THYMIDYLATE SYNTHETASE CATALYZED DEHALOGENATION OF 5-BROMO-AND 5-IODO-2'-DEOXYURIDYLATE

Yusuke Wataya and Daniel V. Santi

Department of Biochemistry and Biophysics and
Department of Pharmaceutical Chemistry
University of California
San Francisco, California 94143

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SUMMARY: Thymidylate synthetase catalyzes a facile dehalogenation of 5-bromo-and 5-iodo-2'-deoxyuridylate in the presence of dithiothreitol. The chloro and fluoro nucleotides are not dehalogenated under similar conditions. A mechanism for this reaction is proposed which is in complete accord with previously ascertained features of the catalytic mechanism as well as model chemical reactions. This reaction is likely an important pathway in the biological dehalogenation of 5-halogenated uracil derivatives.

Thymidylate synthetase catalyzes the reductive methylation of 2'-deoxyuridylate (dUMP) to thymidylate with the concomitant conversion of 5,10-methylenetetrahydrofolic acid (CH_2FAH_4) to 7,8-dihydrofolic acid. Studies of chemical counterparts (1-3) of this reaction and the interaction of the quasi-substrate 5-fluoro-2'-deoxyuridylate (FdUMP) (4,5) have yielded much information regarding the mechanism of the enzymatic reaction. From such investigations, it has become apparent that a primary event in catalysis involves the addition of a nucleophilic group of the enzyme to the 6-position of the nucleotide substrate.

Although other 5-halogenated derivatives of dUMP have been reported to inhibit this enzyme (6,7), the interactions have not been examined in detail. Since 5-halogenated pyrimidine derivatives undergo facile addition of nucleophiles to their 6-positions (8-10), we suspected that they might interact with thymidylate synthetase in a more complicated manner than simply as reversible inhibitors. In this report we describe results which demonstrate that 5-bromo-and 5-iodo-2'-deoxyuridylate (BrdUMP and IdUMP) are rapidly dehalogenated by thymidylate synthetase to yield dUMP. In addition to the interesting mechanistic aspects of this reaction, it is

likely that this reaction represents an important pathway for enzymatic dehalogenation of 5-halogenated uracil derivatives.

MATERIALS AND METHODS

Thymidylate synthetase was obtained from an Ametopterin resistant strain of *Lactobacillus casei* (11). Partially purified enzyme was purchased from the New England Enzyme Center and further purified by recrystallization from 25% $(NH_4)_2SO_4$ solution at pH 7.1 and 4°. Using assay conditions previously described (12) this enzyme showed a specific activity of 3.1 μmole/min/mg using ε_{278} 1.07 x 10^5 for protein determination at 30°. BrdUMP and IdUMP were obtained from Sigma Chemical Company. 5-CldUMP was prepared by the method of Michelson (13). Solutions used for assays of the dehalogenation reaction (Buffer A) contained 6.5 m*M* DTT (dithiothreitol), 25 m*M* $MgCl_2$, 1 m*M* EDTA and 50 m*M* N-methylmorpholine·HCl (pH 7.4) in a total volume of 1.0 ml. Assays were performed at 25° and uv spectra were measured using a Cary 118 ultraviolet spectrophotometer.

Reaction products were separated by two dimensional paper chromatography on Whatmann 3 MM paper. The first dimension was isopropanol-28% NH_4OH-H_2O (7:1:2) and the second dimension was isobutyric acid - 0.5*N* NH_4OH (5:3). Polyethyleneimine (PEI) thin layer chromatography was performed with Polygram Cel 300 UV_{254} (Macherey-Nagel & Co.) with 0.05 M $(NH_4)_2CO_3$ pH 9.1 as eluant. 5-Trifluoromethyl-2'-deoxyuridylate was prepared from the nucleoside (P-L Biochemicals, Inc.) with thymidine kinase from *E. coli* (14). All other materials have been previously described (4).

RESULTS

Figure 1 shows the ultraviolet spectral changes which occur upon treatment of 5-BrdUMP or 5-IdUMP with thymidylate synthetase. In both cases there is a time dependent change of the absorption maximum of the halogenated substrates to 262 nm. No spectral changes were observed when the enzyme was omitted, or with 5-Cl or 5-FdUMP as potential substrates;

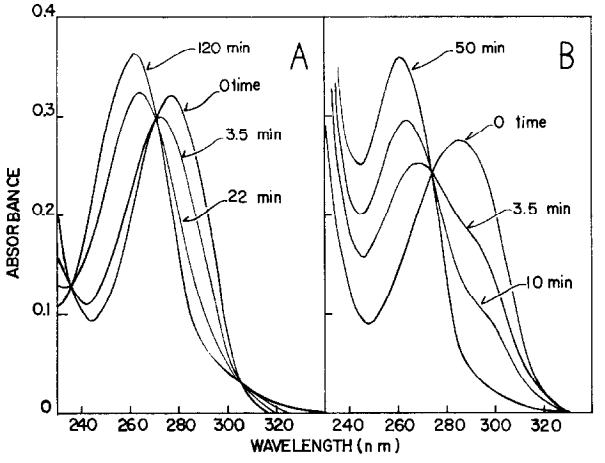


Figure 1. Ultraviolet spectral changes of (A) 40 μM BrdUMP and (B) 38 μM IdUMP in 1.0 ml of Buffer A containing 0.6 μM thymidylate synthetase.

similarly, destruction of the enzyme (0.8 μM) by pre-treatment with 5-trifluoromethyl-2'-deoxyuridylate (3.7 μM) for 30 min. at room temperature decreased the rate of reaction of 5-IdUMP by at least 95%. That the reaction is *catalyzed* by the enzyme is demonstrated by the fact that complete conversion occurs at levels of enzyme which are approximately 500 times lower than that of the substrate used. The initial velocity, as monitored by the decrease in optical density at 290 nm was found to be linear over the range of 0.01-1 μM thymidylate synthetase, using 0.1 mM substrate (BrdUMP). The K_m for BrdUMP, under the conditions described here is *ca.* $8 \times 10^{-6} M$; dUMP is a competitive inhibitor with $K_i \simeq 3 \times 10^{-6} M$.

The reaction product obtained upon treatment of BrdUMP and IdUMP with thymidylate synthetase was ascertained to be dUMP by the following criteria: (*i*) The ultraviolet maximum of the product is identical to that of dUMP (λ_{max} 262 nm). (*ii*) The product of the reaction of thymidylate synthetase and BrdUMP was analyzed by two-dimensional paper chromatography. It moved as a single major ultraviolet absorbing spot, having R_f values identical to that of dUMP. Elution of this spot gave a product which had an ultraviolet spectrum identical to that of dUMP in acidic and alkaline pH regions

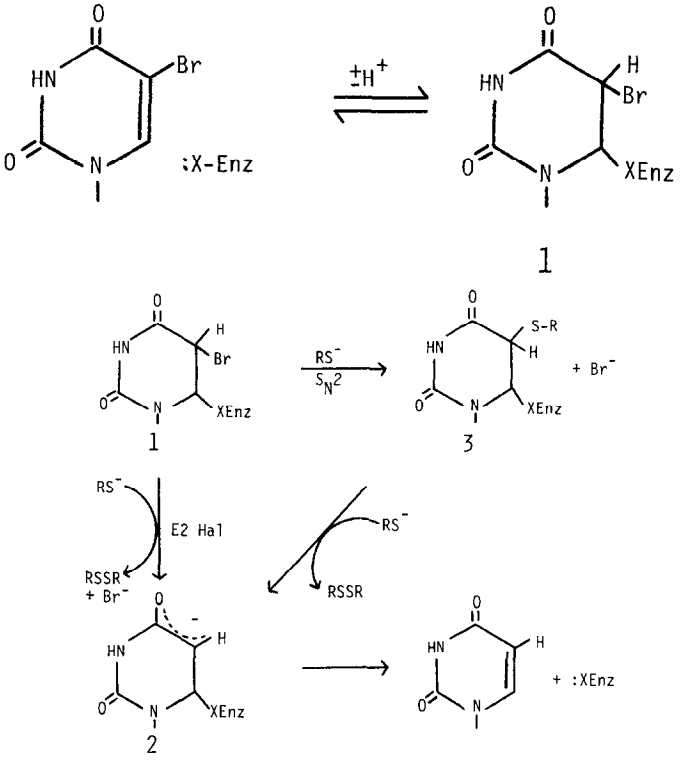
and gave the same R_f value as dUMP on PEI-thin layer chromatography. (*iii*) After completion (2hrs.) of the reaction depicted in Figure 1, excess CH_2FAH_4 was added to the reaction mixture to give a final concentration of 0.18 m*M* and the increase in absorbance at 340 nm which is characteristic of the formation of TMP and FAH_2 from dUMP and CH_2FAH_4 was monitored. From $\Delta\varepsilon_{340}$ = 6400 (15), the products obtained from BrdUMP and IdUMP were ascertained to be 89 and 100% dUMP, respectively. It is also noted that the dehalogenation of IdUMP is accompanied by a corresponding increase in absorbance between 230-260 nm which is assignable to end absorption of I^-; Br^- does not absorb in this region and the conversion of BrdUMP to dUMP shows a well-defined isosbestic point at 236 nm.

DISCUSSION

The data presented here demonstrates that thymidylate synthetase catalyzes a facile dehalogenation of BrdUMP and IdUMP to give dUMP, the natural substrate of the enzyme. Although model reactions have permitted proposals for biological dehalogenation of 5-bromo and 5-iodouracil derivatives (8-10,16), the enzymatic pathways which are operative in vivo have not been elucidated. It appears likely that one important pathway involves dehalogenation at the deoxyribonucleotide level by thymidylate synthetase. With CH_2FAH_4, the overall reaction involves conversion of these halogenated nucleotides to TMP and represents a route by which they may be utilized as precursors of DNA. This fact warrants consideration and care in experiments involving in vivo incorporation of 5-bromouracil into nucleic acids.

A primary event of the thymidylate synthetase reaction involves attack of a nucleophile of the enzyme to the 6-position of dUMP (1-3). Since a similar reaction occurs with FdUMP (4,5), and the chemical dehalogenation of 5-bromo and 5-iodouracils require nucleophilic attack at the 6-position (8-10,16), it is likely that a similar reaction initiates the thymidylate synthetase catalyzed dehalogenation of 5-Br-and 5-IdUMP. Supporting this is the observation that dUMP is a competitive inhibitor of the debromina-

tion of BrdUMP. Thus, we propose that the 5-bromo (iodo)-5,6-dihydro-dUMP derivative, 1, is the first important intermediate formed in these reactions.



As proposed for the chemical models, there are two general mechanisms, with variations, which may account for the subsequent steps. The first (E2 Hal) involves abstraction of bromonium (Br^+) or iodonium (I^+) by DTT, or an intermediary, to provide intermediate 2 and a sulfenyl halide. The latter will rapidly disproportionate to the halide ion and oxidized thiol, and 2 will undergo a β-elimination to yield dUMP and the unmodified enzyme. The second mechanism (S_N2) involves nucleophilic displacement of Br^- from 1 by thiolate to give the intermediate 3. Further reaction with RS^- would yield the oxidized thiol (R-SS-R) and intermediate 2 which is common with the E2 Hal mechanism and would yield dUMP upon β-elimination of the enzyme.

Clearly, the simplicity of this reaction should permit investigations of aspects of the mechanism and interactions of thymidylate synthetase

which were not previously possible. Such studies are currently in progress.

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