text{calc}}$ and ($F_o - F_c$)$\sigma_{\text{calc}}$ electron density maps using FRODO (Jones, 1985) or CHAIN (Sack, 1988) then running

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* $R_\text{calc} = \Sigma |I_o - I_{\text{meas}}(0)| 	imes 100\%$, calculated for 1σ data.
molecular dynamics coupled with R-factor and energy minimization (XPLOR, Brünger et al., 1987).

Comparison of structures

Several of the crystal structures we report here had different cell constants from each other and from wild-type TS-dUMP. The c-axes for TS N229C-dUMP-CB3717, N229A-dUMP, N229A-dUMP-CB3717, N229S-dUMP-CB3717 and N229G-dUMP were 230 Å, compared to 242 Å for N229S-dUMP, N229I-dUMP and N229C-dUMP and 240 Å for wild-type TS-dUMP. For the purpose of comparison, coordinates of TS variant complexes were overlapped with wild-type TS-dUMP coordinates by a least-squares fit of the backbone atoms. The significance of the differences between atoms in the overlapped structures was then evaluated as a function of the atomic B-factors (B) of the compared atoms using an equation of the form $\sigma(x, y, z) = (aB^2 + bB + c)$, where $a$, $b$, and $c$ are empirically determined constants (Chambers & Stroud, 1979; Perry et al., 1990; Stroud & Fauman, 1995).

For the structures with identical cell constants, direct difference electron density maps were computed $(F_{ref} - F_{calc})$ maps. The relative positional shifts of atoms in two compared structures were accurately calibrated by comparison of integrated difference electron density with computed r.m.s. density in the featureless regions of the map. This procedure gives accuracy in computed shifts of position that are about tenfold more accurate than the positional uncertainty of atoms themselves.

Deposition of coordinates and structure factors

Coordinates and structure factors for these structures are deposited in the Brookhaven Protein Data Bank. The access codes are 1MFJ, 1MGJ, 1MJH, 1JMI, 1TVU, 1TVW and 1TVV.

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