A Model for Hydride Transfer in Thymidylate Synthase Based on Unnatural Amino Acid Mutagenesis

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Thymidylate synthase (TS) catalyzes the reductive methylation of 2′-deoxyuridine-5′phosphate (dUMP) by *N*5,10-methylene-5,6,7,8-tetrahydrofolate (CH_2H_4folate) to form thymidine-5′-phosphate (dTMP) and 7,8-dihydrofolate (H_2folate). Hydride transfer is the last and least understood step in the reaction catalyzed by TS.[1] Previous studies in our lab have shown that mutants at the invariant residue Trp82 in *Lactobacillus casei* TS produce a β-mercaptoethanol nucleotide adduct **2**, which results from the trapping of the exocyclic methylene intermediate **1** (Figure 1).[2] This product indicates that these mutants are specifically stalled at the hydride transfer step. In wild type *Escherichia coli* TS (which is highly homologous to *L. casei* TS), the primary isotope effect observed for (6*R*)-5,10-CH_2-[6-^{2}H]H_4-folate is large, indicating that hydride transfer may also contribute to the rate-limiting step in the wild-type enzyme.[3] To further investigate the catalytic role of Trp82, two series of unnatural aromatic amino acids were incorporated at site 82 and their catalytic properties characterized.

To minimize the effects of steric perturbation, a variety of tryptophan analogues containing fluorine, methyl, or ring heteroatom substituents were substituted for Trp82. Because the Phe82 mutant also retains significant catalytic activity, a series of fluorinated phenylalanine derivatives was also incorporated (Table 1). Incorporation of the analogues into N-terminal histidine-tagged TS was accomplished by in vitro suppression of a Trp82TAG amber mutation with a chemically aminoacylated suppressor tRNA derived from Tetrahymena tRNA(Gln).[4−6] The suppression efficiencies of the analogues ranged between 30 and 90%. With un-acylated $tRNA_{CUA}$, less than 1.5% full length TS was produced, compared to in vitro expression of wild-type TS lacking the TAG codon. The protein produced was 10 to 15% homogeneous after affinity chromatography on His-bind resin[6] as measured by densitometry on a coomassie blue stained gel.

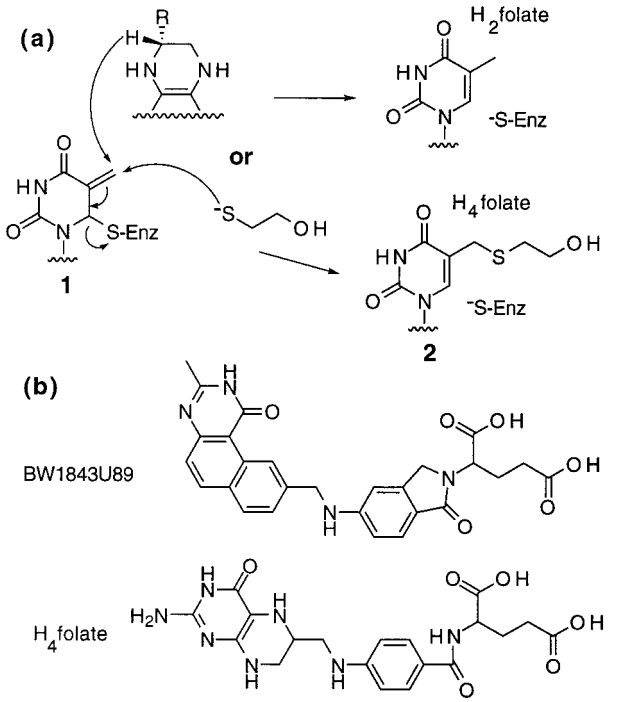

Figure 1. (a) Hydride transfer step of TS and production of β-mercaptoethanol adduct. (b) Structure of H_4folate and the inhibitor BW1843U89.

Table 1. Kinetic Constants of Trp82 Mutants and Theoretical Binding Energies of Na^+ to the Aromatic Side Chains of the Amino Acids[9,10]

	substitutions at position 82	k_{cat} (s^{-1})	K_m (μM) CH_2H_4folate	K_m (μM) dUMP	Na^+ binding energy (kcal/mol)[a]
3	tryptophan(WT)	10	34	3	−32.6
4	5-methylTrp	3.5	35	3	−33.4
5	4-fluoroTrp	1.3	29	3	−27.4
6	5-fluoroTrp	1.5	31	4	−27.5
7	6-fluoroTrp	2.2	35	3	−27.9
8	3-(3-benzothienyl) alanine	0.17	61	20	−26.9
9	7-azaTrp	0.17	23	3	−26.0
10	phenylalanine	0.92	50	23	−27.1
11	2-fluoroPhe	0.53	50	19	−20[a]
12	3-fluoroPhe	0.55	49	19	−20[a]
13	4-fluoroPhe	0.18	50	20	−20[a]
14	3,4-difluoroPhe	0.13	52	19	−17[a]
15	3,4,5-trifluorophe	0.045	55	23	−12[a]

[a] The Na^+ binding value of −20 kcal/mol listed for the 2-, 3-, and 4-flourophenylalanine mutants is from calculations on fluorobenzene; the value used for the 3,4-difluorophenylalanine mutant is from calculations on 1,4-difluorobenzene; and the value for the 3,4,5-trifluorophenylalanine mutant was calculated from 1,3,5-trifluorophenylalanine.[18]

To ensure that no contaminating enzymatic activity was present, a control in vitro reaction containing inactive *L. casei* tagged mutants was shown to have no appreciable activity. The k_{cat} and K_m values for each mutant were determined using a TLC assay with radiolabeled dUMP.[3]

The K_m values for both the Phe and Trp analogues for either CH_2H_4folate or dUMP substrates varied by less than a factor of 2 relative to those of Phe82 or Trp82 TS, respectively (with the exception of 3-(3-benzothienyl)alanine in which the hydrogen bonded NH group of Trp is replaced with a sulfur). This suggests that in general the ring substituents do not significantly perturb the active-site structure. In contrast, the k_{cat} values ranged over 2

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(6) To simplify the purification of TS, a pAED4 T7 expression vector was constructed with a 6-His Tag on the N-terminus using synthetic primers. The protein was purified on HisoBindTM metal chelation resin purchased from Novagen using their procedure, except that the column was washed with 25 mM imidazole, 0.5 M NaCl, and 20 mM Tris acetate, pH 7.9, in place of the recommended wash buffer. After elution of the protein from the His resin, the buffer was exchanged to 10 mM potassium phosphate, pH 7.4, and 1 mM EGTA on G25 Sephadex, and the protein was concentrated with an Amicon Centriprep-30 concentrator. The in vitro protein synthesis reactions were run in the presence of ^{35}S methionine. Protein concentration was determined by using the known concentration of cold methionine, the number of methionines present in thymidylate synthase, and the concentration and activity of the labeled methionine used in the protein synthesis reaction. All of the amino acids were commercially available.

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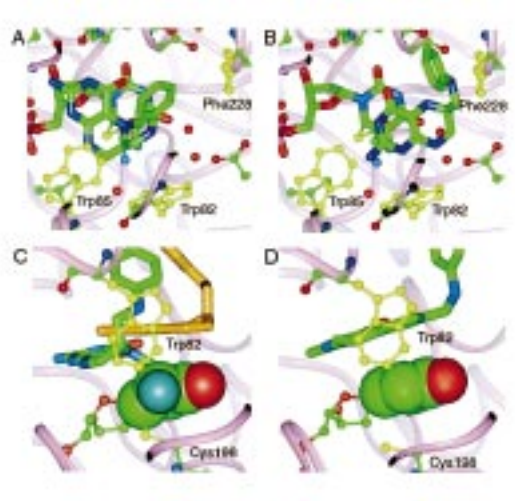

Figure 2. (A) Crystal structure of ternary complex of FdUMP and CH_2H_4-folate at the active site.[11] (B) Proposed binding mode of the H_4folate pterin ring after breakdown of the ternary complex, modeled visually with Insight II into the ternary complex structure. (C) Crystal structure of ternary complex at the active site[11] with the proposed binding mode of H_4folate after the breakdown of the ternary complex overlaid in orange. (D) Crystal structure of inhibitor BW8943U89 bound at the active site of TS.[12]

orders of magnitude, consistent with impaired hydride transfer. The values of log(k_{cat}) of the mutants were compared to both the octanol−water partitioning coefficients[7,8] for the corresponding aromatic moieties and to the theoretically calculated values of the binding energy of Na^+ to the π-systems of tryptophan and phenylalanine analogues.[9,10] While the correlation between log(k_{cat}) and the octanol−water partitioning coefficients was poor, the correlation between log(k_{cat}) and the theoretically calculated values for Na^+ binding energies was significant among both the Phe and Trp analogues; the residues with lower binding energies to Na^+ have, in general, lower catalytic rates.

The observed trends of increasing log(k_{cat}) with increasing Na^+ binding energies among both the Trp and Phe mutants indicates that Trp82 may play a role in binding to a positively charged transition state. In the crystal structure of the ternary complex of TS with FdUMP and methylene tetrahydrofolate (CH_2H_4folate),[11] the indole ring of Trp82 stacks against C9 and C7 of folate making such a stabilizing π-cation interaction unlikely (Figure 2A and C). However, in the crystal structure of TS complexed with dUMP and the three-ringed inhibitor BW1843U89 (Figure 2D),[12] a new binding mode is observed. If tetrahydrofolate were to undergo a conformational shift to this same binding mode (Figure 2B and C), then Trp82 would contact the amine group on C2 and N1 of the pterin ring, and the pterin ring would stack over the uracil ring. These stacking interactions should not only facilitate electron transfer from the pterin ring to the exocyclic methylene intermediate **1** but should also stabilize the net positive charge distributed over the amine on C2, N1, and N5 of the pterin ring. A second conformational shift would then allow the abstraction of the hydrogen on C6 of the pterin ring by the radical anion of the exocyclic methylene intermediate. This postulated mechanism would explain Trp82's importance in catalyzing the hydride transfer step as well as account for the observed trend in catalysis with Na^+ binding. In the structure of BW1843U89 and dUMP bound in the active site of TS, Cys198 and C5 of the uracil ring are 3.23 Å apart vs 1.95 Å in the ternary structure (Figure 2C and D).[11,12] If the distance between the sulfur atom of Cys198 and C5 of the exocyclic methylene, intermediate **1**, lengthens as the H_4folate moves into the proposed binding pocket[13] (increasing the partial positive charge on the nucleotide ring of the exocyclic methylene intermediate and lowering the energy of its LUMO orbital), electron transfer from the pterin ring to the exocyclic methylene might become even more favorable. This postulated binding mode for electron transfer does not place the C6 hydride in proximity to the exocyclic methylene, making a single-step hydride transfer unlikely. A single-step hydride transfer is also unlikely, given the chemical model systems which provide strong evidence that H_4folate and the exocyclic methylene undergo oxidation and reduction, respectively, in several separate mechanistic steps beginning with single-electron transfer.[14,15]

Hydroxymethylases (HM) have been shown to catalyze the addition of a hydroxymethyl group to pyrimidine rings by a mechanism almost identical to that of TS, up to and including the formation of the exocyclic methylene intermediate **1.** Instead of reducing the exocyclic methylene through the transfer of a hydride from the H_4folate, HMs catalyze the addition of water to the exocyclic methylene.[16] The geometry of the active-site residues in the crystal structure of dCMP HM complexed with dCMP is also very analogous to that of TS.[17] However, the proposed electron-transfer binding site present in the TSs for H_4folate appears to be blocked by the side chain of Asn 178 in HM (which is hydrogen-bonded to Arg 177). This observation is also consistent with the theory that this site is involved in the hydride transfer step. Structural studies of TS with bound H_4folate will provide additional insights into these mechanistic possibilities.

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