Kinetic and Equilibrium α-Secondary Tritium Isotope Effects on Reactions Catalyzed by dCMP Hydroxymethylase from Bacteriophage T4†

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ABSTRACT: Deoxycytidylate (dCMP) hydroxymethylase (CH) catalyzes the formation of 5-(hydroxymethyl)-dCMP, essential for DNA synthesis in phage T4, from dCMP and methylenetetrahydrofolate (CH_2THF). The nucleotide analog 5-fluorodeoxuridylate (FdUMP) stoichiometrically inactivates CH by formation of a covalent complex containing enzyme, FdUMP, and CH_2THF. Similar FdUMP complexes are formed by dTMP synthase and dUMP hydroxymethylase, enzymes which are homologous to CH. Both the association and the dissociation rate of the FdUMP complex are shown to be increased by the mutation of active site Asp^{179} to Asn. The mutated enzyme, CH(D179N), has an altered substrate preference, favoring dUMP rather than dCMP [Graves, K. L., et al. (1992) *Biochemistry 31*, 10315]. A value of 0.8 was determined for the α-secondary tritium equilibrium isotope effect on the binding of [6-^{3}H]FdUMP to wild-type CH and to CH(D179N), using a mixture of 2-^{14}C- and 6-^{3}H-labeled FdUMP. These effects, similar to that found for TS, indicate that C6 of the nucleotide is saturated (*i.e.*, sp^3 hybridized) in the covalent complex of CH, FdUMP, and CH_2THF. This strongly suggests that catalysis by CH proceeds *via* sequential $sp^2 \rightarrow sp^3 \rightarrow sp^2$ hybridization changes at C6 of substrate nucleotides, and it is consistent with a transient covalent linkage of C6 to the thiol of an essential CH residue, Cys^{148}. The values of the α-secondary ^{3}H kinetic isotope effect (KIE) on k_{cat}/K_M for CH-catalyzed formation of Hm^5dCMP caused by 6-^{3}H-substitution of dCMP, with both wild-type CH and CH(D179N), were very close to 1.0. However, the KIE for CH-(D179N) with dUMP was 0.82. The latter value is the expected inverse effect for sp^2 to sp^3 rehybridization of C6 either accompanying or preceding the first irreversible step in catalysis by CH(D179N). The value of 1.0 for the observed KIE with dCMP indicates that either (i) nucleophile addition to C6 occurs after the first irreversible step in catalysis or (ii) dissociation of the product Hm^5dCMP from the enzyme is the first irreversible step of the reaction. Either case indicates significant differences in the relative rates of individual catalytic steps in the turnover of dCMP *versus* turnover of dUMP, catalyzed by CH(D179N).

The product of phage T4 gene *42*, deoxycytidylate (dCMP)[1] hydroxymethylase (EC 2.1.2.8), is responsible for the formation of the unusual T4 DNA component 5-(hydroxymethyl)-cytosine (Flaks & Cohen, 1957). dCMP hydroxymethylase (CH) catalyzes the transfer of a methylene to dCMP from methylenetetrahydrofolate (CH_2THF) and the hydration of the methylene group, producing 5-(hydroxymethyl)-dCMP (Hm^5dCMP) and tetrahydrofolate (THF). In many respects, this reaction is analogous to the methylene group transfer to deoxyuridylate (dUMP) catalyzed by thymidylate synthase (TS). TS is a drug target enzyme intensively studied both structurally (Hardy et al., 1987; Matthews et al., 1990a,b; Monfort et al., 1990) and mechanistically (Santi & Danenburg, 1984). A catalytic mechanism for CH has been proposed, by analogy with that for TS (Subramaniam et al., 1989; Graves et al., 1992; see Scheme 1). Arguments favoring the structural and mechanistic homology of TS and CH have been presented previously (Graves et al., 1992; Butler et al., 1994; Graves, 1994). CH differs from TS in that the reaction catalyzed by CH is reversible (Butler et al., 1994). In contrast, TS catalyzes an essentially irreversible reduction of the transferred methylene, using a hydride contributed by tetrahydrofolate, to produce a methyl group and dihydrofolate.

A central feature of the proposed mechanisms of both TS and CH is the initial attack of an enzymic nucleophile (Cys^{148} in CH) upon C6 of the pyrimidine of substrate nucleotides (Pogolotti & Santi, 1977; Graves et al., 1992). This activates C5 of the pyrimidine for addition to the electrophilic methylene group of CH_2THF. The result is the transient formation of a 5,6-dihydropyrimidine intermediate in which carbon 6 is rehybridized from sp^2 to sp^3 (Scheme 1). Secondary hydrogen isotope effects provide useful tools to ascertain whether an enzyme-catalyzed reaction, or the covalent interaction of an inhibitor with an enzyme, involves rehybridization of the isotopically substituted carbon atom during or prior to the first irreversible step (Kirsch, 1977). The presence of an inverse α-secondary tritium isotope effect of 15% or greater (maximum $k_H/k_T = 0.80$) on a reaction, measured using 2-^{14}C,6-^{3}H-substituted pyrimidine nucleotides, is strong evidence for a rehybridization at C6 and the formation of a 5,6-dihydropyrimidine intermediate (Ivanetich & Santi, 1992). Such α-secondary tritium isotope effects have provided support for the proposed mechanism of catalysis by TS. The experiments described here provide measurements of α-secondary tritium isotope effects on equilibria and reaction

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[1] Abbreviations: CH, dCMP hydroxymethylase; CH_2THF, N^5,N^{10}-methylenetetrahydrofolate; dCMP, 2′-deoxycytidylate; dpm, disintegrations per minute; dUMP, 2′-deoxyuridylate; EIE, equilibrium isotope effect; FdUMP, 5-fluoro-2′-deoxyuridylate; FdCMP, 5-fluoro-2′-deoxycytidylate; Hm^5dCMP, 5-(hydroxymethyl)-2′-deoxycytidylate; Hm^5dUMP, 5-(hydroxymethyl)-2′-deoxyuridylate; KIE, kinetic isotope effect; RV, retention volume; TS, thymidylate synthase.

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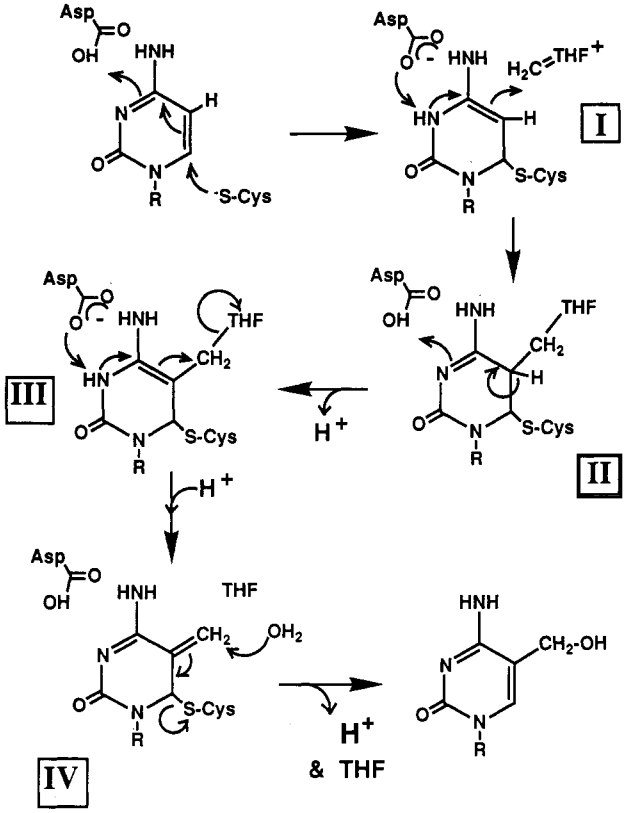
Scheme 1: Proposed Mechanism for Catalysis by dCMP Hydroxymethylase



kinetics involving CH. These effects support the mechanism shown in Scheme 1.

Many of the mechanistic details for catalysis by TS have been obtained by experiments with the substrate analog 5-fluoro-dUMP (FdUMP; Lewis & Dunlap, 1981; Santi & Danenburg, 1984). FdUMP is the metabolically activated form of the anticancer drug 5-fluorouracil. Like TS, CH is inactivated by FdUMP and binds FdUMP covalently in the presence of CH_2THF. A similar interaction with FdUMP has been reported for dUMP hydroxymethylase encoded by *Bacillus subtilis* bacteriophage SP01 (Kunitani & Santi, 1980). Secondary isotope effects have been used previously to study the covalent interactions of TS with FdUMP and CH_2THF (Bruice et al., 1980; Bruice & Santi, 1982). For TS complexed with FdUMP and CH_2THF, there is an inverse equilibrium isotope effect (EIE) and a normal kinetic isotope effect (KIE) on complex dissociation (Bruice et al., 1980) caused by ^3H substitution at C6 of FdUMP. The isotope effects seen with TS are consistent with saturation at C6 in the complex caused by covalent attachment of a Cys thiol. This approach has been extended in the present studies to the interaction of FdUMP with wild-type CH and a variant of the enzyme, CH(D179N),[2] containing an active site mutation which increases the avidity of the enzyme for FdUMP.

MATERIALS AND METHODS

Materials. ^3H- and ^{14}C-labeled nucleotides and nucleosides were obtained from Moravek Biochemicals, Brea, CA. [6-^3H]-dCMP was obtained by phosphorylating 0.013 μmol of [6-^3H]-deoxycytidine ([6-^3H]dCtd) (19 Ci/mmol) using recombinant *Varicella zoster* thymidine kinase (a kind gift from Wellcome Laboratories) as described (Avertt et al., 1991) and purified by anion-exchange HPLC (Graves, 1994). Unlabeled CH_2-THF was prepared as previously described (Graves et al., 1992). [11-^{14}C]CH_2THF was prepared from THF and [^{14}C]formaldehyde (Butler et al., 1994). The stated concentrations of CH_2THF have been corrected for the purity of the L-(6*R*) isomer. CH (wild type and CH(D179N)) were prepared and assayed as previously described (Graves et al., 1992; Graves, 1994). All CH concentrations are given as the monomer.

Inhibition of T4 Phage Growth by 5-Fluorodeoxyuridine (FdUrd). Escherichia coli JM101 cells were grown in minimal (thymidine-free) media to a density of 2×10^8 cells/mL. To samples of these cells was added (i) 122 μM FdUrd and 50 μg/mL thymidine, (ii) FdUrd only, (iii) thymidine only, or (iv) nothing. After incubation for 20 min at 37 °C, the cells were infected with bacteriophage T4 (10^9 plaque forming units per 2-mL cell sample), rolled for 60 min, and lysed with chloroform. Phage progeny were titered on mid-log JM101 cells.

Inactivation of CH by FdUMP. Reaction mixtures (0.5 mL) contained 0.6 mM CH_2THF, 100 μM FdUMP, and either 0.037 μM wild-type CH or 10 μM D179N in standard assay buffer (80 mM potassium phosphate and 20 mM β-mercaptoethanol, pH 7.4). After incubation at 30 °C for varied times, [5-^3H]dCMP (in a volume of less than 20 μL) was added to each reaction mixture to give a final concentration of 1 mM (7.4×10^7 Bq/mmol). Tritium release reactions, quenched after 15 min for wild-type CH and 30 min for D179N, were analyzed as previously described (Graves et al., 1992). In this and all other experiments, radioactivity was quantitated in disintegrations per minute (dpm) by scintillation counting to an accuracy of $2\sigma = 0.5\%$.

Isolation of Complexes Formed with [6-^3H]FdUMP and [11-^{14}C]CH_2THF. Complexes to be analyzed by nitrocellulose binding were formed by reacting 36 μM [6-^3H]FdUMP (5.1×10^9 Bq/mmol), 36 μM [11-^{14}C]CH_2THF (1.8×10^9 Bq/mmol), and 3.6 μM enzyme in standard assay buffer at 30 °C. Samples (100 μL) were filtered on BA85 nitrocellulose filters (Schleicher and Schuell), and the filters were washed five times with 2 mL of 80 mM potassium phosphate, pH 7.4. Filters were dissolved in 1 mL of DMSO and counted in 9 mL of Aquasol scintillation fluid to determine the ratio of ^3H to ^{14}C bound to the enzyme. In order to be sure the reactions had reached equilibrium, reaction mixtures containing wild-type CH were filtered after 45, 90, and 135 min. Reactions containing CH(D179N) were filtered after 10, 20, and 30 min.

Samples to be analyzed by SDS–PAGE contained 0.3 mM [6-^3H]FdUMP (1.0×10^{10} Bq/mmol) or 0.3 mM unlabeled FdUMP, 0.13 mM [11-^{14}C]CH_2THF (1.9×10^9 Bq/mmol) or 0.13 mM unlabeled CH_2THF, and 28 μM enzyme in standard assay buffer. All reaction mixtures were incubated at 30 °C for 60 min. Samples (25 μL) were quenched by the addition of 25 μL of SDS sample buffer and heated for 2 min at 80 °C. Denatured samples were electrophoresed on a 1% SDS/12% polyacrylamide gel with a 3% stacking gel. The gel was treated and exposed to film as described (Graves et al., 1992).

Association and Dissociation Rates of FdUMP Measured by Filtration on Nitrocellulose. Complexes were formed using 7 μM enzyme, 70 μM [6-^3H]FdUMP (3.5×10^6 Bq/μmol), and 0.6 mM CH_2THF in standard assay buffer. Complex formation was measured by filtering triplicate samples (25

[2] Variants of CH created by mutagenesis are indicated by the amino acid residue number preceded by the single-letter code for the wild-type residue and followed by the single-letter code for the new residue.

μL) on BA85 nitrocellulose filters (*vide supra*). Filter binding efficiencies, determined as previously described (Graves et al., 1992), were 25–30%. First-order association rate constants were calculated by analysis, using eq 1, of the fraction of

$$E_{free} = \frac{[E]_{total} - [E]_{bound}}{[E]_{total}} = e^{-kt} \quad (1)$$

enzyme not bound to [6-^3H]FdUMP (E_{free}) as a function of time. $[E]_{total}$ represents the average maximum radioactivity (dpm) bound to the nitrocellulose filters after at least 10 half-lives. In a typical experiment, the maximum tritium bound to the filter corresponded to 30 000–40 000 dpm. $[E]_{bound}$ represents the radioactivity bound to the filter at a given time.

Dissociation rates of the [6-^3H]FdUMP complex for both wild-type CH and CH(D179N) were determined from the time-dependent decrease in the amount of tritium-labeled complex after the addition of excess unlabeled FdUMP. A reaction mixture identical to that described above was incubated at 30 °C until maximal complex formation had occurred, at which time a 50-fold excess of unlabeled FdUMP (3.5 mM) was added. Triplicate timed samples (25 μL) were subsequently assayed for tritium-labeled FdUMP still bound to the enzyme by nitrocellulose filter binding. First-order rate constants for dissociation were calculated using eq 2,

$$E_{bound} = \frac{[E]_{bound}}{[E]_{total}} = e^{-kt} \quad (2)$$

where $[E]_{total}$ represents the radioactivity bound to the filter before the addition of excess unlabeled FdUMP.

Association of FdUMP with CH Measured by SDS–PAGE. Complexes to be analyzed by SDS–PAGE were formed by reacting 35 μM enzyme, 3.5 μM [6-^3H]FdUMP (5.9×10^8 Bq/μmol), and 1 mM CH_2THF in standard assay buffer at 30 °C. Timed samples (20 μL) were quenched by the addition of 20 μL of SDS sample buffer, frozen on dry ice, and subsequently analyzed by polyacrylamide gel electrophoresis and autoradiography as described above. Band intensities were quantitated by scanning densitometry. First-order association rates were calculated from the time dependence of covalent binding of [6-^3H]FdUMP, using eq 1 above, where $[E]_{total}$ represents the band intensity after at least 7 half-lives of association and $[E]_{bound}$ represents the band intensity at a given time.

Equilibrium Isotope Effects. Complexes were formed by reacting 0.6 mM CH_2THF, 0.87 mM FdUMP, and 0.16 mM enzyme in standard assay buffer at 30 °C until isotope ratios were stable (measured by nitrocellulose filter binding). The starting ^3H:^{14}C ratio of the FdUMP was *ca.* 5. Free and bound FdUMP were separated by chromatography on Sephadex G25 and quantitated by liquid scintillation counting. Values for the equilibrium isotope effect (EIE) for complex formation, K_H/K_T, were calculated from two separate reactions using eq 3.

$$K_H/K_T = \frac{(^3H:^{14}C \text{ ratio of free FdUMP})}{(^3H:^{14}C \text{ ratio of bound FdUMP})} \quad (3)$$

Kinetic Isotope Effects. Reaction mixtures (60 μL) contained (in standard assay buffer) 0.5 mM CH_2THF, 1.5–1.9×10^3 Bq/mmol 6-^3H-labeled nucleotide, and 0.25–0.32 $\times 10^3$ Bq/mmol 2-^{14}C-labeled nucleotide. Mixtures for reactions catalyzed by CH(D179N) contained either 20 μM enzyme plus 2 mM dUMP or 100 μM enzyme plus 0.4 mM dCMP. Mixtures for reactions catalyzed by wild-type CH contained 0.18 μM enzyme and 0.4 mM dCMP. Reactions were incubated at 30 °C and quenched at less than 20% conversion by removing the enzyme and most of the folate from samples using 300-mg C18 cartridges (Waters Sep-Pak). Hm^{5}dCMP and dCMP, separated by HPLC on a Lichrosorb C18 column (4.6 × 250 mm, Alltech) isocratically with an aqueous solvent of 5 mM each potassium phosphate and tetrabutylammonium sulfate (pH 7.0), had RV of, respectively, 26 and 30 mL. Hm^{5}dUMP and dUMP, separated on a Microsorb MV C18 column (4.6 × 250 mm, Rainin) isocratically with the same solvent containing 5% methanol, had RVs of 21 and 27 mL, respectively.

The α-tritium KIEs (k_H/k_T) on formation of 2-^{14}C,6-^3H-labeled Hm^{5}dCMP or Hm^{5}dUMP were calculated from the ratio of ^{14}C to ^3H in substrates and products using eq 4,

$$k_H/k_T = [\log(1-x)]/[\log(1-x(R_p/R_o))] \quad (4)$$

where x is the fraction of ^{14}C-labeled product formed, R_p is the ^{14}C:^3H ratio of the product formed, and R_o is the ^{14}C:^3H ratio of the substrate (dCMP or dUMP) at time zero.

Table 1: Rates of FdUMP Inactivation and Ternary Complex Formation and Dissociation

enzyme	k_{inact}[a] (min^{-1})	k_{assoc}[b] (min^{-1})	k_{assoc}[c] (min^{-1})	k_{dissoc}[b] (min^{-1})
wild type	0.08(0.02)	0.090(0.004)	0.009(0.001)	0.0004–0.0025[e]
D179N	>0.7[d]	>0.7[d]	5.0(0.3)	0.0050(0.0004)

[a] Measured by loss of activity in the tritium release assay, as described in Materials and Methods. Inactivation reaction mixtures contained 100 μM FdUMP, 0.6 mM CH_2THF, and enzyme [0.037 μM wild-type CH or 10 μM CH(D179N)]. [b] Determined by nitrocellulose filter binding, as described in Materials and Methods; representative data are shown in Figure 2. [c] Determined by analysis of complexes by SDS–PAGE (denaturing conditions) as shown in Figures 3 and 4. [d] For CH(D179N), the rate of FdUMP inactivation and the association rate with FdUMP measured by nitrocellulose binding are minimal estimates, based on half-lives for these processes of much less than 1 min. [e] This range was estimated from the data in Figure 2B. For details, see Results.

RESULTS

FdUMP Inactivates CH by Ternary Complex Formation. The CH_2THF-dependent tritium release from [5-^3H]dCMP catalyzed by wild-type CH is inactivated, in a time-dependent manner, by FdUMP. The loss of CH activity is a first-order process. In the presence of 0.2 mM CH_2THF and 50 μM FdUMP, inactivation occurs at a rate of 0.06 (±0.01) min^{-1}. A similar inactivation rate (0.08 min^{-1}) was obtained at 0.6 mM CH_2THF and 100 μM FdUMP (Table 1), indicating that these concentrations are near saturation. No inactivation by FdUMP is observed in the absence of CH_2THF. Inactivation of CH is accompanied by formation of a covalent FdUMP–enzyme complex, which also requires the presence of CH_2THF. Previous studies using [6-^3H]FdUMP showed that the complex contains 1 mol of FdUMP per enzyme monomer and that substitution of Cys^{148} in CH (by mutagenesis) prevents FdUMP binding (Graves et al., 1992).

In order to determine directly whether the CH–FdUMP complex also contains CH_2THF, complexes were formed using radiolabeled CH_2THF and analyzed by SDS–PAGE (Figure 1). Complexes formed using wild-type CH, [6-^3H]FdUMP, and unlabeled CH_2THF (lane 1) or using wild-type CH, [11-^{14}C]CH_2THF, and unlabeled FdUMP (lane 3) showed radiolabeled bands which migrate at the molecular weight of the CH monomer (28.6 kDa). No radiolabeled protein bands could be detected when enzyme was reacted with only [6-^3H]FdUMP in the absence of CH_2THF (lane 2) or with

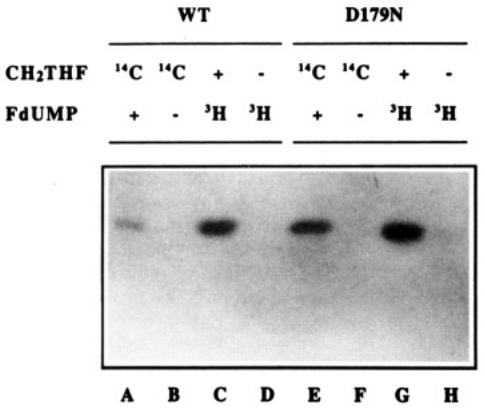


FIGURE 1: SDS–PAGE analysis of complexes formed between [6-^{3}H]FdUMP, [11-^{14}C]CH_2THF, and CH (wild type, lanes A–D; D179N, lanes E–H). All lanes contain 28 μM enzyme monomer. Lanes A and E contain 0.13 mM [11-^{14}C]CH_2THF and 0.3 mM unlabeled FdUMP. Lanes B and F contain 0.13 mM [11-^{14}C]CH_2THF and no FdUMP. Lanes C and G contain 0.3 mM [6-^{3}H]FdUMP and 0.13 mM unlabeled CH_2THF. Lanes D and H contain 0.3 mM [6-^{3}H]FdUMP and no CH_2THF.

only [11-^{14}C]CH_2THF in the absence of FdUMP (lane 4). These data demonstrate that both FdUMP and CH_2THF are covalently bound in a ternary complex with CH.

To establish the stoichiometry of FdUMP and CH_2THF bound to CH, complexes formed using a mixture of the radiolabeled substrates, [6-^{3}H]FdUMP and [11-^{14}C]CH_2THF, were isolated by filtration on nitrocellulose filters. The ^{3}H:^{14}C ratio in the ternary complex bound to the filters indicated that 1 mol of CH_2THF binds per mole of FdUMP.

Wild-type CH does not seem to be substantially inhibited *in vivo* by FdUMP. *Escherichia coli* cells were grown in the presence of a precursor of FdUMP, FdUrd, prior to infection with bacteriophage T4. Growth of host cells in the presence of 0.122 mM FdUrd and 50 μg/mL thymidine did not decrease the plating efficiency of wild-type bacteriophage T4. (In contrast, the viability of the bacterial cells was decreased 100-fold by treatment with FdUrd alone.) This suggests either that the inhibition of CH *in vivo* by FdUMP occurs too slowly to affect the rate of DNA precursor synthesis significantly, or that the FdUMP produced from FdUrd is not accessible to CH *in vivo*.

Effect of Conversion of Asp^{179} to Asn upon FdUMP Binding. Our previous studies showed that conversion of Asp^{179} to Asn results in a reversal of the substrate preference of CH from dCMP to dUMP, due to an increase in the turnover rate of dUMP and a large decrease in the turnover rate of dCMP (Graves et al., 1992). The Asp^{179}-to-Asn mutation also increases, by at least 10-fold, the rate at which the enzyme is inactivated by FdUMP (Table 1). The D179N variant requires less than 1 min of exposure to 50 μM FdUMP and 0.2 mM CH_2THF to be completely inactivated, corresponding to an inactivation rate substantially greater than 0.7 min^{-1}. As with wild-type CH, inactivation of CH(D179N) is a time-dependent, stoichiometric process which requires CH_2THF and is accompanied by the formation of a covalent CH–FdUMP complex (Graves et al., 1992). The data in Figure 1 demonstrate that this complex also contains CH_2THF. Covalent CH(D179N) complexes were detectable on SDS–polyacrylamide gels, using either [6-^{3}H]FdUMP or [11-^{14}C]CH_2THF, only when both FdUMP and CH_2THF were present. (Preliminary experiments using CH_2THF prepared from [3′,5′,7,9-^{3}H]folate (M. M. Butler and L. W. Hardy, unpublished data) gave similar results with both wild-type CH and CH(D179N).) CH complexes which were labeled

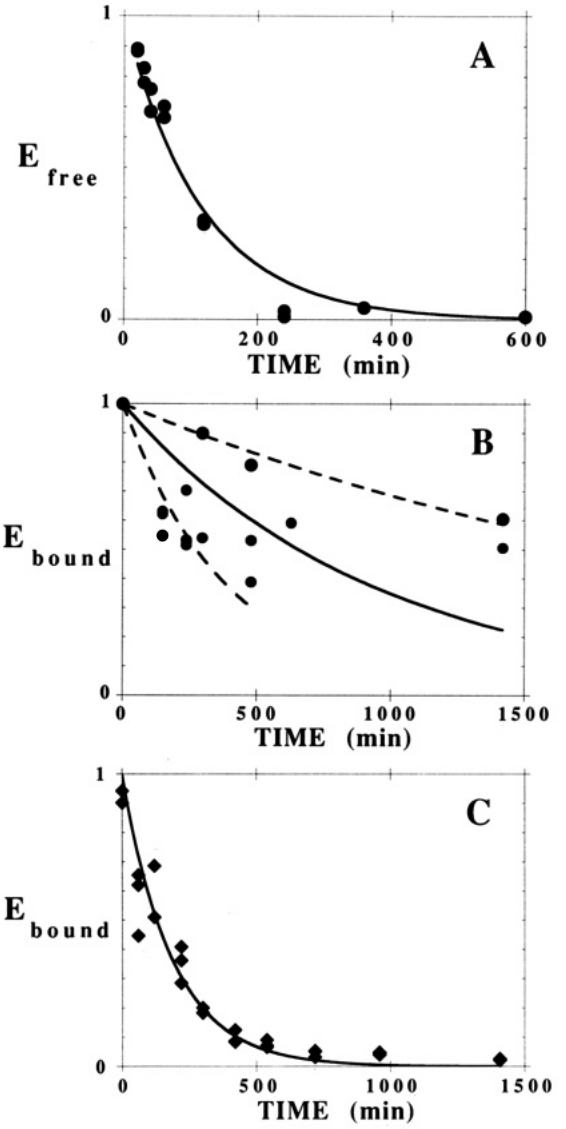


FIGURE 2: Kinetics of association (A) and dissociation (B, C) of complexes between [6-^{3}H]FdUMP, CH_2THF, and CH (wild type and D179N), monitored by nitrocellulose binding as described in Materials and Methods. Panel A: Fraction of free (unbound) wild-type CH remaining (E_{free}). Samples contained 7 μM CH monomer, 70 μM FdUMP, and 0.6 mM CH_2THF. Panels B and C: Fraction of wild-type CH (B) or CH(D179N) (C) associated with [6-^{3}H]-FdUMP after addition of excess (3.5 mM) unlabeled FdUMP (E_{bound}). The solid lines are the theoretical fits of all of the data to eq 1 (A) or eq 2 (B, C). The broken lines in (B) represent estimates of the maximum and minimum rates of dissociation of the complex containing the wild-type enzyme; see text for details. Rates are listed in Table 1.

with both [6-^{3}H]FdUMP and [11-^{14}C]CH_2THF and bound to nitrocellulose filters contained equimolar amounts of FdUMP and CH_2THF.

Rates of Complex Formation and Dissociation. Two methods were used to measure the rates at which complexes form between [6-^{3}H]FdUMP, CH_2THF, and CH (wild type or D179N). The first method, filtration of complexes on nitrocellulose filters, monitored the formation of all enzyme–FdUMP complexes (Figure 2A), whether covalent or non-covalent. This method was also used to measure rates of dissociation of preformed CH complexes containing [6-^{3}H]-FdUMP after the addition of a large excess of unlabeled FdUMP (Figure 2B,C). The nitrocellulose filtration method was too slow to give an accurate value for the association rate of the complex containing CH(D179N), since samples could

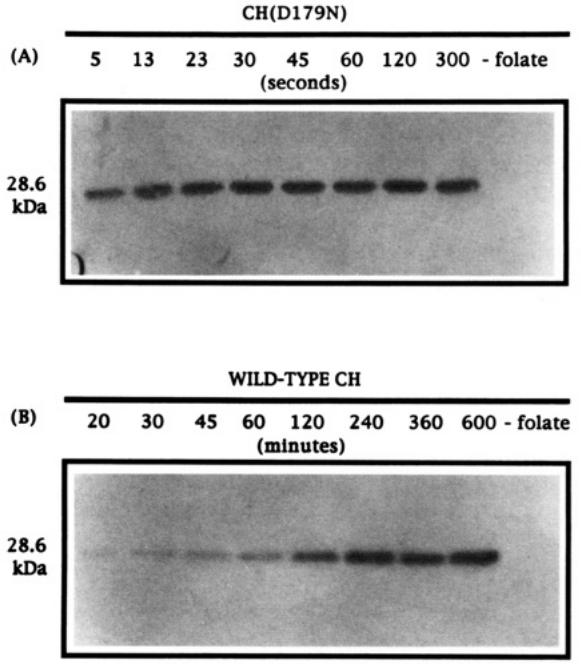


FIGURE 3: Covalent complex formation between [6-^3H]FdUMP, CH_2THF, and CH (D179N and wild type), analyzed by SDS–PAGE as described in Materials and Methods. Autoradiogram A shows complex formation between 35 μM CH(D179N) monomer, 3.5 μM [6-^3H]FdUMP, and 1 mM CH_2THF. Autoradiogram B shows complex formation between 35 μM CH (wild type) monomer, 3.5 μM [6-^3H]FdUMP, and 1 mM CH_2THF. The quench time of each sample is indicated at the top of each lane in seconds (A) or in minutes (B). All samples were heated for 2 min at 80 °C before loading. Band intensities were determined as described in Materials and Methods.

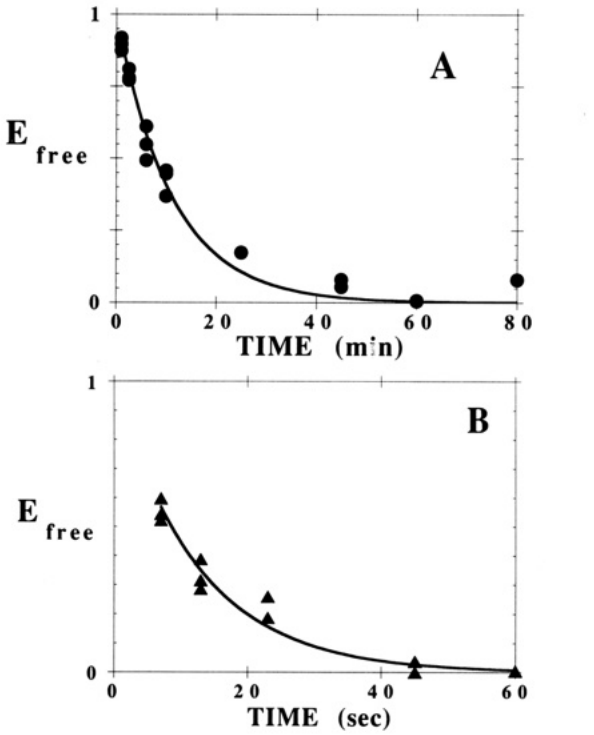


FIGURE 4: Kinetics of covalent association of CH (wild type, panel A, and D179N, panel B) with [6-^3H]FdUMP and CH_2THF. Complexes were formed as described in Materials and Methods and analyzed by SDS–PAGE as shown in Figure 3. The intensities of the bands shown in Figure 3 were determined by scanning densitometry and represent the fraction of tritium-labeled complex formed at each time. These intensities were used to calculate the remaining fraction of free enzyme, which is plotted as a function of time. The solid lines are the theoretical fits of the data to eq 1 (note the difference in scale). Rates are listed in Table 1.

not be quenched in less than 1 min. In the second method, timed samples containing complex were denatured with SDS prior to gel electrophoresis, autoradiography, and quantitation of covalent complexes by densitometry (Figures 3 and 4). The rapid quenching of samples with SDS allowed the determination of an association rate for the CH(D179N) complex. The association rates determined using the two methods are not directly comparable, due to differences in FdUMP and protein concentrations required by the techniques. The first method was done under conditions of excess FdUMP, and the second, under conditions of excess enzyme.

The time course of [6-^3H]FdUMP binding to wild-type CH, monitored by nitrocellulose filtration, exhibits first-order kinetic behavior (Figure 2A). The association rate at 70 μM FdUMP, calculated using eq 1, was 0.09 min^{-1}. The rate of binding to wild-type enzyme at 100 μM FdUMP was not significantly higher (0.11 ± 0.01 min^{-1}), indicating saturation. The binding of [6-^3H]FdUMP to CH(D179N), at either concentration, occurred much more rapidly and was complete in less than 1 min (data not shown). This corresponds to a pseudo-first-order association rate much greater than 0.7 min^{-1}. The dissociation of [6-^3H]FdUMP from ternary complexes containing the wild-type and D179N enzymes (Figure 2B,C) occurs at rates of *ca.* 0.001 and 0.005 min^{-1}, respectively. The analysis of the dissociation kinetics of the wild-type CH complex was difficult due to considerable scatter in the data and an inability to replace all of the [6-^3H]FdUMP bound to the wild-type enzyme with unlabeled FdUMP (Figure 2B). Some of this difficulty may be due to competing denaturation of the enzyme during the prolonged period necessary for the slow dissociation process. Limits for the dissociation rate of the wild-type complex were determined, using eq 2 and those data which would give the fastest and slowest rates. These limits, 0.0025 and 0.0004 min^{-1}, are represented by the broken lines plotted in Figure 2B.

The formation of covalent complexes, monitored by SDS–PAGE (Figure 3), occurs with first-order kinetics (Figure 4). Autoradiograms showing the time dependence of complex formation with wild-type CH and CH(D179N) are shown in Figure 3. The fractions of complex formed at various times were determined by comparing the intensity of each band to the intensity after complex formation was complete. The time-dependent decreases in the fraction of free wild-type CH and CH(D179N) are shown in Figure 4. The rates of covalent complex formation, obtained by least squares regression on eq 1 of the data in Figure 4, are 0.009 and 5.0 min^{-1} for the wild-type and D179N enzymes, respectively.

α-Secondary Isotope Effect on the Equilibrium for Formation of Ternary Complex with [6-^3H]FdUMP. The mechanism which we propose for the inhibition by CH by FdUMP (Scheme 2) requires sp^2 to sp^3 rehybridization of carbon 6 of FdUMP upon ternary complex formation. To determine whether this requirement is met, complexes were prepared between enzyme, CH_2THF, and a mixture of [2-^{14}C]-FdUMP and [6-^3H]FdUMP, with nucleotide in large excess. The equilibrium isotope effects (EIEs) on ternary complex formation, due to tritium substitution at C6, were calculated from the differences in the ratio of ^3H to ^{14}C in free and CH-bound FdUMP using eq 3. (The assumption in this method is that 2-^{14}C substitution of FdUMP does not affect the equilibrium for binding, and is merely a remote label for [6-^1H]FdUMP.) The EIE values, listed in Table 2, are similar for ternary complex formation by wild-type CH and by CH-(D179N). Both binding reactions occur with an inverse EIE

Scheme 2: Proposed Mechanism for the Inactivation of dCMP Hydroxymethylase by FdUMP [R = 1-β-(5′-phosphoribofuranosyl)]

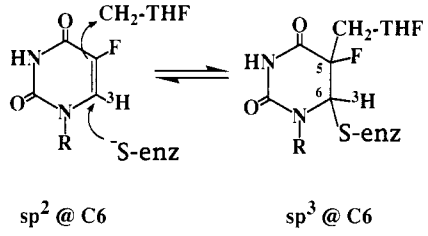


Table 2: Values of the α-Secondary Equilibrium Isotope Effect (EIE) on FdUMP Binding to CH and the Kinetic Isotope Effect (KIE) on CH-Catalyzed Reactions, Due to 6-Tritium Substitution[a]

		KIE (k_H/k_T)[c]	
enzyme	EIE[b] (K_H/K_T)	dCMP	dUMP
wild type	0.829(0.003), $N = 2$	0.99(0.01), $N = 6$	not determined
D179N	0.803(0.003), $N = 2$	0.98(0.02), $N = 5$	0.82(0.02), $N = 4$

[a] Values listed are the mean(SE) of N independent determinations. [b] EIEs on ternary complex formation, using a mixture of 2-^{14}C- and 6-^{3}H-labeled FdUMP, were determined as described in Materials and Methods. [c] KIEs for formation of hydroxymethylated nucleotide products, using a mixture of 2-^{14}C- and 6-^{3}H-labeled substrates, were determined as described in Materials and Methods.

near 0.8, consistent with the hypothesis that carbon 6 of [6-^{3}H]-FdUMP is tetrahedral and sp^3 hybridized.

α-Secondary Kinetic Isotope Effects on Turnover. To determine whether either sp^2-to-sp^3 or sp^3-to-sp^2 rehybridizations are cleanly rate-limiting for CH-catalyzed hydroxymethylations, values of the α-secondary KIE due to ^{3}H substitution at C6 were determined using mixtures of 2-^{14}C- and 6-^{3}H-labeled nucleotide substrates (Table 2). Again, 2-^{14}C substitutions were used as remote labels for the [6-^{1}H] nucleotides. The values of the KIE for the formation of Hm^5-dCMP, catalyzed by either wild-type CH or CH(D179N), were close to 1.0. However, a near maximal inverse α-secondary KIE ($k_H/k_T = 0.82$) was observed for the formation of Hm^{5}dUMP catalyzed by CH(D179N).

DISCUSSION

The observations reported here confirm the proposed mechanism for inhibition of dCMP hydroxymethylase by FdUMP. The inactivated enzyme, analyzed by either nitrocellulose filtration or SDS–polyacrylamide gel electrophoresis, contains both FdUMP and CH_2THF, in quantities stoichiometric with enzyme monomer. This demonstrates that CH is inhibited by the formation of a covalent complex containing both the inhibitory nucleotide and CH_2THF (Figure 1). Formation of the complex requires the catalytically essential Cys^{148} (Graves et al., 1992). During complex formation, the thiol of Cys^{148} is likely to form a covalent bond to C6 of FdUMP, which becomes saturated and sp^3 hybridized in the ternary complex (Table 2). The structure of the analogous ternary complex formed by *Escherichia coli* dTMP synthase has been shown to contain such a covalent bond (Matthews et al., 1991b). In the TS complex, C5 of FdUMP is linked to N5 of THF *via* the methylene moiety which is transferred during normal catalytic turnover. The complexes probably mimic early intermediates in catalysis by CH (II in Scheme 1) and TS and accumulate due to the inability of these enzymes to extract a fluorine cation.

The rate of inactivation of wild-type CH by FdUMP is the same as the rate of association of [6-^{3}H]FdUMP measured by nitrocellulose filtration. These rates are approximately 10-fold faster than that measured by SDS–PAGE (Table 1). This possibly indicates the rapid formation of a non-covalent complex, followed by a slower process involving covalent bond formation. This idea is attractive, because it offers an explanation of the lower stoichiometry of non-covalent binding of FdUMP to wild-type CH: the D179N mutation both accelerates complex formation and shifts the internal equilibrium toward the covalent complex. Another possibility is that the differences in rate are simply due to the higher concentrations of FdUMP (50–100 μM) used in the inactivation and nitrocellulose binding assays than that used in the SDS–PAGE analysis (3.5 μM FdUMP). Our data cannot discriminate between these two possible explanations. We have not systematically examined the effect of FdUMP concentration on the rates of inactivation or complex formation, although the stoichiometries of the two clearly correlate (Graves et al., 1992).

Replacement of Asp^{179} by Asn in CH produces a CH variant with a slight preference for dUMP as a substrate (Graves et al., 1992), as opposed to the pronounced preference of the wild-type enzyme for dCMP. This mutation also increases the yield of the complex between CH(D179N) and FdUMP after denaturation, compared to that obtained with wild-type enzyme (Figure 1; Graves et al., 1992). The Asp^{179}-to-Asn mutation increases both the association rate and the dissociation rate of the complex formed between CH, CH_2THF, and FdUMP (Figures 2, 3, and 4; Table 1). Since the concentrations of FdUMP were sufficiently high to give nearly maximal rates of association and inactivation of wild-type CH, the increased association and inactivation rates for CH(D179N) cannot be due to a difference in the affinity of FdUMP for the two enzymes in an initial (non-covalent) binding step. (Although such a difference in initial binding affinity may exist, this is not addressed by the data presented here.) It may be that a proton transfer, by Asp^{179} to a transient O4 oxyanion in FdUMP, slows complex formation and breakdown in wild-type CH. A similar proposal has been made to explain transient inactivation of the dTMP synthase variant, TS(N177D), by the substrate dUMP (Hardy & Nalivaika, 1992). Such a mechanism would not explain our previous observations that replacement of Asp^{179} with Ala or Ser prevents CH from binding FdUMP (Graves et al., 1992) or the lack of inactivation of wild-type CH by dUMP (K. L. Graves and L. W. Hardy, unpublished observations). The proposed role of Asp^{179} in catalysis by CH is to protonate N3 of covalently bound dCMP, thereby acting as a sink for the charge developed on various intermediates (Scheme 1).

A large inverse α-tritium secondary EIE was observed for the ternary complex formed with CH (wild type and D179N) and [2-^{14}C,6-^{3}H]FdUMP ($K_H/K_T = 0.83$–0.80; Table 3). This is consistent with the hypothesis that the 6-position of FdUMP is sp^3 hybridized and covalently bound to the enzyme (Scheme 2). A study with *Lactobacillus casei* TS demonstrated a similar value of the EIE (0.80) for ternary complex formation with [2-^{14}C,6-^{3}H]FdUMP (Bruice et al., 1980). A large α-tritium secondary KIE ($k_H/k_T = 1.23$) was observed for the dissociation of [2-^{14}C,6-^{3}H]FdUMP from the TS ternary complex (Bruice et al., 1980). Since the magnitude of the KIE on dissociation is explained fully by the EIE, covalent bond cleavage in dissociation of the TS ternary complex occurs prior to the first irreversible step of the reaction. Thus, during the association of the TS ternary complex, covalent bond formation must occur after the first irreversible step. That step has been postulated (Bruice & Santi, 1982) to be the

protein conformational change which accompanies complex formation with TS (Montfort et al., 1991). With CH, we have not been able to obtain sufficiently accurate data to determine the KIE on FdUMP complex formation or dissociation.

The validity of the isotope ratio method with [2-^{14}C,6-^{3}H]-FdUMP relies upon the assumption that the 2-^{14}C substitution of FdUMP has a negligible effect upon FdUMP binding. It is unlikely that significant bond making or breaking at C2 occurs during either catalytic turnover or inactivation by FdUMP, although some changes in electron density at O2 may accompany these processes. Moreover, the magnitude of any ^{14}C isotope effects would likely be much smaller than those reported in Table 2. Of greater concern is the possibility that at least a portion of the binding of FdUMP to wild-type CH is irreversible (Figure 2B). This raises some doubt about the validity of the analytical treatment of this binding as an equilibrium. However, if this irreversibility is due, as we suspect, to denaturation during the extended period required for dissociation, it is unlikely that the denaturation would show any isotopic preference. The isotope ratio which we observe is established rapidly (K. L. Graves, unpublished data), so that the ^{14}C:^{3}H ratio is likely to be a valid indicator of the C6 hybridization changes which accompany ternary complex formation with wild-type CH. The similar value obtained for the EIE for interaction of FdUMP with CH(D179N), which is fully reversible, also supports the validity of the EIE measurement for the wild-type enzyme.

The values of the α-secondary ^{3}H kinetic isotope effect (KIE) on k_{cat}/K_M at position 6 of dCMP with wild-type CH and with CH(D179N) were very close to 1.0 (Table 2). However, the value of this KIE with dUMP and CH(D179N) was 0.82. The latter value is the expected inverse effect, with some sp^2-to-sp^3 rehybridization at C6 either accompanying or preceding the first irreversible step in catalysis by CH-(D179N). The lack of any KIE significantly different from 1.0 for dCMP (Table 2) has several possible explanations. The possibility that there is no change in hybridization at carbon 6 during CH-catalyzed hydroxymethylation of dCMP seems unlikely. The value of the EIE on the formation of the FdUMP complex with both enzymes, combined with the fact that FdUMP binding, Hm^{5}dCMP product formation, and several CH-catalyzed isotope-exchange reactions (Butler et al., 1994) all require Cys^{148}, strongly suggests that sequential sp^2-to-sp^3 and sp^3-to-sp^2 rehybridizations occur during normal catalysis.

Another possible explanation for the KIE of 1.0 with dCMP is that nucleophilic attack upon C6 of dCMP, with its accompanying hybridization change, follows the rate-limiting event for the first irreversible step in CH-catalyzed formation of Hm^{5}dCMP. This second explanation, which is suggested by the behavior of TS with FdUMP (*vide supra*), also seems unlikely given the facile reversibility of the hydroxymethylation reaction (Butler et al., 1994). Moreover, CH-catalyzed tritium exchange from [5-^{3}H]dCMP into solvent water in the presence of THF occurs more rapidly than does the complete reaction in the presence of CH_2THF (Yeh & Greenburg, 1967). This suggests that the initial nucleophilic attack by Cys^{148} precedes the first irreversible step in catalysis and argues against the second explanation for the lack of a KIE caused by [6-^{3}H]-substitution of dCMP.

A third possibility to explain the KIE with dCMP is that the sequential sp^2-to-sp^3 and sp^3-to-sp^2 rehybridizations give rise to compensating opposite KIEs and occur prior to the first irreversible step in CH-catalyzed formation of Hm^5-dCMP. If the first irreversible event were coicident with the final sp^3-to-sp^2 rehybridization (Scheme 1, intermediate IV to product Hm^{5}dCMP), then the observed value of the KIE would be the EIE on forming the sp^3 species multiplied by the KIE on the sp^3 to sp^2 process. This value would likely be inverse, which is not observed. Thus, enzyme–product dissociation is probably rate-limiting for the first irreversible step during CH-catalyzed formation of Hm^{5}dCMP.

The value of 0.82 for the KIE with dUMP and CH(D179N) indicates that sp^2-to-sp^3 rehybridization at C6 accompanies or precedes the first irreversible step in catalysis by CH-(D179N) with this substrate. This value is near the theoretical maximum for this KIE (Bruice & Santi, 1982). Therefore, the subsequent sp^3-to-sp^2 rehybridization, which must accompany the aromatization to form product Hm^{5}dUMP, occurs after the rate-limiting event for the first irreversible step. This would allow the intrinsic KIE arising from sp^2-to-sp^3 rehybridization to be fully evident in the observed KIE.

The results in Table 2 clearly indicate a substrate-dependent change in the relative values of microscopic rate constants in catalysis by CH(D179N). To our knowledge, this is the first report of an α-secondary tritium KIE for alkylation of dUMP by any enzyme. Similar KIE values have been reported for TS-catalyzed transformations of dUMP analogs such as 5-bromo-dUMP and 5-ethynyl-dUMP (Pogolotti & Santi, 1977; Bruice et al., 1980).

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