Trapping of the C5 Methylene Intermediate in Thymidylate Synthase

J. E. Barrett,[†] D. A. Maltby,[‡] D. V. Santi,[‡] and P. G. Schultz*,[†]

Department of Chemistry, University of California at Berkeley, Berkeley, California 94720
Departments of Pharmaceutical Chemistry and Biochemistry and Biophysics, University of California at San Francisco, San Francisco, California 94143

Received September 12, 1997

The enzyme thymidylate synthase (TS) catalyzes a key step in DNA biosynthesis, the conversion of 2′-deoxyuridine-5′-phosphate (dUMP) to thymidine-5′-phosphate (dTMP) using the cofactor *N*5,10-methylene-5,6,7,8-tetrahydrofolate (CH_2H_4folate). This highly conserved enzyme has been a target for the development of anticancer drugs, including 5-fluorouracil and 5-fluoro-2′-deoxyuridine which are precursors to the mechanism-based inhibitor 5-fluoro-2′-deoxyuridylate (FdUMP).[1] In addition, detailed mechanistic and structural studies have provided considerable insight into the mechanism of the methylation reaction (Figure 1).[2] The first step involves Michael addition of an active site cysteine to the C6 position of dUMP, as evidenced by tritium exchange at the C5 position of the base in the absence of cofactor, dehalogenation of 5-bromo-2′-deoxyuridine, and a crystal structure of the enzyme covalently bound to dUMP through the conserved active site cysteine.[3−5] The second step of the reaction, attack of C5 on CH_2H_4folate, is supported by the formation of a stable covalent adduct between the 5-position of FdUMP and the one carbon unit attached to N5 of H_4folate and trapping of the steady-state intermediate **2** in Glu60 mutants.[6−8] It has been proposed that this covalent adduct breaks down to yield the exocyclic methylene intermediate **3**, followed by hydride transfer from 5,6,7,8-tetrahydrofolate (H_4folate) and subsequent elimination of cysteine to produce dTMP and 7,8-dihydrofolate (H_2folate).[2]

To date, there is no direct evidence for the C5 exocyclic methylene intermediate **3** in the TS reaction. However, its formation is supported by nonenzymatic reactions of 5-(hydroxymethyl)uracil derivatives[9] and mechanistic studies of 2′-deoxyuridylate hydroxymethylase.[10] 5-(Hydroxymethyl)uracil esters and ethers undergo facile substitution reactions as a result of addition of nucleophiles to the C6 position to form the C5 enolate which then undergoes a rapid elimination−addition reaction at C7. The presence of an exocyclic methylene intermediate in this substitution reaction is evidenced by its trapping and formation of the C5 methyl derivative when substitution reactions are carried out in the presence of sodium borohydride.[9] The enzyme 2′-deoxyuridylate hydroxymethylase converts dUMP to 5-hydroxymethyl dUMP. Tritium exchange experiments and the isolation of a FdUMP covalent adduct suggest that this enzyme uses the same mechanism as thymidylate synthase.[10] However, instead of hydride transfer from the H_4-folate to the methylene group of intermediate **3**, H_4folate is released from the enzyme and water is added to the putative C5 exocyclic methylene group. Here, we provide evidence for the formation of the C5 methylene intermediate **3** in the TS reaction from trapping experiments using the Trp82Tyr mutant of the *Lactobacillus casei* TS.

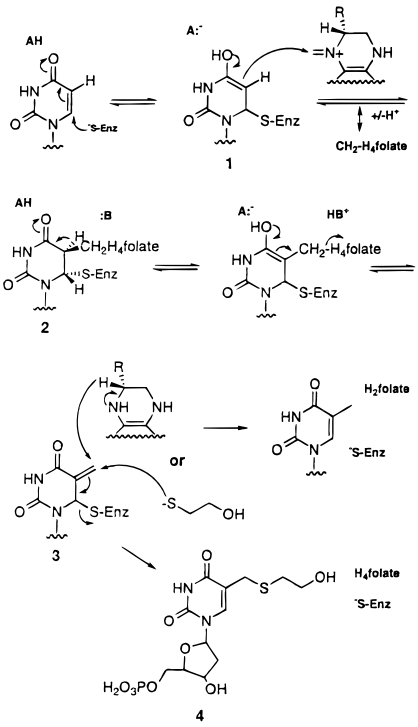

Figure 1. A simplified mechanism for the reaction catalyzed by thymidylate synthase includes the putative intermediates **1**−**3** and the new reaction product **4**.

In the course of studying the kinetics of unnatural amino acid mutants of TS containing tryptophan analogues at position 82, a new product was discovered by reverse phase TLC analysis of the products arising from [2-C^{14}]dUMP.[11] To produce larger amounts of this product for characterization, we screened a series of natural amino acid mutants of Trp82 whose kinetics, but not product composition, had been previously characterized.[12] Under the assay conditions (25 °C, 300 μM CH_2H_4folate, 13 mM formalin, 50 mM $MgCl_2$, 2 mM EDTA, 150 mM β-mercaptoethanol, 10 μM dUMP, and 50 mM TES at pH 7.4), Trp82Gly produced 9-fold more of this new product than dTMP, Trp82Met and Trp82Tyr produced 4-fold more of the new product, and Trp82Asp, Trp82Ser, Trp82Val, and Trp82Ala produced roughly equal amounts of the new product and dTMP. Trp82His and Trp82Phe produced small but detectable quantities of this product, and Trp82Arg produced no new product or dTMP. Trp82Tyr, which has a k_{cat} of 0.034 s^{-1} for the production of dTMP (100-fold slower than wild-type enzyme), was determined to be the most efficient producer of the new product. The product was

* Author to whom correspondence should be addressed at the following: Department of Chemistry, University of California at Berkeley, Berkeley, CA 94720.
[†] Howard Hughes Medical Institute and University of California at Berkeley.
[‡] University of California at San Francisco.

(1) Santi, D. V.; Danenberg, P. V. *Folates and Pterins*; Wiley: New York, 1984; Vol. 1, pp 345−398.
(2) Carreras, C. W.; Santi, D. V. *Annu. Rev. Biochem.* **1995**, *34*, 721−62.
(3) Roberts, D. *Biochemistry* **1966**, *5*, 3546−3548.
(4) Montfort, W. R.; Perry, K. M.; Maley, E. B.; Stroud, R. M. *Biochemistry* **1990**, *29*, 6964−6977.
(5) Wataya, Y.; Santi, D. V. *J. Am. Chem. Soc.* **1977**, *99*, 4535−36.
(6) Matthews, D. A.; Villafranca, J. E.; Janson, C. A.; Smith, W. W.; Welsh, K.; Freer, S. *J. Mol. Biol.* **1990**, *214*, 937−938.
(7) Santi, D. V.; McHenry, C. S.; Sommer, H. *Biochemistry* **1974**, *13*, 471−481.
(8) Huang, W.; Santi, D. V. *J. Biol. Chem.* **1994**, *269*, 31327−31329.
(9) Santi, D. V.; Pogolotti, A. L. *J. Heterocycl. Chem.* **1971**, *8*, 265−172.
(10) Kunitani, M. G.; Santi, D. V. *Biochemistry* **1980**, *19*, 1271−1275.

(11) Aliquots of enzyme assays with dUMP [2-^{14}C]dUMP were quenched with an equal volume of aqueous 1.5 M HCl and then spotted onto a C18 silica plate and developed for 30 min in a TLC chamber with aqueous 2 M NaH_2PO_4. The plates were then dried and exposed overnight on phosphorimager plates for quantitation: dUMP had an R_f of 0.7 and dTMP of 0.5.

(12) Kealey, J. T.; Echstein, J.; Santi, D. V. *Chem. Biol.* **1995**, *2*, 609−614.

S0002-7863(97)03210-1 CCC: $15.00
Published on Web 01/21/1998

isolated from a 30 mL reaction containing 500 μg (0.5 μM) of Trp82Tyr, 300 μM CH_2H_4folate, 13 mM formalin, 50 mM $MgCl_2$, 2 mM EDTA, 150 mM β-mercaptoethanol, 160 μM dUMP, and 50 mM TES at pH 7.4. The product was purified by reverse phase C18 HPLC using a linear gradient of aqueous 0.1% TFA and acetonitrile (0 to 100%), followed by purification on the same column using a linear gradient of aqueous 10 mM acetic acid and acetonitrile (0 to 100%).

NMR and electrospray mass spectroscopy were used to identify the purified product as the β-mercaptoethanol adduct **4** of the exocyclic methylene intermediate **3**. The ^{1}H NMR spectrum in D_2O reveals the presence of an anomeric proton at 6.39 ppm and the C3′, C4′, C5′ and C2′ protons at 4.62, 4.24, 4.13, and 2.24 ppm, respectively.[13] The C6 proton of the base at 7.96 ppm is shifted slightly downfield from that in dTMP due to the through σ bond inductive effect from the substitution of one of the hydrogens on C7 with a sulfur. The singlet at 3.59 ppm corresponds to the C7 methylene protons of the thymidine ring of **4** and the two triplets at 2.74 and 3.78 ppm correspond to the ethylene protons of the mercaptoethanol moiety. Electrospray mass spectrometry reveals a peak at 399 corresponding to the parent MH^+ ion of structure **4**. A series of high-resolution electrospray experiments produced a mean mass of 399.0627 with a standard deviation of 0.0015 (6 measurements), precisely in agreement with the calculated exact mass of 399.0627.[14] Derivatization of the reaction product with *N*,*O*-bis(trimethylsilyl)-trifluoroacetimide (BSTFA) and 1% trimethylchlorosilane (TMCS) resulted in a low-resolution FAB spectrum with an intense peak at 615 and a weaker one at 687, corresponding to the addition of three and four silyl groups to structure **4**, respectively.[15] High-resolution FAB of the 687 peak gave a mass of 687.2221, very close to the predicted mass of 687.2208. The λ_{max} of 270 nm in the UV spectrum of the product is at a slightly longer wavelength than that of dTMP, consistent with enhanced hyperconjugation of the C5 methylene group with the pyrimidine ring. The above analysis indicates that the new product **4** results from the trapping of the proposed intermediate **3** with β-mercaptoethanol which is present in the reaction mixture. Trapping of intermediate **2** with β-mercaptoethanol by an S_N2 mechanism to yield **4** is mechanistically unlikely within the steric constraints of the enzyme active site. Moreover, Glu60 mutants which are known to stall TS at intermediate **2** do not produce detectable levels of **4**. Thus, the observation of trapped product **4** provides the first direct experimental evidence for the exocyclic methylene intermediate **3** in the TS reaction. It rules out homolytic cleavage of the bond between N5 of the folate and the methylene in intermediate **2**, which has previously been proposed as a possible mechanistic pathway.[16]

Wild-type thymidylate synthase does not produce detectable levels of product **4**, while nine mutants at site 82 produce this product. The tryptophan at position 82 in the active site of *L. casei* thymidylate synthase is conserved in all thymidylate synthase enzymes characterized to date.[2] In crystal structures of the complex of the enzyme with substrate and cofactor this tryptophan is in direct contact with C7 and C9 of the pterin moiety of the folate cofactor.[4,17,18] Kinetic analysis of Trp82 mutants suggested that this residue is not essential for catalysis, although the mutants did have increased K_m values for CH_2H_4folate of 2- to >25-fold relative to WT enzyme.[12] However, the above results suggest that the interaction of Trp82 with folate plays a key role in the final transfer of hydride to intermediate **3** to produce the dTMP−enzyme adduct. In the tryptophan mutants, the H_4folate cofactor may be bound weakly or positioned suboptimally for hydride transfer. Alternatively, Trp82 may play an electronic role in facilitating the redox reaction. In either case, the likely result of mutations at Trp82 is premature release of unoxidized H_4folate followed by binding of β-mercaptoethanol in the active site and adduct formation with the exocyclic methylene group of intermediate **3**. Surprisingly, we have found that substitution of close steric analogues of tryptophan, such as 7-azatryptophan, into position 82 results in the formation of significant amounts of product **4**. The role this tryptophan residue plays in catalyzing the last, and least understood, hydride transfer step in the conversion of dUMP to dTMP by thymidylate synthase will be the focus of future work.

Acknowledgment. We are grateful for financial support for this work from the National Institute of Health (grant no. R02GM49220). We also thank D. S. King for the initial low-resolution electrospray MS and advice on characterization and David Wemmer for advice on the NMR. P.G.S. is a Howard Hughes Medical Institute Investigator and W. M. Keck Foundation Investigator. High-resolution mass spectrometry was provided by the UCSF Mass Spectrometry Facility (A. L. Burlingame, Director) supported by the Biomedical Research Technology Program of the National Center for Research Resources, NIH NCRR BRTP 0614.

JA973210T

(13) ^{1}H NMR (500 MHz, D_2O): δ 2.42 (m, 2H), 2.74 (t, 2H, J = 6.5 Hz), 3.59 (s, 2H), 3.78 (t, 2H, J = 6.5 Hz), 4.13 (m, 2H), 4.24 (m, 1H), 4.62 (m, 1H), 6.39 (d, 1H, J = 7 Hz), 7.96 (s, 1H).

(14) High-resolution mass spectrometry data were obtained on a PerSeptive Biosystems Mariner Biospectrometry Workstation, an electrospray time-of-flight mass spectrometer operating at approximately 5500 resolution (full-width half-height definition). The exact masses were obtained using an internal calibration of the data with adenosine diphosphate (MH^+ = 428) and guanosine monophosphate (MH^+ = 364) as internal standards.

(15) Less than 2 nM of **4** was derivatized with 10% pyridine, 1% TMCS, and 89% BSTFA for 20 min at 60 °C; the products were then directly injected into the mass spectrometer.

(16) Kagel, J. R.; Wang, B.; Mertes, M. P. *J. Org. Chem.* **1993**, *58*, 2738−2746.

(17) Matthews, D. A.; Appelt, K.; Oatley, S. J.; Xuong, N. H. *J. Mol. Biol.* **1990**, *214*, 923−936.

(18) Fauman, E. B.; Rutenber, E. E.; Maley, G. F.; Maley, F.; Stroud, R. M. *Biochemistry* **1994**, *33*, 1502−1511.