INHIBITION OF THYMIDYLATE SYNTHETASE BY 5-FORMYL- AND 5-HYDROXYMETHYL-2'-DEOXYURIDYLATE

Daniel V. Santi and Ted T. Sakai

Department of Chemistry
University of California
Santa Barbara, California 93106

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SUMMARY: 5-Formyl-2'-deoxyuridylic acid has been found to be an extremely potent inhibitor of thymidylate synthetase from Escherichia coli B, competitive with 2'-deoxyuridylate and showing a K_i value of 1.3×10^{-8} M. The related 5-hydroxymethyl-2'-deoxyuridylate has no substrate properties, but is a competitive inhibitor with respect to 2'-deoxyuridylate ($K_i = 1.05 \times 10^{-5}$ M). The data presented argue against the possibility of free formaldehyde as the electrophilic reactant.

Thymidylate synthetase catalyzes the conversion of 2'-deoxyuridylate (dUMP) to thymidylate (TMP) by transfer of the methylene group of 5,10-methylenetetrahydrofolic acid (5,10-CH_2FAH_4) to the 5-position of the pyrimidine followed by a disproportionation which leads to 7,8-dihydrofolate (7,8-FAH_2). Because this enzyme represents one of the two anabolic pathways for cellular synthesis of thymidylate, considerable efforts have been devoted to an understanding of its mechanism as well as methods for its blockage (1-3). We describe herein our preliminary studies of 5-formyl-2'-deoxyuridylate (formyl-dUMP) which appears to be one of the most potent inhibitors of thymidylate synthetase. 5-Hydroxymethyl-2'-deoxyuridylate (HMdUMP) has also been examined as an inhibitor and possible intermediate and the data permit some conclusions regarding the enzymic reaction.

Materials and Methods

Thymidylate synthetase from E. coli B was prepared by the method of Wahba and Friedkin (4) with the exception that DEAE-cellulose chromatography was performed with a linear gradient from 0.05 M potassium phosphate (pH 7.0) to 0.3 M potassium phosphate (pH 6.5). The preparation produced 50 mμmoles TMP/mg protein/min at 25°. The standard assay contained 0.82 mM dUMP, 0.12 mM

FAH_4, 6.5 mM formaldehyde, 25 mM $MgCl_2$, 1 mM EDTA, 75 mM 2-mercaptoethanol, and 50 mM N-methylmorpholine-HCl buffer (pH 7.40) in a total volume of 1.1 ml. The reaction was initiated by addition of a limiting amount of enzyme and monitored spectrophotometrically (5) at 25°. dUMP was omitted for controls and when one of the components was varied all others were held constant at the concentrations listed above. Velocities are given in mμmoles TMP produced/mg protein/min. 5-Hydroxymethyl-2'-deoxyuridine (HMUdR) was obtained by base-catalyzed hydroxymethylation of 2'-deoxyuridine (6). 5-Formyl-2'-deoxyuridine (formyl-UdR) was prepared by oxidation of 3',5'-di-O-(p-chlorobenzoyl)-5-hydroxymethyl-2'-deoxyuridine (7) with activated MnO_2 in methylene chloride followed by removal of the blocking groups in methanol in the presence of sodium methoxide catalyst. Spectral and chromatographic properties of the product obtained were identical to those reported (8,9). Both HMUdR and formyl-UdR were converted to their 5'-mononucleotides with carrot phosphotransferase (10). Concentrations of the nucleotides were measured by uv spectroscopy using the extinction coefficients of the corresponding nucleosides (9, 11).

Results and Discussion

Thus far, the most useful inhibitors of thymidylate synthetase have been those which bind competitively with dUMP for its binding site. These compounds are generally 5-substituted 2'-deoxyuridylates and are exemplified by TMP (12), 5-mercapto-dUMP (13), 5-fluoro-dUMP (FdUMP) (14), and 5-trifluoromethyl-dUMP (F_3TMP) (15). It appears that optimal inhibitory effects may be obtained if the 5-substituent is sufficiently small to avoid detrimental steric interactions at the binding site and if the substituent is electron-withdrawing; for example, FdUMP and F_3TMP bind two or three orders of magnitude tighter than dUMP or TMP. Inspection of CPK molecular models indicate that formyl and hydroxymethyl groups, both of which are electron-withdrawing, are no larger than the trifluoromethyl group, suggesting that both would be sterically accommodated by the dUMP binding site. Based on these rationalizations, 5-formyl- and 5-hydroxymethyl-2'-deoxyuridylate were prepared and evaluated as inhibitors

of thymidylate synthetase.

It was first shown that in the absence of dUMP, formyl-dUMP is not a substrate under standard assay conditions at concentrations up to 7 x 10^{-6} M, either in the presence or absence of formaldehyde. In Figure 1 are given double reciprocal plots of the inhibition data obtained with formyl-dUMP at varying dUMP (Figure 1A) and varying 5,10-CH_2FAH_4 (Figure 1B) concentrations. Formyl-dUMP is found to be a very good competitive inhibitor of dUMP with a K_i of 1.3 x 10^{-8} M and is a non-competitive inhibitor with respect to 5,10-CH_2FAH_4. Inhibition in both cases is linear with inhibitor concentration. It is noted that the inhibition produced is of the same magnitude as that of FdUMP and F_3TMP. Further studies are underway to determine the nature of the strong inhibition produced by formyl-dUMP which will hopefully provide insight into the mechanism of the enzymic reaction.

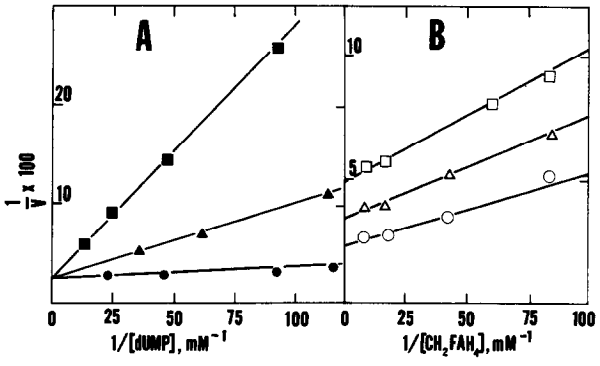

Figure 1. (A) Double reciprocal plots of data for formyl-dUMP at varying dUMP; inhibitor concentrations are 0 (●), 1.36 x 10^{-8} M (▲), and 4.5 x 10^{-8} M (■). (B) Double reciprocal plots at varying CH_2FAH_4 concentrations; inhibitor concentrations are 0 (○), 1.36 x 10^{-7} M (△), and 2.72 x 10^{-7} M (□).

One of the variations of the mechanism proposed for thymidylate synthetase involves the reaction of dUMP with free formaldehyde transported to the active site by FAH_4 to give HMdUMP as an intermediate which undergoes subsequent reduction by FAH_4. This has been rejected on the basis that HMdUMP is not detected in the course of reaction and that there is no synthesis of TMP from HMdUMP in the presence of thymidylate synthetase from phage-infected E. coli

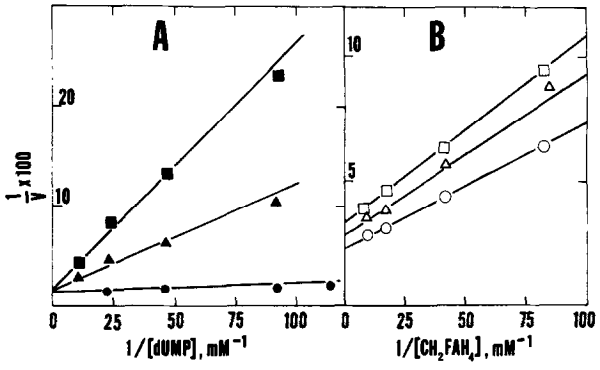

Figure 2. (A) Double reciprocal plots of data for HMdUMP at varying dUMP and inhibitor concentrations of 0 (●), 4.97 x 10^{-5} M (▲), and 8.94 x 10^{-5} M (■). (B) Double reciprocal plots at varying CH_2FAH_4; inhibitor concentrations are 0 (○), 2.15 x 10^{-5} M (△), and 4.3 x 10^{-5} M (□).

(16) or rat embryo (17). However, it occurred to us that an intermediate formed in an enzymic reaction which is not released may, for numerous reasons, not bind to the active site if supplied to the enzyme as an external substrate.

Although earlier workers have observed that HMdUMP inhibits thymidylate synthetase (16,17), the nature and magnitude of inhibition was not ascertained. As shown in Figure 2, HMdUMP is a good competitive inhibitor with respect to dUMP (K_i = 1.05 x 10^{-5} M) and noncompetitive with respect to 5,10-CH_2FAH_4, suggesting that this analog is bound to the same site of thymidylate synthetase as is dUMP. In the presence or absence of formaldehyde, substitution of HMdUMP for dUMP in the standard assay mixture did not result in the formation of any detectable amounts of TMP. These results support the earlier conclusions that HMdUMP is not an intermediate in the thymidylate synthetase reaction. Thus, if free formaldehyde is proposed to be the reactive electrophilic species in the enzymic reaction, a mechanism must be forwarded which does not include the formation of HMdUMP as an intermediate.

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