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Mechanism and Thermodynamics of Interaction of Thymidylate Synthase with 5-Fluoro-2′-deoxyuridylate

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I. Introduction

Thymidylate synthase (TS) catalyzes the conversion of dUMP and 5,10-methylenetetrahydrofolate (CH_2-H_4folate) to dTMP and 7,8-dihydrofolate (H_2 folate). The amino acid sequences of TS from some 16 sources are known, and the primary sequences of TS have revealed that it is the one of most conserved proteins known[1]. 5-Fluoro-2'-deoxyuridylate (FdUMP) behaves as a mechanism-based inhibitor in the TS reaction. Because of its importance in chemotherapy of neoplastic disease, the interaction of FdUMP with TS has been extensively studied. Furthermore, since the interaction mimics several steps in the normal enzymatic reaction, it provides an approach toward studying individual steps of the reaction that are otherwise inaccessible.

Now that the crystal structure of TS is known[2], this capability has taken on new importance. However, our knowledge of the kinetics and thermodynamics of the formation and disruption of the covalent TS =FdUMP=CH_2-H_4folate complex is incomplete. Previously reported kinetics of formation were indirectly determined and superficially treated as a simple bimolecular reaction[3]. Finally, we do not have a good understanding of the contribution that covalent bond formation provides to the stability of the ternary complex.

Because of source limitations, most of biochemical work on TS has been performed with the enzyme obtained from methotrexate-resistant strains of L. casei that produced TS to levels of about 1% of the total protein. Recently, recombinant L. casei TS expressed in E. coli provides 10-20% of the total soluble protein as TS[4]. Since the recombinant enzyme is highly overexpressed, large quantity of the pure enzyme has been obtained with no difficulties in purification. Catalytically active recombinant TS is indistinguishable from that isolated from L. casei. In the present work, we describe data which permit us to deduce the mechanism of interaction of 5-fluorodeoxyuridylate with L. casei TS expressed in E. coli.

II. Materials and Methods

Enzyme Purification and Assay

Expression and purification of the synthetic L.casei TS have been previously described elsewhere[5]. TS activity was assayed spectrophotometrically at 25℃ using the conditions of Santi et al[6]. One unit of activity is the amount of TS that catalyzes the formation of 1 μmol of product per min. Protein concentration was determined by the method of Bradford[7] using bovine serum albumin as a standard.

Filter Binding Assay

The formation of the enzyme-[^{3}H]FdUMP complex was performed in a standard assay mixture containing 50 mM TES(pH 7.4), 25 mM $MgCl_2$, 6.5 mM

HCHO, 1 mM 2-mercaptoethanol, 0.15 mM FAH_4, and [6-^{3}H]FdUMP(New England Nuclear). The reaction was initiated by addition of enzyme in a total assay volume of 100ul. After 45 minutes incubation, 40ul aliquots were assayed in duplicate for complex formation and 10ul was removed for determination of [^{3}H]FdUMP concentration in the assay mixture. Nitrocellulose membranes were soaked before use in 75mM potassium phosphate(pH 7.4). Filters which were not wetted within 2 min were discarded. The filter discs were placed on a filter manifold(Hoeffer-Scientific) and a gentle vacuum was applied to remove excess moisture. Without removing the vacuum, filters were washed with 2ml of the same buffer and then 40ul aliquots of the reaction mixture were applied the discs and allowed to permeate the membrane. After washing the filters with 6ml of the same buffer, the damped filters were placed in scintillation vials and dissolved in 10ml of Aquasol. Optimal solubilization of filters required approximately 24h. Counting efficiencies were determined using external standards. Filtration efficiencies were routinely between 88 and 94% and constant within one same experiment.

UV Difference Spectra

Ultraviolet difference spectra were obtained by adding 20ul of a 1.8 x 10^{-4} M solution of FdUMP or an equivalent amount of water to two previously balance cuvettes containing in 1.0ml: TS(3nmol), DTT (6.5umol), formaldehyde(7.0umol), $MgCl_2$(25umol), EDTA(1umol), and N-methylmorpholine-HCL (50umol, pH 7.4). The spectrum was scanned and after 5min CH_2FAH_4 was introduced into both cuvettes to give a final concentration of 7.2uM and a total volume of 1ml.

III. Results

Stoichiometry of [^{3}H]FdUMP Binding to TS

Figure 1 showed that the bound radioactivity reaches a maximum when the enzyme-[^{3}H]FdUMP ratio is 1:1.8. For comparison, theoretical curves were presented which were constructed for one and two binding sites with the assumption of stoichiometric binding. The equivalence point for complete inhibition of catalytic activity required an average of 1.9mol of FdUMP per mol of enzyme as shown in Fig. 2.

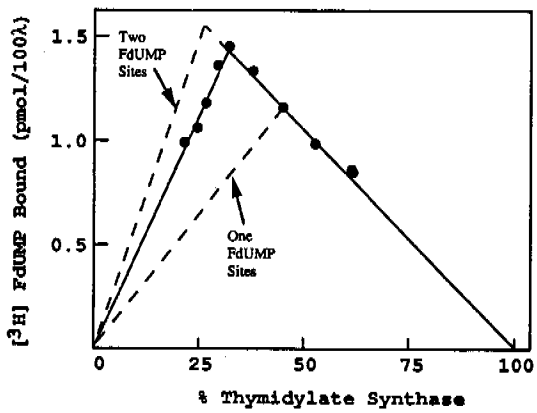


Fig 1. Determination of stoichiometry of FdUMP binding to TS. The experimentally obtained curve was normalized to 100 % filtration efficiency. The theoretical curves calculated for one and two FdUMP binding sites are shown.

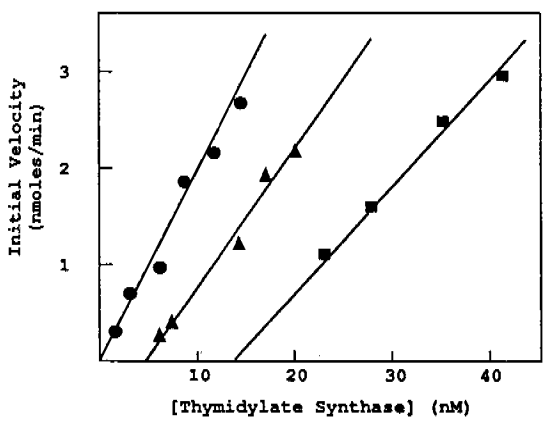


Fig 2. Titration of FdUMP by TS. The indicated amount of the enzyme was incubated for 70 min at room temperature with 8.75 nM FdUMP(triangle) or 26.3 nM FdUMP(square) and standard assay mixture except dUMP ; the control(circle) contained no FdUMP.

Release of Active Enzyme and FdUMP from The Complex

To demonstrate that association−dissociation of the E $-CH_2FAH_4-$FdUMP complex was not accompanied by modification of the enzyme, we performed an experiment to show that [^{3}H]FdUMP could be incorporated into the performed complex. The initial complex was formed with a saturating level of [^{3}H]FdUMP of low specific activity and freed from unbound ligands by gel filtration. The complex in the void volume was quantitated, treated with CH_2FAH_4, and permitted to equilibrate with [^{3}H]FdUMP of high specific activity. As shown in Figure 3, could be exchanged into at least 92% of the preformed complex; moreover, over the time period examined numerous association−dissociation events occur and it may be concluded that such events do not modify the enzyme.

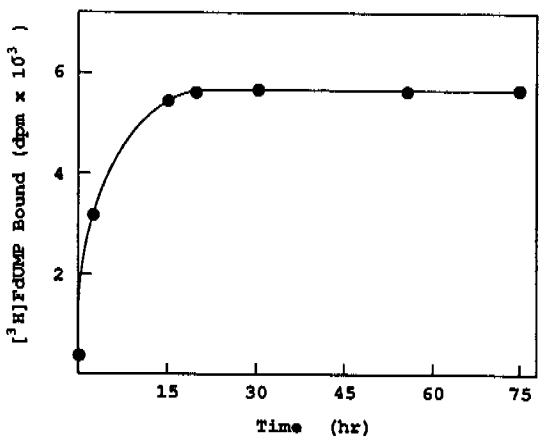


Fig 3. Incorporation of [^{3}H]FdUMP into the E−CH_2FAH_4−FdUMP complex. The E−CH_2−FAH_4−FdUMP complex was formed as described in Materials and Methods. The mixture was filtered through Sephadex G−25 and the three leading fractions were combined and treated with 5,10−CH_2FAH_4 (0.13 mM for 30 min); the mixture contained 7.7 pmol of bound [^{3}H]FdUMP. After the addition of [^{3}H]FdUMP (10.6nM), aliquots were filtered through nitrocellulose filters. At equilibrium the specific activity of [^{3}H]FdUMP was 8 x 10^{3}dpm/pmol; the data shown are corrected for filtration efficiency (91%).

UV Difference Spectra

Figure 4 showed the difference spectrum obtained when FdUMP was added to sample and reference cuvetts containing equal amounts of TS. The spectrum obtained was that of FdUMP and shows no change for at least 4min. Also shown was the scan obtained when equal amounts of CH_2FAH_4 are then added to the sample and reference cuvettes. There was a complete loss of absorbance at 269 nm, the absorbance maximum of FdUMP. In addition, there were also marked spectral changes which can only be attributed to the cofactor, CH_2FAH_4; notably, there was a loss of absorbance in the region below 295nm, and an appearance of a differential peak at 330nm. From the concentration of enzyme, a minimum $\Delta\varepsilon$330 is calculated to be 1.8 x 10^4 or 0.9 x 10^4, respectively, depending on whether 1 or 2mol of ligand are bound per mol of enzyme.

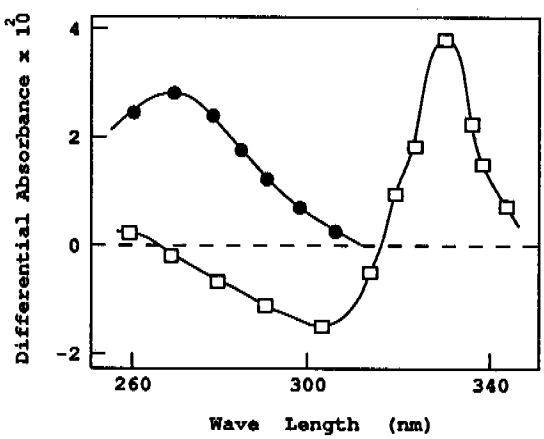


Fig 4. Difference spectra showing the loss of FdUMP and CH_2FAH_4 absorbance, and appearance of a new peak at 330nm. FdUMP is added to the sample cuvette (circle) and then equal amounts of CH_2FAH_4 are added to both cuvattes(square) as described in Materials and Methods.

Thermodynamic and Kinetic Parameters

To obtain *k*obsd for the formation of the covalent complex, several points were obtained at appropriate times for each experiment and plotted as shown in Figure 5A. For each of the temperatures, *k*obsd values were obtained at different CH_2−H_4folate concentrations ranging from about 10 to 50 uM. The values at each temperature were fitted to the non−linear

form, and data were replotted as $1/k$obsd versus $1/[CH_2-H_4folate]$. As shown in Figure 5B, plots of $1/k$ obsd versus $1/[CH_2-H_4folate]$ were linear; k_1 was obtained from the vertical intercept and corresponds to the rate constant at extrapolated infinite concentration of $CH_2-H_4folate$. Values for K_A at each of the temperatures were calculated by using the reported temperature dependence of K_A[8] adjusted to the K_A determined at 30℃ under the conditions used in this study. With these values for k_1 and K_A and the concentrations of TS binding sites, K_B values were calculated from the slope at each temperature.

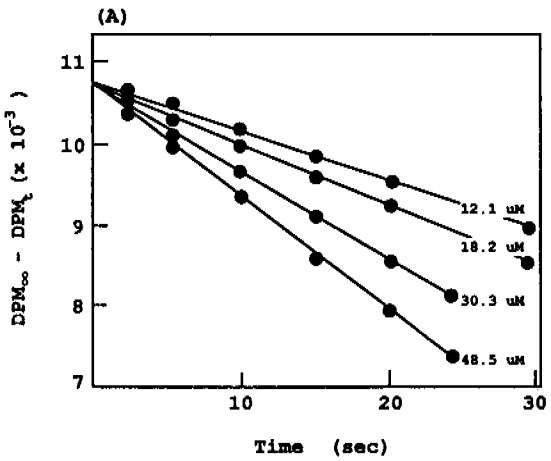
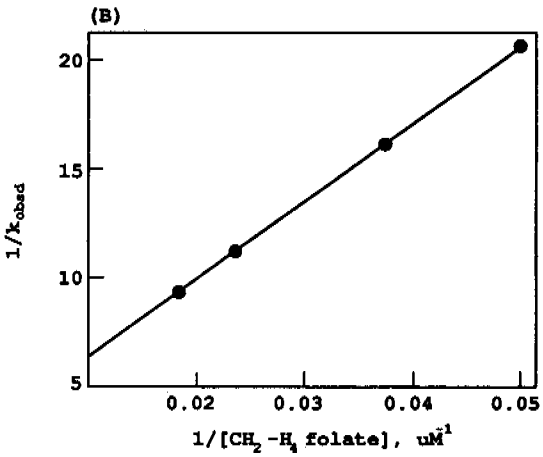


Fig 5. (A) First-order plots for appearance of filter-bound [6-^{3}H]FdUMP accompanying TS=FdUMP=CH_2-H_4folate complex formation with varing (6R)-L-CH_2-H_4folate concentrations.



(B) Replot of the pseudo-first-order rate constants from panel A versus the concentration of (6R)-L-CH_2-H_4folate.

Table I gave values for k_1 and K_B determined at several temperatures, as well as those extrapolated to 25℃. From the Arrhenius plot of these data(not shown), the energy of activation (E_a) for formation of the covalent complex is calculated to be 19.9kcal/mol. Table I also shows i) values of the rate constants for the dissociation of [6-^{3}H]FdUMP from the covalent complex at specified temperatures which might be assumed to reflect k_{-1}; ii) the equilibrium constants for the covalent versus noncovalent complex, determined as k_{-1}/k_1; and iii) the thermodynamic activation parameters for the formation and dissociation of the covalent complex.

Table 1. Thermodynamic and kinetic parameters for interaction of FdUMP and CH_2-H_4folate with TS

Temp (℃)	[Fdump] (nM)	[TS] (nM)	K_A (uM)	K_B (uM)	k_1 (s^{-1})	E_a	$\Delta G^{\ddagger}$	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	k^{-1} ($10^{-6}s^{-1}$)	E_{a-1}	$\Delta G_{-1}^{\ddagger}$	$\Delta H_{-1}^{\ddagger}$	$\Delta S_{-1}^{\ddagger}$	k_{-1}/k_1
3	3.4	177	3.9	3.0	0.037	19.9	17.9	19.3	0.005	0.62	29	24.0	28.5	0.016	1.7
10	2.6	130	5.7	2.3	0.320		17.2	19.3	0.007	2.38		23.8	28.4	0.016	0.8
20	2.6	122	8.9	1.04	0.241		17.9	19.3	0.005	13.7		23.6	28.4	0.016	5.7

TS concentration refers to total FdUMP binding sites as determined by active site titration. K_A values are extrapolated from K_A=15uM at 30℃ by using the remperature dependence of Mittelstaedt and Schimerlik(8). E_a, $\Delta G^{\ddagger}$, and $\Delta H^{\ddagger}$ values are in kcal/mol; $\Delta S^{\ddagger}$ values are in kcal/(mol·deg).

Ⅳ. Discussion

It was previously demonstrated that in the presence of CH_2FAH_4, FdUMP forms a tight complex with TS whereas in the absence of the cofactor binding is undetectable[10]. These results indicate that formation of the high affinity complex only occurs in the presence of all components, and contains both FdUMP and CH_2FAH_4 bound to the enzyme. The interaction of FdUMP(or dUMP), CH_2H_4folate, and TS appears to proceed by an ordered mechanism with nucleotide binding first[3,9], at least under the conditions used here to study the reactions. Subsequent to formation of the reversible, noncovalent ternary complex, enzyme catalyzed conversions occur that result in formation of a covalent bond between C−6 of the nucleotide and the thiol of Cys−198 of TS and a covalent bond between C−5 of the nucleotide and the one−carbon unit of the cofactor. The importance of this interaction to an understanding of catalysis is that it mimics several steps of the normal enzymatic reaction, up to and including formation of the covalent adduct, which is a stable analogue of a steady−state intermediate. We might reasonably assume that the individual chemical conversions in this interaction proceed in a sequence analogous to the normal enzymatic reaction. Further, secondary α−hydrogen kinetic isotope studies of the FdUMP interaction have permitted the placement of the putative intermediates relative to the rate−determining step of the reaction[10]. The FdUMP interaction provides an entree to studies of individual steps of catalysis that are otherwise inaccessible.

In the present work, we report experiments that led to determination of the dissociation constants of CH_2H_4folate from the noncovalent TS−FdUMP−CH_2H_4folate complex and the rate constants for conversion of the noncovalent ternary complex to the covalent TS=FdUMP=CH_2H_4folate complex. Together with previously reported data, these constants permit a complete kinetic and thermodynamic description of the interaction of FdUMP, CH_2H_4folate, and TS. In addition these parameters have been determined at several temperatures, thereby allowing calculation of the corresponding activation parameters.

Dissociation constants of the binary TS−FdUMP complex have previously been reported[8]. From the ratio k_{-1}/k_1 determined here we can calculate the equilibrium constant between the noncovalent and covalent ternary complexes; at 25℃, this number is 5.8 x 10^{-5}, which corresponds to a G of −6.0kcal/mol; K_B is about 1uM at 25℃ and shows little temperature dependence. Knowing K_B and k_{-1}/k_1, we calculate the overall equilibrium constant between the covalent TS=FdUMP=CH_2H_4folate complex and the binary TS−FdUMP complex to be about 5 x 10^{-11} M at 25℃. The extraordinarily low dissociation constant of the covalent complex formally defines the dissociation of CH_2H_4folate from this complex, and CH2H4folate does "induce" the formation of the tight complex. However, the ligands act with apparent synergism in forming the tight complex, and their contributions to stability of the complex are not currently known.

In the TS reaction, the reactants are relatively rigid and the enzyme is mobile. As indicated by hydrodynamic, circular dichroism, and fluorescent quenching studies[11,12], a large cofactor−dependent conformational change of the enzyme occurs upon formation of the TS−FdUMP−CH_2H_4folate complex. A large absorbance peak at 320−340 nm also appears upon formation of the covalent TS=FdUMP=CH_2H_4folate complex as shown in Figure 4. These properties seem to result from interactions of the protein with the *p*−aminobenzoic acid (PABA) moiety of the cofactor and concomitant changes in their environments. Resonance Raman changes indicate that there is a significant change in the electronic structure of the PABA moiety in the ternary FdUMP complex[13], and it has been proposed that a negative charge(or H−bond acceptor) may reside near 10−NM. Intriguingly, the crystal structure of TS shows an unusual cluster of seven aromatic amino acid sid

chains adjacent to the active site cavity that may be the site of this interaction[2].

Two reasonable general mechanisms can be envisioned for the TS-catalyzed conversion of the imidazolidine ring of 5,10-CH_2H_4folate to the 5-iminium ion. The first involves general-acid-catalyzed cleavage of the N-10-CH_2-11 bond, a mechanism suggested previous workers[1-4]. The second general mechanism for this conversion involves enzyme-induced perturbations of the five-membered ring of the cofactor within the noncovalent TS-FdUMP-CH_2H_4folate complex; that is, binding forces between the enzyme and cofactor could directly assist formation of the 5-iminium ion. In accord with this proposal, there is evidence that the enzyme interacts favorably with the pteridine and *p*-aminobenzoic acid (PABA) moieties of the cofactor, causing large pertubations in the environment of the PABA group and involving a conformational change of the enzyme.

With a complete kinetic and thermodynamic description in the reaction of FdUMP and with TS and the recently reported three-dimensional structure of the enzyme, we are now able to approach questions as to how each of the steps is catalyzed. An understanding of the interaction of the FdUMP with TS is necessary to design of even more potent inhibitors, thus anticancer drugs, either by optimizing favorable interactions or by using the heterocycle as a scaffold for other groups which may interact favorably with the enzyme.

V. Acknowledgment

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References

1. Climie SC, Carreras CW, Santi DV : Complete replacement set of amino acids at the C-terminal of thymidylate synthase. Biochemistry 1992 ; 31 : 6032-6038.
2. Hardy LW, Finer-Moore JS, Monfort WR, Jones MO, Santi DV, Stroud RM : Atomic structure of thymidylate synthase: Target for rational drug design. Science 1987 ; 235 : 448-455.
3. Danenberg PV and Danenberg KD : Effect of 5, 10-methylenetetrahydrofolate on the dissociation of 5-fluoro-2'-deoxyuridylate from thymidylate synthase. Biochemistry 1978; 17 : 4018-4024.
4. Climie S, Santi DV : Chemical synthesis of the thymidylate synthase gene. Proc Natl Acad Sci USA 1990; 87: 633-637.
5. Climie S, Ruiz-Perez L, Gonzalea-Pacanowska D, et al : Saturation site-directed mutagenesis of thymidylate synthase. J Biol Chem 1990; 265: 18776-18779.
6. Santi DV, Pinter K, Kealey J, Davisson VJ : Site-directed mutagenesis of Arg 179 of thymidylate synthase. J Biol Chem 1990; 265: 6770-6775.
7. Bradford MM : A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248-254.
8. Mittelstaedt DM, Schimerlik MI: Stopped-flow studies of 2'-deoxynucleotide binding to thymidylate synthase. Arch Biochem Biophys 1986; 245: 417-425.
9. Lu Y, Aiello PD, Matthews RG : Studies on the polyglutamate specificity of thymidylate synthase from fetal pig liver. Biochemistry 1984; 23: 6870-6876.
10. Santi DV, Danenberg PV : Folates in pyrimidine nucleotide biosynthesis. In Folates and Pterins (eds. Blakley RL, Benkovic SJ), New York, John Wiley, 1984; 345-398.
11. Lewis CA, Dunlap RB : Thymidylate synthase

and its interaction with 5−fluoro−2'−deoxyuridylate. In Topics in molecular biology(eds. Burgen ASV, Roberts GCK), Amsterdam, Elsevier, 1981; 169−219.

12. Lockshin A, Mondale K, Danenberg RG : Spectroscopic studies of ternary complexes of thymidylate synthase, deoxyribonucleotides, and folate analogs. J Biol Chem 1984; 259; 113436−11352.

13. Fitzhugh AL, Fodor S, Kaufman S, Spiro TG : Resonance raman spectroscopic evidence for alternative structures in the native ternary complex formed with thymidylate synthase. J Am Chem Soc 1986; 108: 7422−7424.

14. Benkovic SJ : On the mechanism of action of folate− and biopterin−requiring enzymes. Ann Rev Biochem 1980; 49: 227−251.

=국문초록=

Thymidylate synthase와 5−fluoro−2′−deoxyuridylate의 작용기전

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본 논문에서는 항암제로서 널리 쓰이는 5−fluoro−2'−deoxyuridylate(FdUMP)와 그 target이 되는 thymidylate synthase(TS)의 상호작용의 기전을 열역학적인 방법을 이용하여 연구하였다. 이들의 작용은 우선 비공유결합 상태의 복합체를 이룬뒤 다시 공유결합적 형태를 이루는 단계를 거치는데 이때의 전환속도는 25℃에서 $0.6s^{-1}$이었다. 또한 이때 비공유결합 상태의 복합체에서 CH_2H_4folate가 떨어져 나가는 유리 상수의 크기는 1uM이었다. 공유결합적 형태를 이루는데 쓰이는 열역학적 변수들은 각각 활성화 에너지 변화가 20kcal/mol, 자유 에너지 변화가 17.9kcal/mol, 엔탈피 변화가 19.3kcal/mol, 그리고 엔트로피 변화가 0.005kcal/mol/deg 였다. 비공유결합 상태의 복합체와 공유결합적 형태 사이의 평형상수는 2×10^4이었으며 공유결합 상태의 복합체에서 CH_2H_4folate가 떨어져 나가는 최종 유리 상수의 크기는 10^{-11}M이었다. 이상의 결과 및 UV difference spectrum의 결과로 미루어 볼때 CH_2H_4folate 결합에너지의 일부가 5−iminium ion 중간물질의 형성에 쓰이고 있으며, 이러한 5−iminium ion 중간 물질의 형성 반응은 CH_2H_4folate의 N−10 위치에서의 general−acid catalysis및 비공유결합 상태의 TS−FdUMP−CH_2H_4folate 복합체내의 특정 부위의 변화에 의해서 촉진되어지는 것으로 추정된다.