Biochemistry

 Volume 19, Number 7 April 1, 1980

On the Mechanism of 2′-Deoxyuridylate Hydroxymethylase†

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ABSTRACT: dUMP hydroxymethylase from SP01-infected *Bacillus subtilis* has been purified 160-fold by chromatography on DEAE-cellulose and ethylagarose. The enzyme catalyzes exchange of the 5-hydrogen of dUMP for protons of water in the presence or absence of the cofactor CH_2-H_4folate. Upon treatment with FdUMP and CH_2-H_4folate, an isolable covalent complex is formed which is believed to be structurally similar to a steady-state intermediate of the normal reaction. The FdUMP–CH_2-H_4folate–dUMP hydroxymethylase complex is stable toward denaturation with sodium dodecyl sulfate and shows a subunit molecular weight of 46 000. By analogy with chemical models and studies of dTMP synthetase, a mechanism is proposed for the reaction catalyzed by dUMP hydroxymethylase.

dUMP hydroxymethylase catalyzes the conversion of dUMP and CH_2-H_4folate to HmdUMP and H_4folate. The enzyme is found in *Bacillus subtilis* infected with certain bacteriophage (Hemphill & Whiteley, 1975) and is analogous to the dCMP hydroxymethylase found in T-even phage infected *Escherichia coli*. dTMP synthetase also catalyzes a similar reaction except that after transfer the 1-carbon unit is reduced by H_4folate, resulting in the products dTMP and H_2folate. Because all three enzymes utilize the same cofactor and all involve electrophilic substitution at the 5 position of the pyrimidine heterocycle, it is reasonable to believe that they might share common mechanistic features. In particular, model chemical studies and precedent established with dTMP synthetase have resulted in the proposal that all such reactions proceed by initial nucleophilic attack at the 6 position of the pyrimidine heterocycle; in this manner, the 5 position is made sufficiently electronegative for subsequent reaction with the electrophile [for a review see Pogolotti & Santi (1977)].

We have partially purified dUMP hydroxymethylase from SP01-infected *B. subtilis* and have examined aspects of its catalytic properties and interaction with FdUMP. The results described here demonstrate that salient features of the mechanism of this enzyme are quite similar to those which have been established with dTMP synthetase. Further, an analogous mechanism is proposed for dCMP hydroxymethylase.

Materials and Methods

Bacillus subtilis 3610 *thy tlr* and *B. subtilis* phage SP01 were a gift from A. R. Price. Nitrocellulose filters (Bac-T-flex; 2.4 cm) were purchased from Schleicher & Schuell. Ethylagarose (Er-el et al., 1972) was obtained from Miles Biochemicals and HmdUrd was obtained from Calbiochem. dUMP was obtained from P-L Biochemicals and FdUrd was a gift from the Drug Development Branch of the National Cancer Institute. (±)-L-H_4folate was prepared by the method of Hatefi et al. (1960). The (±)-L-[6-^{3}H]H_4folate used was the preparation previously described (Santi et al., 1976). [2-^{14}C]dUrd, [5-^{3}H]dUrd, [6-^{3}H]dUrd, and [6-^{3}H]FdUrd were obtained from Moravek Biochemicals. Nucleoside 5′-monophosphates were prepared by using dThd kinase and purified as previously described (Wataya & Santi, 1977); further purification was performed by high-performance liquid chromatography (LC) using the systems described by Garrett et al. (1977) or by reverse-phase LC on a Lichrosorb C_{18} column (Altex; 4.6 × 250 mm) using 5.0 mM $(n\text{-Bu})_4N^+$-HSO_4^- and 5.0 mM potassium phosphate (pH 7.0) as the eluant; the flow rate was 1.0 mL/min and the effluent was monitored at 254 nm with an ISCO UA-5 spectrophotometer. Protein concentration was determined by the method of Bradford (1976). Buffer A refers to a solution containing 20 mM potassium phosphate (pH 7.0) and 10 mM 2-mercaptoethanol. Radioisotopes were counted in a Nuclear Chicago Isocap 300 using Aquasol II (New England Nuclear) or 0.4% Omnifluor (New England Nuclear) in 25% (v/v) Triton X-114 in xylene.

Enzyme Assays. dUMP hydroxymethylase activity was assayed at ambient temperature (21–23 °C) in a mixture containing in 0.25 mL of 0.1 M Tris-HCl (pH 7.5), 2.5 mM $MgCl_2$, 75 mM 2-mercaptoethanol, 6 mM formaldehyde, 0.5 mM (±)-L-H_4folate, 0.04 mM [5-^{3}H,2-^{14}C]dUMP (10 mCi/mmol ^{14}C; ^{3}H/^{14}C dpm = 6.7), and (1–50) × 10^{-5} unit of enzyme. Five to eight (50–30 μL) aliquots were removed at intervals and assayed for tritium release as previously described (Pogolotti et al., 1979). One unit of enzyme activity is defined as the amount required to catalyze release of tritium from 1 μmol of dUMP per min.

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0006-2960/80/0419-1271$01.00/0

The radioactive ternary FdUMP-CH_2-H_4folate–enzyme complex was formed in the mixture described above except specified amounts of [6-^{3}H]FdUMP (3.8 Ci/mmol) or [6-^{3}H]H_4folate (1.0 Ci/mmol) were used instead of labeled dUMP and unlabeled H_4folate, respectively; mixtures utilizing [6-^{3}H]H_4folate contained 0.08 mM unlabeled FdUMP instead of dUMP. Unless otherwise specified, incubations were performed for 30 min at ambient temperature. Controls omitted the nonradioactive ligand, either FdUMP or CH_2-H_4folate. The complex was isolated by adsorption to nitrocellulose filtration as previously described for dTMP synthetase (Santi et al., 1974) except 50 mM phosphate was used for washing and filters were dissolved and counted in Aquasol II. Alternatively, gel filtration was used to separate bound and free ligands. This was performed by applying ~0.12 mL of the reaction mixture to a Sephadex G-25 column (1.0 × 28 cm) equilibrated with 10 mM potassium phosphate (pH 7.0), 90 mM NaCl, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, and 10% (v/v) glycerol; elution was performed with the same buffer system. Fractions (0.5 mL) were collected and counted in the Triton X-114 system. For assays of the denatured complex, a 10% solution of $NaDodSO_4$ was added to the preformed complex to give a final concentration of 3%; after 30 min at room temperature, bound and free radioactive ligands were separated by gel filtration.

Enzyme Purification. Twelve liters of *B. subtilis* cells was grown to a density of 10^8 colony-forming units (A_{660} = 0.9) and infected with SP01 phage at a multiplicity of 5 phages/cell (Dunham, 1973). After 25 min at 37 °C, cells were rapidly chilled, harvested, and washed with cold 20 mM potassium phosphate (pH 7.0) as described by Alegria et al. (1968). The cells (31 g) were resuspended in 35 mL of buffer A and disrupted by using an Eaton press. After centrifugation at 10^4g for 10 min, 20 mL of 5% streptomycin was added to the supernatant, and the precipitate was removed by centrifugation at 20000*g* for 10 min. The cell extract (75 mL; 6.4 mg of protein/mL) was diluted with 65 mL of cold water to give a solution of 5 mmho/cm and applied to a DEAE-cellulose column (1.5 × 30 cm) previously equilibrated with buffer A. After being washed with 100 mL of buffer A, the column was eluted with 300 mL of a linear NaCl gradient (0–0.70 M) in the same buffer. Fractions (5 mL) possessing over 8×10^{-3} unit/mg (22–25 mmho/cm) were combined (30 mL; 1.05 mg of protein/mL) and diluted with water to give a solution with a conductivity of 5.5 mmho/cm. This was applied to an ethylagarose column (1.0 × 10.5 cm) previously equilibrated with buffer A–10 mM EDTA. After being washed with 16 mL of the equilibration buffer, protein was eluted with 40 mL of a linear NaCl gradient (60–640 mM) in the same buffer. Fractions (2 mL) were collected, and those containing dUMP hydroxymethylase activity (13–16 mmho/cm) were combined to give a 160-fold purified preparation. Preparations were usually stored at −80 °C after DEAE-cellulose chromatography and further purified in smaller portions shortly before use (see Results). In such cases protein was adsorbed to a smaller ethylagarose column (0.7 × 2.6 cm), washed with 7 mL of equilibration buffer, and eluted with 20 mL of the aforementioned NaCl gradient.

Gel Electrophoresis. Protein (10–20 μg/0.1 mL) was precipitated by addition of 0.25 volume of cold 50% trichloroacetic acid and collected by centrifugation at 3000*g* for 5 min. The precipitate was washed with 0.2 mL of cold acetone, collected by centrifugation, and dried under vacuum. The sample was dissolved in 50 μL of a solution containing 7% $NaDodSO_4$, 10% 2-mercaptoethanol, 20% glycerol, and 0.25 M Tris-HCl (pH 6.8) heated at 85 °C for 2 min. After twofold dilution with water, portions of the sample were applied to wells of a vertical slab gel apparatus (14 cm^2 × 0.8 mm) containing 0.1% $NaDodSO_4$–9% acrylamide as described by Laemmli (1970) and run for 2.5 h at 20 mA. Fluorography was performed as described by Bonner & Laskey (1974) using Kodak X-Omat R film. Gels were stained with Coomassie Blue (Laemmli, 1970) and scanned with an E-C densitometer. Prereplicative bacteriophage T4 proteins, a gift from P. Z. O'Farrell, were used as molecular weight standards (O'Farrell & Gold, 1973).

Table I

	total protein (mg)	sp act. (units/mg)	purifn	yield (%)
crude cell extract	480	1.10×10^{-3}		100
DEAE-cellulose	31.5	1.38×10^{-2}	12.5	82
ethylagarose	0.82	1.74×10^{-1}	158	27

Results

dUMP hydroxymethylase from SP01-infected *B. subtilis* was purified 158-fold in 27% yield by chromatography on DEAE-cellulose and ethylagarose (Table I). Other procedures attempted for purification were unsuccessful, largely because of losses encountered during manipulations or storage. As examples, chromatography on Sephacryl-200 or phenylagarose resulted in over 80% loss in yield and large decreases in specific activity. Likewise, NH_4SO_4 precipitation, dialysis and ultrafiltration led to large losses in recovery and specific activity. The stability of enzyme activity on storage was variable but in general decreased with increasing purity of the preparation. Preparations of highest purity lost significants amounts of activity within 2 weeks at 0, −20 (with or without 20% glycerin), and −80 °C. At −80 °C, the DEAE-cellulose purified preparation lost 20 and 50% activity at 2 and 4 weeks, respectively. We found it most advantageous if the enzyme was stored at −80 °C after DEAE-cellulose purification and chromatographed on ethylagarose shortly before use (see Materials and Methods). The enzyme preparations used in the experiments described below had a specific activity of at least 0.05 unit/mg of protein.

The assay described in this paper is based on the obligatory release of the 5-hydrogen of dUMP into water which accompanies hydroxymethylation. By use of [2-^{14}C,5-^{3}H]dUMP, tritium release is monitored as the $^3H/^{14}C$ remaining after evaporation of water; this double-label technique, utilized for dTMP synthetase (Pogolotti et al., 1979), is highly sensitive and not subject to manipulative errors. Excess formaldehyde is included in the assay mixture to minimize the reverse reaction; as the product H_4folate is formed, it is rapidly converted to the substrate CH_2-H_4folate. Under the conditions described, initial velocities of tritium release were linear vs. time to ~20% reaction and vs. enzyme to at least 5×10^{-4} unit. When enzyme was omitted or preincubated with excess FdUMP (see below), there was no detectable tritium release; omission of CH_2-H_4folate decreased the rate of tritium release by over 98%. Under the standard assay conditions for the tritium release assay, the K_m for dUMP was 4.6 μM.

The only other enzyme which is known to catalyze a CH_2-H_4folate-dependent release of tritium from the 5 position of dUMP is dTMP synthetase. Although the strain of *B. subtilis* used should not possess this activity, product analysis was performed to ensure that HmdUMP was the sole reaction product. [2-^{14}C,5-^{3}H]dUMP, excess CH_2-H_4folate, and hydroxymethylase were incubated for 16 h, mixed with unlabeled

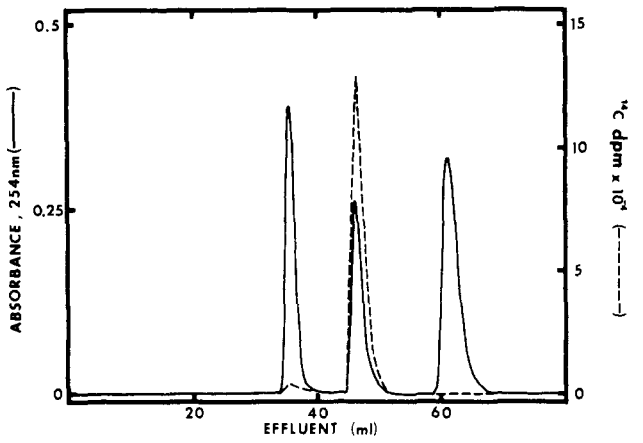

FIGURE 1: Reverse-phase chromatography of dUMP hydroxymethylase incubation. A mixture of [2-^{14}C,5-^{3}H]dUMP (^{3}H/^{14}C = 5.80), excess CH_2-H_4folate, and hydroxymethylase (2.4×10^{-3} unit/mL) were incubated as described under Materials and Methods for 16 h. Radioactivity was adsorbed and eluted from a small DEAE-cellulose column (Pogolotti et al., 1979), lyophilized, mixed with unlabeled markers of dUMP, HmdUMP, and dTMP, and chromatographed on reverse-phase LC. Compounds were monitored by absorbance at 254 nm (—), and radioactivity was monitored by collecting fractions (1.3 mL) and counting (- - -).

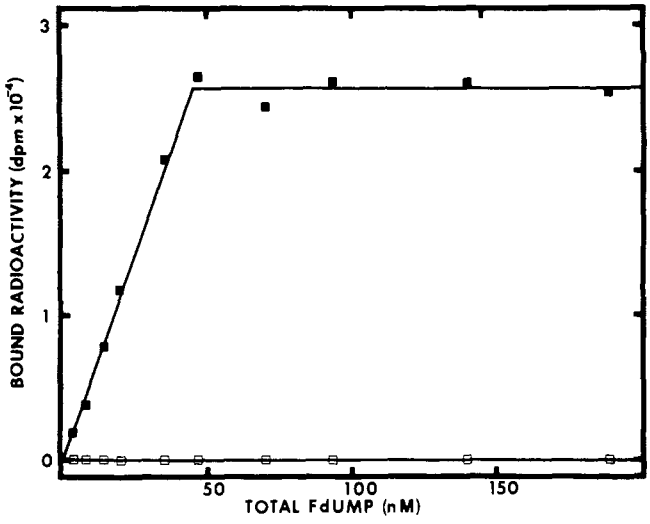

FIGURE 2: Titration of dUMP hydroxymethylase with [^{3}H]FdUMP in the presence (■) or absence (□) of CH_2-H_4folate (3.4×10^{-4} M). [6-^{3}H]FdUMP (3.8 Ci/mM) was incubated with dUMP hydroxymethylase (1.1×10^{-4} unit/mL), and the complex was adsorbed on nitrocellulose filters and quantitated as described under Materials and Methods.

markers of dUMP, HmdUMP, and dTMP, and chromatographed on LC (Figure 1). Of the recovered ^{14}C radioactivity (80% overall yield), 3% eluted as dUMP (RV = 35 mL), 97% with HmdUMP (RV = 46 mL), and less than 0.07% as dTMP (RV = 61 mL). It was also observed that the ^{3}H/^{14}C in the small amount of dUMP recovered had decreased from 5.80 to 0.06 (see below). In another experiment, product analysis by LC was directly compared to the 5-tritium release assay. By use of standard conditions, aliquots were removed at five intervals over a period of 60 min; 25 μL was assayed for tritium release and 50 μL was processed for LC. For the latter, the aliquot was added to 0.5 volume of an aqueous solution containing unlabeled HmdUMP and dUMP markers and 2 volumes of $CHCl_3$. After vortexing and centrifugation, the aqueous layer was chromatographed on reverse-phase LC, and the fractions corresponding to dUMP and HmdUMP were collected and counted. Comparison of the ^{3}H/^{14}C of the combined radioactivity eluting as dUMP and HmdUMP in each aliquot to the ^{3}H/^{14}C obtained by the tritium release assay demonstrated that the assays were in excellent agreement. Four of the five points were sampled over the first 20 min and were within 1% of each other; the error of the last (60 min) aliquot sampled was ~3% and, albeit acceptable, was probably due to counting error as the ^{3}H/^{14}C decreased from 5.8 to 1.6. In this experiment, it was also observed that the ^{3}H/^{14}C of the isolated dUMP decreased by a first-order process with $k = 0.42$ h^{-1}. Over the period used for initial velocity determinations by the tritium release assay, this corresponded to 37% of the rate of hydroxymethylation of dUMP or 27% of the rate of total tritium release.

When H_4folate and formaldehyde were omitted from the reaction mixture, the enzyme catalyzed a slow release of tritium from the 5 position of dUMP which proceeded at 1.5% the rate of tritium release in the presence of all components. This was confirmed by incubation of an excess of dUMP hydroxymethylase (0.01 unit/mL) with 2×10^{-5} M [2-^{14}C,5-^{3}H]dUMP, followed by reverse-phase LC; after 2 h, 70% of the tritium of the isolated dUMP was exchanged for protons of water and HmdUMP was undetectable (i.e., <0.01%). Although unlikely, it was considered possible that this slow exchange might be due to a contaminant of CH_2-H_4folate in the enzyme preparation. *Lactobacillus casei* dTMP synthetase (3.3 units/mL) was incubated with 9.2×10^{-3} unit/mL of the hydroxymethylase preparation and [6-^{3}H]dUMP (1.6 μM; 17 Ci/mmol) for 3 h in the dark to exclude this. As before, unlabeled dUMP and dTMP markers were added, and nucleotides were separated by LC and counted; radioactivity eluting with dTMP was undetectable. Since 2×10^{-11} M dTMP was our lower limit of detection, it was concluded that CH_2-H_4folate was not a significant contaminant of our hydroxymethylase preparation.

Pretreatment of dUMP hydroxymethylase with FdUMP and excess CH_2-H_4folate results in a decrease in the rate of tritium release from [2-^{14}C,5-^{3}H]dUMP with complete inhibition requiring 65 nmol of FdUMP per unit of enzyme. As with the covalent FdUMP–CH_2-H_4folate–dTMP synthetase complex (Santi et al., 1974a,b), when [6-^{3}H]FdUMP and CH_2-H_4folate are incubated with dUMP hydroxymethylase, radioactivity can be adsorbed to nitrocellulose filters under conditions where free [6-^{3}H]FdUMP may be completely removed by washing; no radioactive complex was adsorbed when CH_2-H_4folate was omitted from the mixture. In the presence of limiting [6-^{3}H]FdUMP and excess CH_2-H_4folate, increasing amounts of enzyme result in proportional increases in the radioactivity retained until apparent saturation is reached; from this experiment, the filtration efficiency (Yarus & Berg, 1967; Santi et al., 1974a) was calculated to be 89%. As shown in Figure 2, when [^{3}H]FdUMP was varied in the presence of a constant amount of dUMP hydroxymethylase and excess CH_2-H_4folate, the complex retained was proportional to the [6-^{3}H]FdUMP added, reaching saturation at 62 nmol of FdUMP per unit of enzyme. Since direct titration with [6-^{3}H]FdUMP corresponds well with FdUMP inhibition of the catalytic activity (62 vs. 65 nmol of FdUMP per unit), it is concluded that both assays reflect binding of FdUMP to dUMP hydroxymethylase.

Formation of the [6-^{3}H]FdUMP–CH_2-H_4folate–enzyme complex was complete within 30 s, and no loss of filter-bound radioactivity was observed for at least 3.5 h. The rate of dissociation of [^{3}H]FdUMP from the complex was determined by adding a 200-fold excess of unlabeled FdUMP to the preformed complex in the presence of 0.5 mM CH_2-H_4folate and monitoring the loss of adsorbed protein-bound radioactivity

with time (Santi et al., 1974b); upon dissociation, the bound radioactive ligand is diluted into a large nonradioactive pool and does not significantly recombine with the enzyme. At 25 °C, [^{3}H]FdUMP dissociates from the ternary complex with a first-order rate constant of 8.0×10^{-3} min^{-1}.

The ternary FdUMP–CH_2-H_4folate–dUMP hydroxymethylase complex could also be separated from free ligands by gel filtration on Sephadex G-25. By use of an excess of [^{3}H]FdUMP and CH_2-H_4folate to form the complex, macromolecular bound radioactivity in the void volume corresponded to 1.1 nmol of FdUMP per mg of protein; in the absence of CH_2-H_4folate all radioactivity eluted with free ligand. In a parallel experiment the ternary complex formed with [^{3}H]FdUMP was denatured with 3% $NaDodSO_4$ prior to gel filtration; the radioactivity in the void volume corresponded to 0.97 nmol of FdUMP per mg of protein, in good agreement with the value obtained with the native complex. Similarly, by use of [6-^{3}H]H_4folate to form the ternary complex, the denatured complex isolated by gel filtration possessed 0.95 nmol of H_4folate per mg of protein. $NaDodSO_4$ gel electrophoresis of the complex formed with either [^{3}H]FdUMP or [^{3}H]H_4folate showed a single radioactive band migrating with a M_r of 46 000; in the absence of either unlabeled ligand, the radioactive band could not be detected. When the gel was scanned after staining, it was determined that ~30% of the protein contained in the 158-fold purified preparation migrated with the labeled ternary complex.

Discussion

Release of tritium from [2-^{14}C,5-^{3}H]dUMP serves as a convenient assay for dUMP hydroxymethylase. In the presence of CH_2-H_4folate, 73% of the total tritium released during initial periods was due to HmdUMP formation and 27% resulted from exchange of the 5-tritium of dUMP for protons of water. The exchange reaction could be direct or due to reversal of hydroxymethylation. However, because of the excess formaldehyde used in the assay, almost all (>99.8%) of the product H_4folate would be trapped as the substrate CH_2-H_4folate; if the exchange reaction is due to reversal of hydroxymethylation, it is likely that this occurs while the reactants are still bound to the enzyme. If so, this would suggest that release of products from the enzyme is at least partially rate determining and that the proton released from the 5 position of dUMP exchanges with solvent protons at a rate competitive with or faster than dissociation of substrates. The enzyme also catalyzes exchange of the 5-hydrogen of dUMP for protons of water in the absence of CH_2-H_4folate at a rate which is 6% of the rate of exchange in the presence of CH_2-H_4folate. Thus, at least a portion of the observed 5-hydrogen exchange of dUMP in the presence of CH_2-H_4-folate likely results without the intermediate formation of HmdUMP. In this case, the cofactor may serve to decrease the K_m for dUMP and/or increase the V_{max} for the exchange reaction.

The dUMP hydroxymethylase catalyzed release of tritium from [2-^{14}C,5-^{3}H]dUMP, both from hydroxymethylation and exchange, is completely inhibited by FdUMP. When the enzyme is treated with [6-^{3}H]FdUMP and CH_2-H_4folate or with FdUMP and CH_2-[6-^{3}H]H_4folate, stable radioactive complexes can be isolated on nitrocellulose filters or by gel filtration; in either case, radioactive complexes could not be isolated if the unlabeled ligand was omitted. Confirmation that the complexes are due to binding of FdUMP and CH_2-H_4folate to dUMP hydroxymethylase was obtained by demonstrating a direct correspondence between the amount of ternary complex isolated and the degree of inhibition of

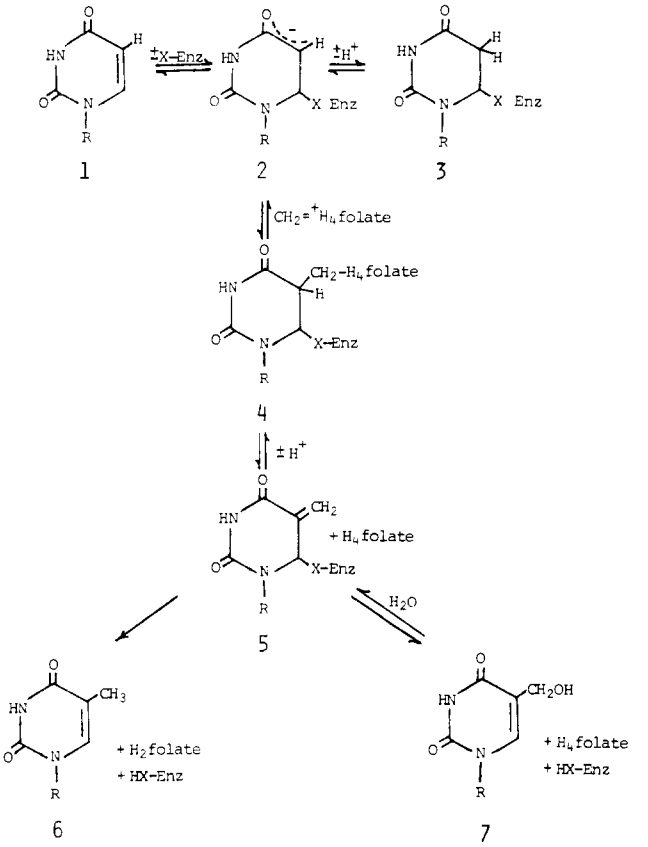

FIGURE 3: Proposed mechanisms for dTMP synthetase and dUMP hydroxymethylase; R = 5-phospho-2-deoxyribosyl.

enzyme activity. Formation of the [^{3}H]FdUMP–CH_2-H_4-folate–enzyme complex is too rapid to monitor by manual methods, and the ternary complex formed is stable for long periods. By use of an isotope dilution technique (Santi et al., 1974b), dissociation of [^{3}H]FdUMP from the ternary complex was shown to be a first-order process with $t_{1/2}$ = 87 min (k = 8.0×10^{-3} min^{-1}).

Denaturation of the ternary complex did not result in dissociation of ligands; indeed, the complex was stable to sequential treatment with Cl_3AcOH, acetone, and 7% $NaDodSO_4$ at 85 °C. $NaDodSO_4$ gel electrophoresis of the ternary complex labeled with either [^{3}H]FdUMP or CH_2-[6-^{3}H]H_4-folate, followed by staining and fluorography, demonstrated that the complex has a subunit molecular weight of 46 000 and that ~30% of the total protein in our purest preparation comigrated with the complex. These results conclusively establish that both FdUMP and CH_2-H_4folate are covalently bound to dUMP hydroxymethylase.

Aspects of the mechanism of dUMP hydroxymethylase may be surmised when the above findings are interpreted in view of what is known regarding model systems and the related enzyme dTMP synthetase. Direct chemical counterparts for the reactions catalyzed by dUMP hydroxymethylase are provided by studies of 5-hydrogen exchange and 5-hydroxymethylation of 1-substituted uracils (Santi & Brewer, 1968, 1973). In all cases examined, these reactions were found to require attack of a nucleophile at the 6 position of the heterocycle to activate the 5 position as an enolate or enol; after reaction with an electrophile (proton or formaldehyde), the resultant 5,6-dihydropyrimidine intermediates undergo β-elimination to provide products. The currently accepted mechanism of dTMP synthetase is shown in Figure 3. An early event in catalysis involves attack at the 6 position of dUMP by a nucleophile of the enzyme to provide the cova-

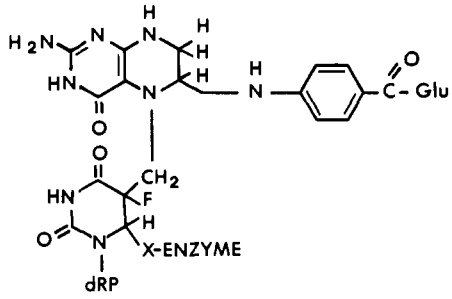

FIGURE 4: Structure of the FdUMP–CH_2-H_4folate–enzyme complex formed with dTMP synthetase or dUMP hydroxymethylase.

lently bound intermediate **2**. While not on the reaction pathway, **2** may react with solvent protons to form **3**; reversal of these steps results in exchange of the 5-H of dUMP for protons of water in the presence or absence (Pogolotti et al., 1979) of CH_2-H_4folate. Thus, there is both chemical precedent and enzymic precedent to support the conclusion that the dUMP hydroxymethylase catalyzed exchange of the 5-H of dUMP requires nucleophilic attack at the 6 position and formation of 5,6-dihydropyrimidine intermediates. Strong support for the mechanism of formation and structure of **4** has been obtained from studies of the interaction of FdUMP with dTMP synthetase. In the presence of CH_2-H_4folate, a nucleophile of the enzyme attacks the 6 position of FdUMP and the 5 position of FdUMP then reacts with the 1-carbon unit of the cofactor. The structure of the resultant complex (Figure 4) is directly analogous to steady-state intermediate **4** formed upon reaction of **2** with CH_2-H_4folate in the normal enzymic reaction.

With exception of the rates of dissociation and the subunit molecular weight, the properties of the FdUMP–CH_2-H_4-folate–hydroxymethylase complex also have the structure depicted in Figure 4, and its formation and relevance to catalysis are analogous to what have been described for dTMP synthetase (Santi et al., 1974a; Pogolotti & Santi, 1977).

From what has been described thus far, it is reasonable to conclude that dUMP hydroxymethylase and dTMP synthetase share a common pathway up to and including formation of the putative exocyclic methylene intermediate **5**. At this stage, the electrophilic carbon generated at the 6 position of the nucleotide reacts with hydride (or equivalent two electron, proton transfer) from C-6 of H_4folate in the dTMP synthetase reaction to provide dTMP (**6**) and H_2folate. In the dUMP hydroxymethylase reaction, intermediate **5** would react with water to provide HmdUMP (**7**) and H_4folate. It is of interest that the related enzyme dCMP hydroxymethylase also catalyzes 5-H exchange of dCMP in the absence of the cofactor CH_2-H_4folate (Yeh & Greenberg, 1967). As chemical studies have shown that this reaction also proceeds via nucleophilic attack at the 6 position of the heterocycle (Wechter & Kelly, 1970; Hayatsu, 1976; Blackburn et al., 1978), it is logical to conclude that the mechanism of dCMP hydroxymethylase is directly analogous to that which is described here for dUMP hydroxymethylase.

Acknowledgments

We thank Dr. Alan Price for providing the *B. subtilis* 3610 *thy tlr* and phage SP01. Dr. R. Meyer is acknowledged for performing computer analysis of kinetic data.

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