Role of the conserved tryptophan 82 of *Lactobacillus casei* thymidylate synthase

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Background: Thymidylate synthase (TS; EC 2.1.1.45) catalyzes the reductive methylation of 2'-deoxyuridine-5'-monophosphate (dUMP) by 5,10-methylene-5,6,7,8-tetrahydrofolate (CH_2H_4folate) to produce 2'-deoxythymidine-5'-monophosphate (dTMP) and 7,8-dihydrofolate (H_2folate). Major advances in the understanding of the mechanism of TS have been made by studying site-specific mutants of the enzyme. Trp82 is completely conserved in all the 20 TS sequences known. It forms part of the CH_2H_4folate binding pocket, is reported to be a component of a catalytically important H-bond network, and is suspected to be the source of an unusual absorbance change at 330 nm when TS forms a ternary complex with 5-fluoro-dTMP and CH_2H_4folate. We therefore prepared and characterized a set of 12 mutants at position 82 of *Lactobacillus casei* TS.

Results: Eight Trp82 mutants were active enough for us to determine their kinetic constants for dTMP production, while four were inactive. The active mutants had higher K_m values for dUMP (2- to 10-fold) and CH_2H_4folate (2- to 27-fold), and lower k_{cat} values (12- to 250-fold) than wild-type TS. The most active mutants were those containing the aromatic side chains Phe and His at position 82. All of the Trp82 mutants catalyzed the debromination of 5-bromo-dUMP with kinetic parameters similar to those of wild-type TS, and all formed ternary complexes with 5-fluoro-dUMP and CH_2H_4folate. The absence of Trp82 did not prevent the absorbance change at 330 nm on ternary complex formation.

Conclusions: Trp82, a completely conserved residue that was shown by X-ray crystallography to interact directly with CH_2H_4folate and indirectly with dUMP, does not appear to be essential for binding or catalysis. We do, however, find a preference for an aromatic side chain at position 82. Trp82 does not contribute to the unique spectral change at 330 nm that accompanies TS ternary complex formation.

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Introduction

Thymidylate synthase (TS; EC 2.1.1.45) catalyzes the reductive methylation of 2'-deoxyuridine-5'-monophosphate (dUMP) by 5,10-methylene-5,6,7,8-tetrahydrofolate (CH_2H_4folate) to produce 2'-deoxythymidine-5'-monophosphate (dTMP) and 7,8-dihydrofolate (H_2folate). The structure and function of TS have been extensively studied. A comparison of over 20 sequences of TS revealed that TS is the most conserved enzyme known [1,2]. Three-dimensional structures of the free enzyme and of several binary and ternary enzyme complexes have been determined [2–5]. The basic features of the catalytic mechanism of TS have also been established (for reviews see [6–8]).

To further our understanding of the structure and mechanism of TS, we have been studying 'replacement sets' of TS mutants, prepared by substituting a key residue of the enzyme with some or all of the other 19 naturally occurring amino acids (see [6]). Trp82 is completely conserved in all TSs sequenced to date [6]. Crystallographic studies have shown that Trp82 forms part of the CH_2H_4folate binding pocket, making van der Waals contact with C7 and C9 of the pterin moiety of the cofactor (see Fig. 1). The indole NH of Trp82 also participates in a cyclic H-bond network consisting of several amino acids at the active site and the pyrimidine ring of dUMP (Fig. 2) [9].

The conservation of Trp82 in TS and its reported participation in important structural elements of the enzyme suggest that this residue may serve one or more important structural and/or functional roles. In an attempt to identify this role or roles, 12 Trp82 mutants of *Lactobacillus casei* TS were constructed, and the mutant proteins were purified and characterized. We found that mutation at position 82 impaired dUMP binding only minimally, but did affect CH_2H_4folate binding and the chemical steps that occur thereafter. In general, Trp82 was not essential for catalysis, but there was a strong preference for an aromatic side chain at position 82 in TS.

Results

Trp82 mutants of TS were prepared by cassette mutagenesis of plasmid pSCTS9Δ *Pst* (*Asu*II/*Pst*I), a derivative of pSCTS9 which contains a 'stuffer' sequence between the *Asu*II and *Pst*I sites of the synthetic TS gene [10]. The stuffer plasmid does not encode an active TS and has unique *Not*I and *Sph*I sites for restriction–purification of mutant plasmids [11]. Use of the stuffer plasmid eliminates the possibility of expression of wild-type TS and allows selective destruction of the parent plasmid, thus

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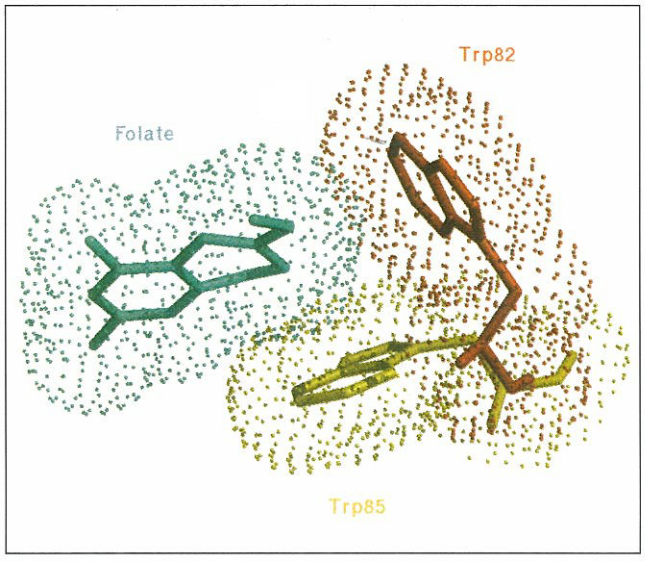

Fig. 1. View of the CH_2H_4folate binding pocket as observed in the TS–H_2folate–dTMP product complex. The pterin moiety of H_2folate is shown.

providing 100 % mutagenesis efficiency. Plasmids were transformed into *Escherichia coli* strain χ2913 recA and transformants were grown on media containing thymine. Plasmids were isolated from randomly chosen colonies, and the TS gene was sequenced through the cassette and flanking regions. After sequencing purified plasmids from 27 colonies, we obtained nine different mutants: W82H, F, A, L, M, N, G, S, or R. W82Y was constructed using an oligonucleotide cassette containing the Y codon (TAC) at codon 82 in the sequence. To increase the number of mutations, W82D and V were constructed using an oligonucleotide cassette containing a two-fold degenerate codon [G(A,T)T] at codon 82 in the sequence.

TS activity was initially assessed by complementation of the Thy^- phenotype of *E. coli* χ2913 recA cells by plasmids containing mutations at codon 82. Failure to complement χ2913 recA indicated that the mutant produced less than 1 % of the TS activity expressed by the wild-type parent plasmid, pSCTS9 (< 0.002 U of TS per mg protein in crude extracts [12]). Of the 12 Trp82 mutants tested, only the three with conservative aromatic amino acid substitutions (TS W82H, F and Y) complemented χ2913 recA. Each mutant TS was purified to homogeneity.

We measured steady-state kinetic parameters for each TS mutant (Table 1). Two mutants with aromatic amino acids, W82F and H, had k_{cat} values that were 12- to 20-fold lower than that of wild-type TS. The K_m values for these two mutants for CH_2H_4folate were two- to three-fold higher than that of wild-type TS, while the K_m values for dUMP were seven-fold higher. W82Y, also a mutant with a structurally conservative amino acid change, had a very low k_{cat} (160-fold lower than for wild-type TS), and displayed K_m values for CH_2H_4folate and dUMP that were three-fold higher than that of wild-type TS. The mutants W82H and F showed the highest values of k_{cat}/K_m for CH_2H_4folate (25- to 64-fold lower than that of wild-type TS), while W82Y displayed a substantially lower value (500-fold lower than wild-type). The k_{cat}/K_m values for the three mutants for dUMP ranged from 95- to 550-fold lower than that of wild-type TS.

The mutants W82S, N, M, L and A were all marginally active, as assessed by k_{cat} values. These mutants displayed k_{cat} values and k_{cat}/K_m values for CH_2H_4folate ranging from 70- to 250-fold and 850- to 5400-fold lower than those of wild-type TS, respectively. The K_m values for CH_2H_4folate and for dUMP ranged from 7- to 26-fold and 2- to 10-fold higher than those of wild-type TS, respectively. The k_{cat}/K_m values for dUMP for three of these mutants (W82S, M and A) were 400- to 700-fold lower than that of wild-type TS. The k_{cat}/K_m values for dUMP for W82N and L could not be obtained under conditions of saturating CH_2H_4folate (i.e., $[CH_2H_4\text{folate}] >> K_m$).

Four mutant enzymes (TS W82D, V, R and G) showed no TS activity in the standard assay for up to 1 h ($< 10^{-3}$ U mg^{-1} with < 0.1 mg enzyme ml^{-1}). These mutants displayed less than 0.02 % of the activity of wild-type TS, which represents the maximum sensitivity of the spectrophotometric assay [13].

TS catalyzes folate-independent chemical reactions at the 5-position of a number of 5-substituted deoxyuridine monophosphates (for a review see [7]). One such reaction is the thiol-dependent debromination of 5-bromo-2'-deoxyuridine-5'-monophosphate (BrdUMP) [14]. We

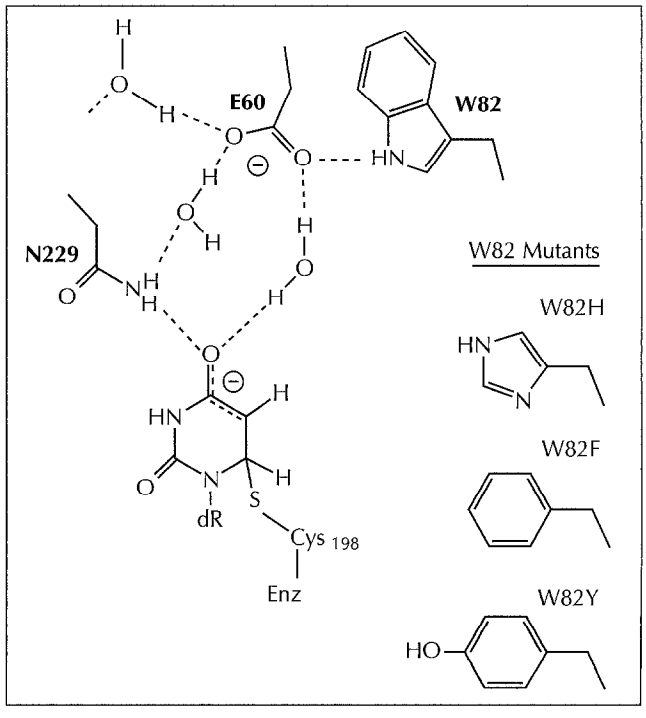

Fig. 2. H-bond network of *L. casei* TS. Mutants at position 82 that complement the Thy^- strain of *E. coli* strain χ2913 recA are shown below the wild-type residue, W.

have previously used this TS-catalyzed reaction as a means of decoupling nucleotide binding and reaction from dTMP formation [13]. Most Trp82 mutants, including those that failed to catalyze dTMP formation, catalyzed the debromination of BrdUMP with k_{cat} and K_m values similar to those of wild-type TS. The exceptions were the mutants with charged residues, W82D and R, which exhibited k_{cat} values 15- and 8-fold lower, respectively, than did wild-type TS. For these mutants, however, the K_m for the debromination reaction was identical to or lower than that of wild-type TS.

When incubated with [6-^{3}H]-5-fluoro-2'-deoxyuridine-5'-monophosphate ([6-^{3}H]FdUMP) and CH_2H_4folate, all 12 mutant enzymes formed TS–[6-^{3}H]FdUMP–CH_2H_4folate ternary complexes which were stable to SDS-PAGE (data not shown). For selected mutants, formation of the TS–FdUMP-CH_2H_4folate complex was also assessed spectrophotometrically. The $\Delta\epsilon_{330}$ values for ternary complex formation for wild-type TS and mutants W82F, H, Y, L and A are shown in Table 1. With the exception of W82A, which displayed a $\Delta\epsilon_{330}$ about five-fold lower than did wild-type TS, the $\Delta\epsilon_{330}$ values were similar for mutant and wild-type enzymes.

Discussion

The crystal structures of TS liganded with substrates, substrate analogs, or products indicate that the conserved Trp82 is involved in several potentially important interactions in the active site of the enzyme [4,5,15]. Along with other residues, Trp82 and Glu60 form an extensive H-bond network, which is believed to help stabilize the enol form of dUMP in the transition state of Michael adduct formation (Fig. 2) [9,16]. The indole NH group of Trp82 is H-bonded to the carboxylate of Glu60, which, in turn, is H-bonded to a conserved water molecule that contacts O4 of dUMP. It is believed that this water molecule assists proton donation/removal at O4 at several steps in the catalytic pathway [16]. Further, the indole of Trp82 makes contact with C7 and C9 of CH_2H_4folate and interacts with Trp85 [4,5,15], a non-conserved tryptophan that also lines the folate binding pocket (Fig. 1). The complete conservation of Trp82, and its position in the TS active site, suggested that it is important in catalysis.

To assess the role of Trp82 in binding and catalysis, we mutated Trp82 to 12 other amino acids and determined the steady state kinetic parameters for these mutants in both the normal enzymic reaction and the dehalogenation of the alternative substrate, BrdUMP.

In the normal TS reaction, the kinetic parameters most affected by mutation at position 82 were those associated with CH_2H_4folate binding and catalysis. Trp82 mutants showed only moderate increases in the K_m for dUMP, indicating that the mutations had little effect on dUMP binding. In contrast, significant differences were observed in the K_m values of many of the mutants for CH_2H_4folate, presumably reflecting differential effects of

Table 1. Characteristics of Trp82 mutants of *L. casei* TS.

Residue 82	FdUMP complex $\Delta\epsilon_{330}$ mM^{-1}cm^{-1}	dTMP formation k_{cat} (s^{-1})	K_m (μM) CH_2H_4folate	K_m (μM) dUMP	k_{cat}/K_m (s^{-1} μM^{-1}) CH_2H_4folate	k_{cat}/K_m (s^{-1} μM^{-1}) dUMP	Dehalogenation of BrdUMP k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} μM^{-1})
W (WT)	20.0	5.5	14	3	3.9×10^{-1}	1.9	0.92	26	3.5×10^{-2}
F	32.3	0.46	28	23	1.6×10^{-2}	2.0×10^{-2}	3.1	27	1.2×10^{-1}
H	29.8	0.28	46	22	6.1×10^{-3}	1.3×10^{-2}	1.1	34	3.2×10^{-2}
Y	24.3	0.034	44	10	7.7×10^{-4}	3.4×10^{-3}	0.71	21	3.4×10^{-2}
S	–	0.045	98	10	4.6×10^{-4}	4.5×10^{-3}	0.26	7	3.7×10^{-2}
N	–	0.054	372[a]	–[b]	1.5×10^{-4}	–[b]	0.27	13	2.1×10^{-2}
M	–	0.078	201[a]	29	3.9×10^{-4}	2.7×10^{-3}	1.9	59	3.2×10^{-2}
L	18.5	0.027	358[a]	–[b]	7.5×10^{-5}	–[b]	2.1	49	4.3×10^{-2}
A	4.2	0.022	305[a]	6	7.2×10^{-5}	3.7×10^{-3}	0.99	82	1.2×10^{-2}
V	–	ND	ND	ND	ND	ND	0.87	41	2.1×10^{-2}
G	–	ND	ND	ND	ND	ND	0.40	25	1.6×10^{-2}
R	–	ND	ND	ND	ND	ND	0.12	26	4.6×10^{-3}
D	–	ND	ND	ND	ND	ND	0.061	12	5.1×10^{-3}

[a]The highest K_m values for CH_2H_4folate could be overestimated, and k_{cat}/K_m values for CH_2H_4folate could be underestimated, since the maximum folate concentration that could be used in the standard TS assay was 300 μM.
[b]Values could not be accurately determined under conditions of saturating CH_2H_4folate.
Values are estimated to be ±10 %.
ND = activity not detectable.

side chains at position 82 on cofactor binding. Mutants with planar, aromatic side chains at residue 82 showed only a two- to three-fold increase in the K_m of the cofactor and W82S showed a seven-fold increase in K_m for cofactor. In contrast, the K_m values for cofactor of other active mutants were increased by 14- to 25-fold. These effects on cofactor binding may be correlated with the structural relationships of the side chain of Trp82 with CH_2H_4folate in the TS–dUMP–cofactor complex. In the ternary complex, the indole moiety of Trp82 is positioned perpendicular to the pterin ring of the folate, making van der Waals contact with C7 and C9. Trp82 and the non-conserved Trp85 form a V-shaped receptacle which serves to bind the folate (Fig. 1). Since the Trp82 mutants that bind cofactor tightest are those with planar aromatic side chains (Phe, His and Tyr), it is reasonable to conclude that the interaction of the planar side chain of Trp82 with CH_2H_4folate, as revealed by crystal structures of the TS–nucleotide–cofactor ternary complexes [4,5,15], does indeed contribute to cofactor binding.

It has been proposed that a Trp residue might interact with the cofactor in the TS–FdUMP–CH_2H_4folate ternary complex, resulting in the unique absorbance of the complex at 330 nm [17]. Since Trp82 is one of only four conserved Trp residues in TS, and since Trp82 appears to have a structural and functional role in cofactor binding, it was a reasonable candidate for the source of the unusual absorbance of the FdUMP complex. All of the Trp82 mutants formed TS–FdUMP–CH_2H_4folate complexes as assessed by SDS-PAGE analysis. However, where examined, all the ternary complexes still showed the absorbance at 330 nm. Further, for the mutants containing planar, aromatic residues, the extinction coefficients were significantly higher than that of the wild-type enzyme, while the $\Delta\epsilon_{330}$ of W82A was significantly lower. Although the nature of this absorbance change remains unexplained, it is clear that the interaction of Trp82 with cofactor is not the source of the absorbance of the TS–FdUMP–CH_2H_4folate complex.

The k_{cat} values of the Trp82 mutants for dTMP formation reflect the reactivity of the ternary Michaelis complex. Most Trp82 mutants retained activity, although k_{cat} values were decreased 12- to 250-fold compared to that of wild-type TS. The most active mutants were W82F and H which had k_{cat} values only 12- to 20-fold lower than did the wild-type enzyme. The other six active mutants with side chains of various sizes and physical properties had k_{cat} values which were 100- to 250-fold lower than that of wild-type TS, but k_{cat} values within the group were similar. Since mutants with this diverse group of side chains (Y, S, N, M, L and A) show comparable activity, it appears that all of these residues in the 'marginally active' mutants affect catalysis equivalently. The planar side chains of Trp in wild-type TS, and to a lesser extent Phe and His in TS mutants, probably interact in a manner that makes a positive contribution to the reaction rate. Also, with the possible exception of His and Tyr, the mutants examined cannot participate in the H-bond network involving Glu60, so we conclude that this interaction is not essential for catalytic activity. However, even the most active mutant, W82F, was some 12-fold less active than the wild-type TS, and it is possible that the H-bond network involving Trp82 and Glu60, although not essential, contributes marginally to activity.

All of the Trp82 mutants, including those unable to catalyze dTMP formation, catalyzed the dehalogenation of BrdUMP. BrdUMP dehalogenation does not require the folate cofactor and thus measures the ability of the enzyme to bind nucleotide, and to catalyze reactions involving (1) attack at C6 of the pyrimidine by Cys198 of TS to form a dihydropyrimidine adduct, (2) a thiol-dependent dehalogenation, and (3) β-elimination of the enzyme to provide products (Fig. 3) [14]. The K_m values

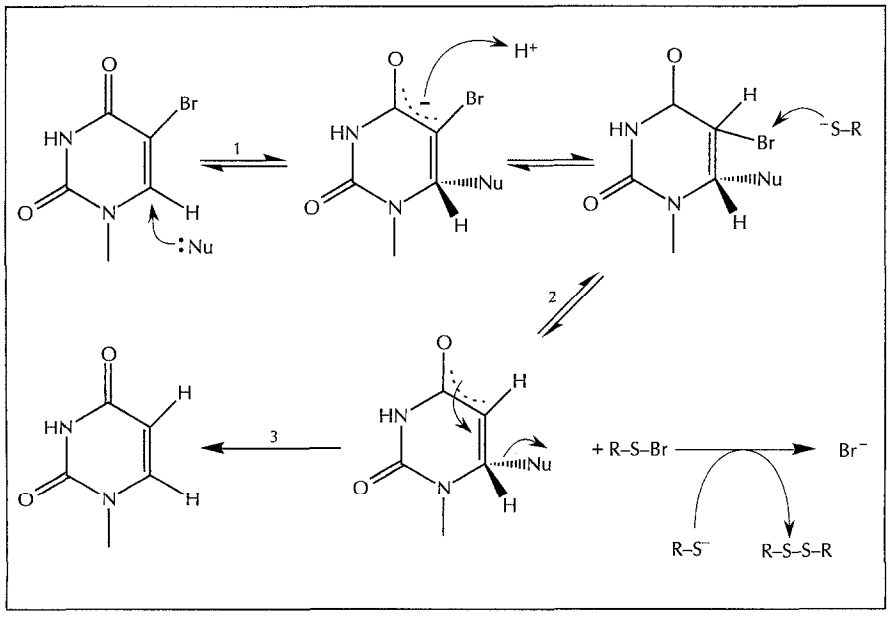

Fig. 3. Reaction scheme for BrdUMP dehalogenation. This reaction does not require cofactor. The enzyme first binds the nucleotide, then catalyzes (1) attack at C6 of the pyrimidine by Cys198 of TS to form a dihydropyrimidine adduct, (2) a thiol-dependent dehalogenation, and (3) β-elimination of the enzyme.

of most Trp82 mutants for BrdUMP are, in general, similar to each other and to that of the wild-type enzyme. The K_m of the BrdUMP dehalogenation has been previously shown to reflect binding of the nucleotide [14], and we may conclude that, as previously discussed for the normal TS reaction, Trp82 does not have a role in nucleotide binding. The initial catalytic step of BrdUMP dehalogenation involving nucleophilic attack of Cys198 is directly analogous to an early step in the TS reaction, and chemical considerations, as well as experimental data, dictate the need for assistance by general acid–base catalysis at the 4-oxo group of the pyrimidine (see [18]). As shown in Table 1, the k_{cat} values for this reaction are generally quite similar for the mutant and wild-type enzymes. The exceptions are W82F, W82M and W82L, which have higher k_{cat} values than wild-type TS, and the mutants with charged side chains, W82R and W82D, which have significantly lower k_{cat} values. Thus, mutations at position 82 did not affect nucleotide binding, nor did they affect the rate-determining step of the subsequent catalytic process in the dehalogenation pathway. Taken together, these results suggest that the disruption of the Glu60–Trp82 H-bond does not affect BrdUMP dehalogenation or, by analogy, the early steps of the TS reaction involving dUMP binding and initial covalent adduct formation. We therefore conclude that the effects of Trp82 mutations in the normal TS reaction are manifested subsequent to nucleotide binding; the mutations mainly affect CH_2H_4folate binding and probably one or more chemical steps that occur thereafter. Such later steps may or may not involve general acid–base catalyzed reactions facilitated by the putative H-bond network involving the Glu60–Trp82 pair.

In summary, two potential roles for Trp82 were proposed from the three-dimensional structure of liganded forms of the enzyme: first, binding to the cofactor in the ternary TS–dUMP–CH_2H_4folate complex, and second, involvement in an H-bond network believed to be important in proton transfer reactions that occur at the 4-oxo group of pyrimidine substrates. Mutation of Trp82, and the resultant disruption of the Glu60–Trp82 H-bond, does not affect nucleotide binding or early steps of catalysis in the TS reaction. This latter conclusion came from observations that Trp82 mutants that disrupt the Glu60–Trp82 interaction have no effect on the dehalogenation of BrdUMP — a reaction pathway that contains steps analogous to early steps of the TS reaction. A major role of Trp82 in the TS reaction appears to be in binding of the cofactor and in subsequent catalytic steps. Here, the contribution of the indole ring to catalysis is about 4 kcal mol^{-1}, representing the difference in k_{cat} between the wild-type TS and W82A or W82S mutants. Compared to other mutants, those with planar side chains Phe and His at residue 82 also contribute in a positive manner to k_{cat}, possibly by maintaining the chemical micro-environment of the cofactor, or by stabilizing enzyme structure. Whatever the exact function of Trp82 is in catalysis, it clearly contributes to, but is not essential for, catalytic activity.

Significance

Thymidylate synthase represents the sole *de novo* pathway for dTMP synthesis and has received much attention as a target for inhibitors with potential chemotherapeutic value. Progress toward inhibitor design will be aided by a thorough understanding of the catalytic mechanism and substrate recognition of the enzyme. To learn more about the enzymatic mechanism of thymidylate synthase, we have been studying properties of mutant enzymes, generated by substituting conserved residues of the enzyme with some or all of the other naturally occurring amino acids. In the present study we changed the conserved Trp82 to 12 other amino acids and determined kinetic parameters for the mutant enzymes. By changing the Trp residue to several alternative residues, we were able to gain an understanding of the contribution to k_{cat} made by the Trp residue. The results indicated that mutation of Trp82 did not significantly affect nucleotide binding, but did impair cofactor binding and the catalytic steps that occur thereafter. Further, we found that there was a preference for an aromatic side chain at position 82.

Materials and methods

Materials

E. coli strain χ2913recA (ΔthyA572, recA56), and the plasmid pSCTS9 have been reported [10,19] . Oligonucleotide synthesis and automated DNA sequencing were performed at the UCSF Biomolecular Resource Center. Other materials were obtained from commercial sources.

Mutagenesis

General procedures for DNA manipulations and the method for constructing TS mutants by cassette mutagenesis have been described [10,20]. The *Asu*II/*Pst*I fragment of pSCTS9ΔPst [10] was replaced by the oligonucleotide 5'-CGAAGCAT-GCGGCCGCCTGCA-3' to give pSCTS9ΔPst (*Asu*II/*Pst*I). All other procedures were as described [12].

Protein purification

TS mutants were routinely purified by sequential chromatography on phosphocellulose and hydroxyapatite as described earlier [21]. The W82A mutant did not bind to phosphocellulose and was purified as follows. The flow-through from the phosphocellulose column was loaded onto a Q-Sepharose column (Pharmacia, 2.5 x 20 cm, 60 ml bed volume) pre-equilibrated with 20 mM Tris/HCl, pH 7.4, 0.5 mM EDTA and 10 % glycerol. The column was washed with 60 ml of equilibration buffer, and TS was eluted with a seven-column-volume linear gradient from equilibration buffer to equilibration buffer containing 0.8 M KCl. Fractions containing TS were pooled, and the enzyme was further purified by hydroxyapatite chromatography as described [21]. Enzyme purifications were monitored by SDS–12% PAGE [22] and, where possible, by specific activity determinations. Pure enzymes were concentrated using Amicon Centriprep-30 concentrators and stored at –80 °C.

Enzyme assays

TS activity was monitored at room temperature by the increase in absorbance at 340 nm which accompanies H_2folate production [23]. One unit of TS activity is the amount of enzyme necessary to produce 1 μmol of product in 1 min. The standard assay buffer contained 50 mM N-tris[hydroxymethyl]-methyl-2-aminoethane sulfonic acid (TES), pH 7.4, 25 mM $MgCl_2$, 6.5 mM formaldehyde, 1 mM EDTA and 75 mM β-mercaptoethanol. For TS mutants that complemented χ2913 recA, the standard assay mixture (1 ml) contained the above buffer plus 100 μM CH_2H_4folate, 100 μM dUMP, and about 1.5 μM mutant TS. For mutants that did not complement, the standard assay mixture (1 ml) contained the above buffer plus 200 μM CH_2H_4folate, 300 μM dUMP and up to 3.6 μM mutant TS. TS-catalyzed dehalogenation of BrdUMP was monitored at room temperature by the decrease in absorbance at 285 nm which accompanies dehalogenation ($\Delta\epsilon_{285}$ = 5320 M^{-1} cm^{-1}) [14]. Steady-state kinetic parameters were obtained by non-linear least squares fit of the data to the Michaelis–Menten equation.

Ternary complex formation with FdUMP and CH_2H_4folate.

Each mutant TS (5 μM) was incubated for 90 min at room temperature in a reaction mixture (95 μl) containing 158 μM FdUMP and 473 μM [^{3}H]-CH_2H_4folate. An aliquot of the reaction mixture was analyzed by SDS-PAGE. Following staining with Coomassie blue, the gel was subjected to autoradiography [24].

For selected mutants, formation of the TS–FdUMP–CH_2H_4folate complex was also monitored by the increase in absorbance at 330 nm [25]. A mixture containing 6 μM of wild-type or mutant TS (W82F, H, A or L), 0–10 μM FdUMP, 15 μM CH_2H_4folate, and standard assay buffer was incubated for 30 min at room temperature, and spectra were collected between 200 and 500 nm. The maximal increase in absorbance at 330 nm for the enzyme–FdUMP complex was used to calculate the extinction coefficient ($\Delta\epsilon_{330}$) for the absorbance change, assuming a ratio of 1 mol FdUMP bound per mol of enzyme monomer.

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