Evidence from ^{18}O Exchange Studies for an Exocyclic Methylene Intermediate in the Reaction Catalyzed by T4 Deoxycytidylate Hydroxymethylase†

Michelle M. Butler, Karen L. Graves, and Larry W. Hardy*

Departments of Pharmacology and Molecular Genetics & Microbiology and Program in Molecular Medicine, University of Massachusetts Medical Center, 55 Lake Avenue North, Worcester, Massachusetts 01655

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ABSTRACT: ^{18}O exchange experiments were designed to identify the final intermediate in the catalytic mechanism of bacteriophage T4 deoxycytidylate (dCMP) hydroxymethylase (CH). CH catalyzes the formation of 5-(hydroxymethyl)-dCMP (HmdCMP) from dCMP and methylenetetrahydrofolate (CH_2-THF). CH resembles thymidylate synthase (TS), an enzyme of known three-dimensional structure, in both amino acid sequence and the reaction catalyzed. The final intermediate in the reaction catalyzed by TS or CH has been proposed to be the nucleotide with an exocyclic 5-methylene group covalently linked to the enzyme. This intermediate is then hydrated to HmdCMP (by CH) or reduced to deoxythymidylate (by TS). We report here that CH catalyzes the incorporation of ^{18}O from solvent water into the product, HmdCMP, in the presence of tetrahydrofolate (THF). The cause of this exchange is a reverse reaction followed by a resynthesis. CH also catalyzes the exchange of ^{18}O from solvent water into HmdCMP in the absence of exogenous THF and in the presence of THF analogues that lack N-5. N-5 is the nitrogen that is likely to be bound to the methylene as it is transferred to dCMP. A CH variant that lacks the nucleophilic Cys 148 is incapable of promoting these ^{18}O exchange reactions. The THF analogues lacking N-5 do not promote a CH-catalyzed reverse reaction. Rather, we propose that the CH-catalyzed ^{18}O exchange reaction promoted by these THF analogues occurs via 5-methylene-dCMP linked to the enzyme through Cys 148. We conclude here that enzyme-bound 5-methylene-dCMP is the final intermediate during catalysis by CH, as has also been proposed for TS and dUMP.

The bacteriophage T4 enzyme, deoxycytidylate hydroxymethylase (CH),[1] catalyzes the conversion of deoxycytidylate (dCMP) to 5-(hydroxymethyl)-dCMP (HmdCMP). The latter nucleotide is an essential precursor for DNA synthesis by bacteriophage T4 (Flaks & Cohen, 1957). The product of T4 gene *42*, dCMP hydroxymethylase is *in vivo* noncovalently associated with several other T4 proteins involved in DNA precursor synthesis (Thylén & Mathews, 1989). The reaction catalyzed by the hydroxymethylase is analogous to the reaction catalyzed by thymidylate synthase (TS), which provides an essential precursor for DNA synthesis in all organisms. Because TS is a drug target enzyme, it has been subjected to considerable mechanistic (Santi & Danenberg, 1984) and structural (Hardy et al., 1987; Matthews et al., 1990a,b; Montfort et al., 1990) studies; the derived information may be relevant to CH. The amino acid sequences of CH and TS have significant regions of similarity (Lamm et al., 1988; Thylén, 1988). The residues in TS that contact nucleotide and folate are especially well-conserved in the sequence of CH (Graves et al., 1992). Both enzymes catalyze the alkylation of carbon 5 of a pyrimidine 2′-deoxyribonucleoside 5′-monophosphate, using methylenetetrahydrofolate (CH_2-THF) as a one-carbon donor. There are two key differences between the reactions catalyzed by these enzymes. (i) Thymidylate synthase exclusively utilizes dUMP as substrate, whereas dCMP hydroxymethylase strongly prefers dCMP. (ii) Thymidylate synthase catalyzes reduction of the transferred methylene to a methyl group and concomitant oxidation of the tetrahydrofolate to dihydrofolate. In contrast, dCMP hydroxymethylase catalyzes hydration of the transferred methylene group, without oxidizing tetrahydrofolate.

A catalytic mechanism has been proposed for CH (Scheme 1) by analogy with that for TS (Subramaniam et al., 1988; Graves et al., 1992). The initial step is Michael addition of the enzymic nucleophile, Cys 148 (Graves et al., 1992), on carbon 6 of dCMP, producing intermediate I in Scheme 1. This activates carbon 5 for addition to the methylene group of CH_2THF, which presumably is in the form of an N-5 iminium ion (Kallen & Jencks, 1966), yielding intermediate II. Removal of the proton at carbon 5 is proposed to result in the cleavage of the bond to THF and the generation of the exocyclic methylene at carbon 5 (intermediate IV). Finally, hydration of the methylene group by a solvent water yields a hydroxymethyl group, and β-elimination of Cys 148 and dissociation from the enzyme provide the products of the reaction, HmdCMP and THF.

A proposed intermediate in catalysis whose structure is still unproven is the 5-exocyclic methylene form of dCMP (IV in Scheme 1). The intermediacy of this structure in the catalytic mechanism for TS is based mainly upon a single chemical model study (Pogolotti & Santi, 1974, 1977) where nucleophilic displacement reactions of 5-[(*p*-nitrophenoxy)methyl]-uracil derivatives were examined. In addition, data from reactions of TS with 5-ethynyl- and 5-bromovinyl-substituted dUMP analogues [reviewed by Ivanetich and Santi (1992)]

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* Author to whom correspondence should be addressed (telephone (508) 856-4900 or 856-6744).

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[1] Abbreviations: CH, dCMP hydroxymethylase; CH_2THF, N^5,N^{10}-methylenetetrahydrofolate; dCMP, deoxycytidylate; dUMP, deoxyuridylate; 5-daTHF, 5-deazatetrahydrofolate; FdUMP, 5-fluorodeoxyuridylate; HmdCMP, 5-(hydroxymethyl)deoxycytidylate; 10-me-5,8-ddaTHF, N^{10}-methyl-5,8-dideazatetrahydrofolate; THF, tetrahydrofolate; TS, thymidylate synthase.

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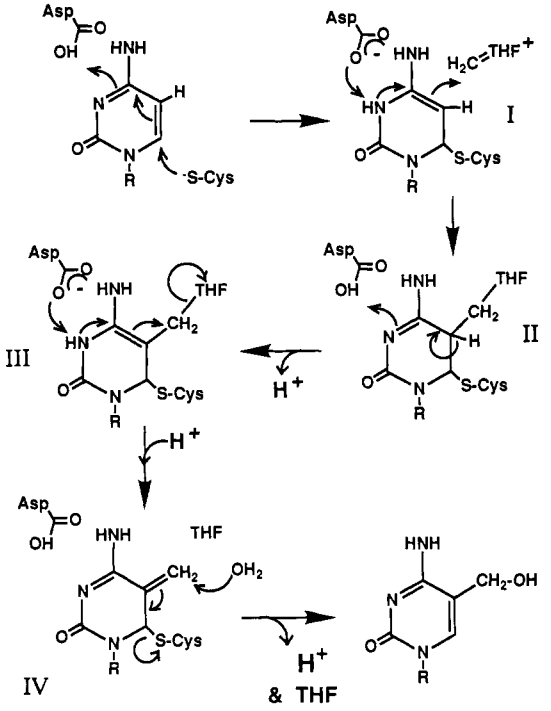
Scheme 1: Proposed Mechanism for Catalysis by dCMP Hydroxymethylase



are consistent with the presence of the intermediate, but do not conclusively prove its existence. The mechanistic proposals suggest that this intermediate is hydrated by solvent water in the CH reaction or reduced by hydride transferred from THF in the TS reaction. An alternate hypothesis for CH catalysis is that a water molecule directly attacks the dCMP-CH_2-N-5-THF bridge (III in Scheme 1). To test which of these hypotheses is more likely, we have examined the ability of CH to catalyze an isotope exchange reaction, involving oxygen exchange between solvent water and product HmdCMP, in the presence of folate analogues or in the absence of any folate derivative. The sensitivity of the ^{13}C chemical shift (during NMR spectroscopic analysis) to attachment to ^{16}O versus ^{18}O (Risley & Van Etten, 1980) was exploited to detect ^{18}O exchange into HmdCMP enriched with ^{13}C at the 5-hydroxymethyl carbon. Our results support the structure shown for III in Scheme 1 for the penultimate catalytic intermediate in catalysis by CH.

MATERIALS AND METHODS

Materials. dCMP was obtained from Sigma. THF was purchased from Sigma, unless otherwise indicated. [5-^3H]-dCMP was obtained from Moravek Biochemicals. Wild-type and mutant CHs were overproduced in an *Escherichia coli* system and purified as described (Graves et al., 1992). Methylene-THF was prepared from THF as described (Graves et al., 1992). 10-Methyl-5,8-dideaza-THF, a gift from Dr. M. G. Nair (Nair et al., 1987), whose melting point was determined to be 240 °C (with a published melting point of 235–238 °C), was dissolved in 0.1 N NaOH. 5-Deaza-THF, a gift from Dr. Joe Shih at Lilly Research Laboratories (Taylor et al., 1989), was dissolved in 0.1 N NaOH. The following extinction coefficients were used to quantitate the indicated compounds: 10-methyl-5,8-dideaza-THF, 22 167 M^{-1} cm^{-1} at pH 13, λ = 312 nm; 5-deaza-THF, 11 900 M^{-1} cm^{-1} at pH 13, λ = 276 nm (Singh et al., 1991).

Tritium Exchange Assays. Unless otherwise noted, reactions were done in a standard reaction buffer which contained 80 mM potassium phosphate (pH 7) and 20 mM β-mercaptoethanol. [5-^3H]dCMP (1 mM, 1.25×10^4 mCi/mmol), 0.5 mM CH_2THF or 1 mM THF, and varying concentrations of CH (wt and mutant forms) were allowed to react in a total volume of 0.5 mL at 20 or 30 °C. The reactions were quenched as described (Graves et al., 1992) and counted in Ready Safe (Beckman) scintillation fluid to obtain the disintegrations per minute (dpm) of tritium released into solvent water. Rate constants for reactions were calculated using data obtained at less than 20% transfer of ^3H from nucleotide to solvent. K_i determinations were performed with varying substrate (dCMP or THF) concentrations and one or more concentrations of competitive inhibitor. For the determination of kinetic parameters, initial velocities were fit directly to the Michaelis–Menten equation using the software package Kaleidagraph.

Enzymatic Preparation and Purification of [hydroxymethyl-^{13}C]-5-(Hydroxymethyl)-dCMP. In 4 mL of standard reaction buffer, dCMP (25 mg, 81.4 μmol), THF (14 mg, 25.3 μmol, from Fluka), and [^{13}C]paraformaldehyde (6 mg, 200 μmol, from Cambridge Isotope Laboratories) were reacted in the presence of 1 mg of CH(wt) at 30 °C for 2 h. The product nucleotide, HmdCMP, was purified by chromatography on Dowex-1 (Sigma) anion exchange resin (bicarbonate form), lyophilized, redissolved in water, and further purified by HPLC. First, to remove residual dCMP, the material was run isocratically on a Lichrosorb C18 column in 5 mM tetrabutylammonium sulfate and 5 mM potassium phosphate (pH 7.0) (buffer A) at 1 mL/min. Fractions containing HmdCMP were pooled and lyophilized. The second column, a SynChropak AX100 (Rainin) column (10 × 250 mm), was employed to remove the tetrabutylammonium ion from the HmdCMP and was run with a 140-mL gradient of 0–1 M ammonium acetate in 30% acetonitrile at 2 mL/min. HmdCMP eluted at approximately 0.35 M ammonium acetate. The pooled fractions were lyophilized from water several times in preparation for the NMR assays.

NMR Analysis of [hydroxymethyl-^{13}C]HmdCMP. All ^{13}C NMR spectra were measured at 75.423 MHz on a Varian Unity 300 spectrometer. In a typical experiment, 4 μmol of sample, [*hydroxymethyl*-^{13}C]HmdCMP (99% ^{13}C enrichment), was dissolved in a final volume of 0.7 mL containing 10% D_2O, 1 mM Tris (pH 7.0), 10 mM β-mercaptoethanol, $H_2{}^{18}O$ (Cambridge Isotope Laboratories) at 63–82% enrichment, 0.015–1.0 mg/mL enzyme (wt or mutant forms of CH), and either no folate, 0.5 or 1 mM THF, 0.5 mM 10-me-5,8-ddaTHF, or 1 or 2 mM 5-deaza-THF. Normal conditions employed a temperature of 20 °C, 128 scans, a 1500-Hz sweep width, and an acquisition time of 22 min. Peak sizes were quantitated by deconvolution and integration using the VNMR software provided by Varian.

Synthesis and Purification of [11-^{14}C]CH_2THF. THF (56 μmol, Fluka) was reacted with 28 μmol of [^{14}C]formaldehyde (Amersham, 50 mCi/mmol) in 66 mM Tris-HCl (pH 8) under argon for 2 min. The product folate, [11-^{14}C]CH_2THF, was purified by chromatography on DEAE cellulose (Sigma) anion exchange resin (Curthoys et al., 1972), lyophilized from deoxygenated H_2O, and redissolved in 10 mM potassium phosphate (pH 7) and 20 mM β-mercaptoethanol.

^{14}C Tracer Experiments. Reactions were designed to mimic conditions used in the NMR experiments and contained 1 mM Tris-HCl (pH 7), 10 mM β-mercaptoethanol, 5.75 mM HmdCMP, 0.45 mM THF, 0.05 mM [11-^{14}C]CH_2THF (52 mCi/mmol), and 0.015 mg/mL CH(wt) in a final volume of 1.2 mL. The above mixture was reacted at 20 °C, and 0.125-mL aliquots were removed at various times. Reactions were quenched by increasing the pH to 11, which effectively

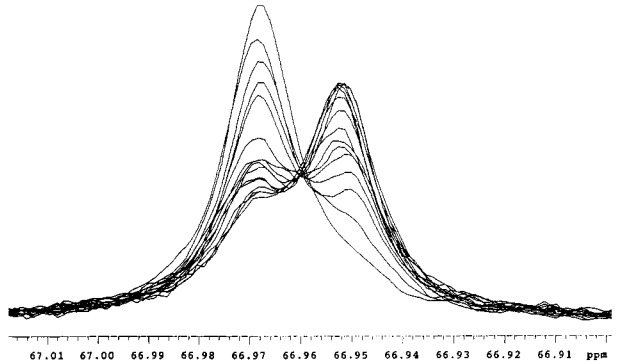


FIGURE 1: Time course of ^{18}O exchange from solvent water into [*hydroxymethyl*-^{13}C]-5-(hydroxymethyl)-dCMP. The reaction contained 0.5 mM 10-me-5,8-ddaTHF, 0.1 mg/mL CH(wt), and 5.75 mM [*hydroxymethyl*-^{13}C]HmdCMP. The first acquisition began at t = 4 min and the last began at t = 348 min, with intervals of 22 min between the start times of each acquisition. Each plotted time corresponds to the mean time for an acquisition interval, where t = 0 was the time enzyme was added. The downfield peak (66.97 ppm) represents the ^{13}C signal associated with a hydroxymethyl group containing a ^{13}C–^{16}O linkage, and the upfield peak (66.95 ppm) represents the ^{13}C signal associated with a hydroxymethyl group containing a ^{13}C–^{18}O linkage.

inactivates the enzyme. Samples were deproteinized prior to HPLC analysis by applying them to Centricon-10 units (Amicon) and centrifuging at 5000*g* for 45 min. The resulting filtrate was lyophilized, resuspended in H_2O, and applied to a Microsorb C18 (Rainin) HPLC column (4.6 × 25 mm). The nucleotides were eluted by running buffer A isocratically at 1 mL/min for 40 min, and the folates were eluted by switching to buffer A plus 50% (v/v) methanol at 0.5 mL/min for an additional 40 min. Fractions containing dCMP, HmdCMP, and CH_2THF, identified by continuous monitoring of the UV absorbance of the effluent, were assayed by liquid scintillation counting to determine the ^{14}C in each sample.

RESULTS

^{18}O Exchange Observed with CH(wt) and Folate Analogues. ^{18}O exchange into enzymatically synthesized [^{13}C,^{16}O]-(hydroxymethyl)-dCMP was followed by ^{13}C NMR. There is a 2–3-Hz change in the chemical shift of the ^{13}C signal that is caused by replacement of the proximal ^{16}O of the hydroxymethyl group of HmdCMP with ^{18}O (Risley & Van Etten, 1980). In the absence of enzyme, the signal, associated with ^{13}C linked to ^{16}O only, remained a single peak (data not shown). Upon the addition of enzyme, a second, upfield peak, one associated with the ^{13}C linked to ^{18}O, appeared. This CH-catalyzed ^{18}O exchange occurred slowly in the absence of folate and was accelerated by the addition of several folate analogues. Figure 1 shows a typical ^{18}O exchange experiment with CH(wt) and the folate analogue, 10-methyl-5,8-dideaza-THF, with the individual spectra at various times superimposed. The first line, that with the most intense downfield peak, represents a spectrum obtained beginning 4 min after the addition of enzyme. (Each spectrum required 22 min for data acquisition.) The remaining spectra were obtained at equal time intervals, with the last few time points representing near completion of the exchange reaction (since the $H_2{}^{18}O$ was only 80% enriched).

Pseudo-first-order rate constants, k, for the exchange reactions were obtained from log plots of the fraction of [^{13}C,^{16}O]-(hydroxymethyl)-dCMP remaining (not exchanged) versus time (Figure 2). The data were fit to the relationship:

$$(C_\infty - C_t)/C_\infty = e^{-kt} \quad (1)$$

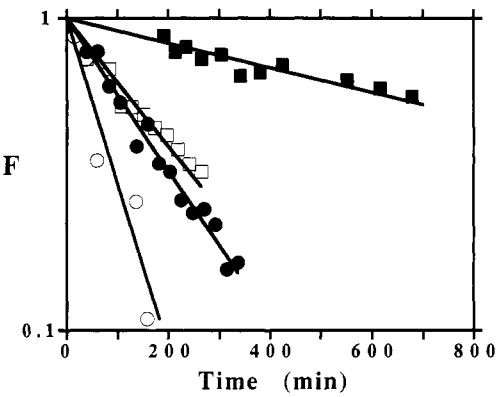


FIGURE 2: Exchange of ^{18}O from solvent water into [*hydroxymethyl*-^{13}C]-5-(hydroxymethyl)-dCMP catalyzed by CH(wt) as a function of time, analyzed by ^{13}C NMR. *F* is the fraction of [^{13}C,^{16}O]HmdCMP remaining (not exchanged). The minus folate reaction (■) and the 10-me-5,8-ddaTHF reaction (●) employed an enzyme concentration of 0.1 mg/mL and 5.75 mM HmdCMP. The THF reaction (○) employed an enzyme concentration of 0.015 mg/mL and 3.8 mM HmdCMP. The 5-daTHF reaction (□) employed an enzyme concentration of 0.02 mg/mL and 5.64 mM HmdCMP. All reactions were performed at 20 °C. The solid lines are the theoretical curves described by eq 1. The apparent pseudo-first-order rate constants, obtained by regression of the data with eq 1, are 0.0009 min^{-1} for the minus folate reaction, 0.0056 min^{-1} for the 10-me-5,8-ddaTHF reaction, 0.012 min^{-1} for the THF reaction, and 0.0047 min^{-1} for the 5-daTHF reaction.

Table 1: Rates of ^{18}O Exchange with CH(wt)[a] and CH(C148G) in the Presence of Folate Analogues[c]

folate analogue	rate of ^{18}O exchange (μmol min^{-1} mg^{-1})
1 mM THF	3.07 ± 0.47
0.5 mM THF	3.45[b]
2 mM 5-deaza-THF	1.31 ± 0.031
1 mM 5-deaza-THF	1.31 ± 0.057
0.5 mM 10-me-5,8-dideaza-THF	0.32 ± 0.0098
no folate	0.052 ± 0.0025
0.5 mM THF + CH(C148G)	<0.00035

[a] All experiments employed CH(wt) unless otherwise indicated. [b] Standard error not given since this value was based on two data points. [c] These rates were measured by monitoring the ^{13}C resonance of [*hydroxymethyl*-^{13}C]HmdCMP in $H_2{}^{18}O$ as a function of time, as described in Materials and Methods. Representative data are shown in Figures 1 and 2.

where C_t and C_∞ are the concentrations of [^{18}O]HmdCMP at time t and after equilibration, respectively (determined either by direct measurement at t > 10 half-times or by calculation from the known composition of the solvent). The exchange rates varied linearly with enzyme concentration (data not shown). The pseudo-first-order rate constants were multiplied by the concentration of HmdCMP and divided by the enzyme concentration to obtain the initial velocities of the ^{18}O exchange reactions (in units of μmol min^{-1} mg^{-1}) listed in Table 1. CH(wt) alone was able to catalyze a slow ^{18}O exchange reaction with no folate present. However, a mutant CH variant, CH(C148G),[2] was unable to catalyze a detectable ^{18}O exchange in either the absence or presence of the folate cofactor, with a maximum possible rate of 3.5×10^{-4} μmol min^{-1} mg^{-1}.

In the presence of THF, the rate of ^{18}O exchange was accelerated 60–70-fold over the rate observed in the absence

[2] Variants of CH created by mutagenesis of the gene 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 the new residue.

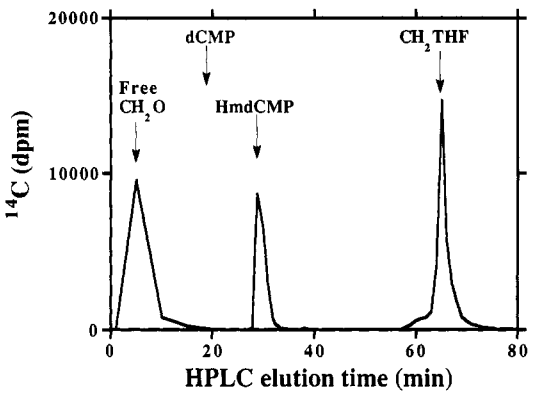


FIGURE 3: ^{14}C tracer analysis of the breakdown and resynthesis of HmdCMP, monitored as described in Materials and Methods. The HPLC elution profile shown here was obtained by analysis of a 40-min reaction containing 5.75 mM HmdCMP, 0.45 mM THF, 0.05 mM [11-^{14}C]CH_2THF, and 0.015 mg/mL CH(wt). The data from these analyses were plotted to obtain Figure 4.

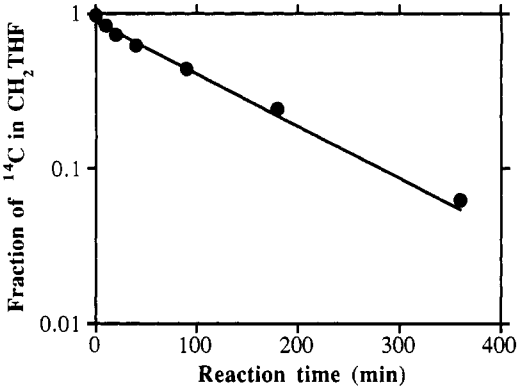


FIGURE 4: Exchange of ^{14}C from [11-^{14}C]CH_2THF into HmdCMP catalyzed by CH(wt) as a function of time, analyzed by HPLC separation of the reaction components (as shown in Figure 3). The reaction contained 5.75 mM HmdCMP, 0.45 mM THF, 0.05 mM [11-^{14}C]CH_2THF, and 0.015 mg/mL CH(wt) and was performed at a reaction temperature of 20 °C.

of added folate (Table 1). The rates with both concentrations of THF were identical (within experimental error), which indicated that THF was saturating.

The folate analogue 5-daTHF accelerated exchange by 25-fold and was nearly as effective as THF at stimulating ^{18}O exchange (Table 1). In our experiments, this analogue was also present at saturating conditions since doubling of the concentration produced no increase in the ^{18}O exchange rate. 5-daTHF behaved as a competitive inhibitor of THF in the tritium exchange assay. Its K_i was 0.11 mM, which is well below the concentrations employed in the ^{18}O exchange assays.

The folate analogue 10-me-5,8-ddaTHF was also able to stimulate ^{18}O exchange at a 10-fold lower rate than that observed in the presence of THF (Table 1). The K_i for this analogue as a competitive inhibitor of CH(wt) in a tritium exchange assay was 0.01 mM.

The K_i for HmdCMP as a competitor of dCMP was obtained in a tritium exchange assay of the forward reaction catalyzed by CH. A value of 0.1 mM for K_i was obtained (data not shown). This indicates that the concentration of [^{13}C]-HmdCMP utilized in the ^{18}O exchange experiments (3.8–5.75 mM) was saturating.

^{14}C Tracer Experiments. The replacement of ^{16}O by ^{18}O, catalyzed by CH in the presence of THF, could occur in two different ways. There could be a full reversal of the reaction to the starting material, dCMP, and a subsequent resynthesis that would incorporate ^{18}O into the product. Alternatively, the replacement could occur via a partial reaction, through a cycle of dehydration and rehydration of enzyme-bound HmdCMP. In order to distinguish between these possibilities, we synthesized [11-^{14}C]CH_2THF, a folate whose donor carbon was labeled with ^{14}C.

Tracer experiments were performed that matched the conditions used for the ^{18}O exchange experiments, *except* that there was a trace amount of [11-^{14}C]CH_2THF present. Were a back reaction occurring, the (forward) resynthesis would incorporate a ^{14}C label into the HmdCMP. We analyzed the distribution of ^{14}C in samples at different times by separating dCMP, HmdCMP, and CH_2THF using an HPLC system.

Figure 3 illustrates a typical HPLC separation of HmdCMP and CH_2THF where ^{14}C radioactivity was monitored. ^{14}C was detected in fractions containing both HmdCMP and CH_2-THF in all samples that were analyzed, except for a control sample that had no enzyme present; this sample contained the ^{14}C label only in fractions containing CH_2THF. Figure 4 shows the time-dependent transfer of ^{14}C from labeled CH_2-THF into HmdCMP. The rate of ^{14}C incorporation into HmdCMP was 2.98 ± 0.24 μmol min^{-1} mg^{-1}, a value identical to the rate of ^{18}O exchange in the presence of THF (see Table 1).

The HPLC analysis of the reactions containing HmdCMP, THF, and CH, monitored by UV absorbance, revealed the formation of dCMP. The amount of dCMP present increased with time, plateauing at about one-third of the total nucleotide amount under the reaction conditions used. The observed rate for this process is the sum of the rates of the forward and reverse reactions. In order to determine the rates for both reactions, we determined, by spectrophotometric quantitation of HPLC peaks, the relative amounts of dCMP and HmdCMP in each of the above samples as a function of time. Analysis of these quantities as the reaction approached equilibrium (Jencks, 1969) yielded rates for the forward and reverse reactions of 4.6 ± 0.9 and 3.1 ± 0.6 μmol min^{-1} mg^{-1}, respectively (data not shown). HPLC analysis of the products of reactions done in the absence of THF or in the presence of the folate analogues, 5-daTHF and 10-me-5,8-ddaTHF, gave no indication of a reverse reaction (*i.e.*, there was no dCMP in these samples).

DISCUSSION

Our interpretation of the results of the isotope exchange data is based largely on the hypothesis that the structure of the CH active site is homologous with the known structure of the TS active site. The amino acid sequences of the two enzymes show significant similarity (Thylén, 1988; Graves et al., 1992); moreover, both enzymes are homodimers of similar size. A stronger argument for the structural homology between the two enzymes is that the mutation of predicted active site residues in CH has yielded the expected results (Graves et al., 1992; Graves, 1994). CH (like TS) is covalently inactivated by 5-fluoro-dUMP (FdUMP) (Graves et al., 1992). This interaction resembles that observed in the covalent complex between TS and FdUMP, in that an α-^3H equilibrium isotope effect indicates that carbon 6 of the FdUMP bound to Cys 148 in CH is sp^3-hybridized (Graves, 1994). Upon mutagenesis of the CH active site cysteine, there is a loss of FdUMP binding and, as expected, a complete loss of enzyme activity (Graves et al., 1992). Using the known three-dimensional structure of TS as a guide, we have been able to alter the substrate specificity of CH (Graves et al., 1992). The variant of CH, CH(D179N), prefers the substrate, dUMP, over dCMP. The opposite mutation in TS, TS(N177D), alters the

substrate preference from dUMP to dCMP (Hardy & Nalivaika, 1992). In TS, mutation of Glu 58 results in dramatic decreases in k_{cat} (Zapf et al., 1993; Nalivaika & Hardy, unpublished results). In CH, mutation of the corresponding residue, Glu 60, also results in severe decreases in the rate of product formation (Graves, 1994). For the Glu 60 (Glu 58) variants of CH (TS), the rates of product formation are affected much more than the rates of partial reactions, including (for CH) the ^{18}O exchange reaction described here.

All of the above results together argue strongly for a structural and mechanistic homology between TS and CH. The following discussion is based upon this hypothetical structural homology.

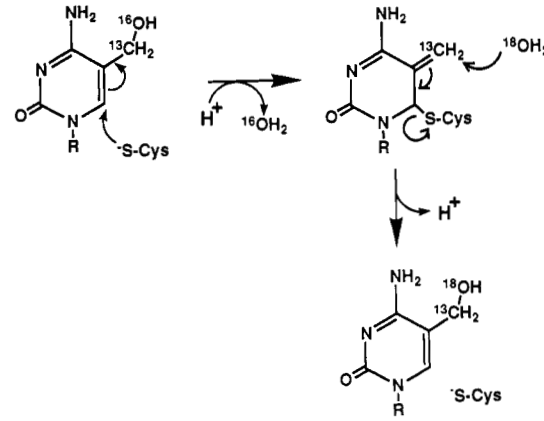
^{18}O Exchange Observed in the Presence of THF. THF stimulates the CH-catalyzed ^{18}O incorporation from solvent water into HmdCMP at a rate of 3–3.5 μmol min^{-1} mg^{-1} (Table 1). This observed rate of ^{18}O incorporation is about 2-fold lower than the rate of forward turnover starting with saturating dCMP, saturating CH_2THF, and CH(wt). The results of the ^{18}O exchange reaction in the presence of THF conclusively indicate that solvent water is the entity that hydrates the final intermediate in the CH reaction mechanism (see Scheme 1).

We attempted to determine whether the observed ^{18}O incorporation was due to a partial reaction or a combination of a reverse reaction and a forward resynthesis. The ^{14}C tracer experiments revealed that ^{14}C is incorporated into HmdCMP from [11-^{14}C]CH_2THF at a rate of 3 μmol min^{-1} mg^{-1}, which is identical to the observed ^{18}O incorporation rate. In addition, we observed the appearance of dCMP in these tracer reactions, thus providing further evidence for the breakdown and resynthesis of HmdCMP in the presence of CH and THF. The equilibrium for these reactions lies slightly in favor of HmdCMP. In other words, the reaction proceeds faster in the direction of HmdCMP formation than it does in the reverse direction, under these conditions (0.5 mM THF).

^{18}O Exchange Observed in the Absence of Added Folate. In the absence of exogenously added THF, slow exchange of ^{18}O from solvent water into HmdCMP was catalyzed by CH, with a rate that is 60–70-fold smaller than the rate for ^{18}O incorporation in the presence of THF. This exchange could arise for one of two reasons. First, it could be a true folate-independent exchange or partial reaction, where the nucleophilic cysteine of CH attacks C-6 of HmdCMP to form a Michael adduct (as it does in the first step of the forward reaction; see Scheme 1). This step would then release the ^{16}OH group from the 5-methylene to yield an exocyclic methylene intermediate (Scheme 1, intermediate IV). This intermediate would then be available for a hydration reaction with $H_2{}^{18}O$. The second option is a slow exchange due to the presence of persistent THF that copurified with CH. We attempted to rule out this possibility by analyzing the reaction products using HPLC. We did not detect the presence of dCMP in any reactions performed without added THF, but any telltale dCMP could only have arisen in a stoichiometric amount with the residual "persistent" THF, an amount not detectable by HPLC analysis. In conclusion, we cannot rule out the possibility that ^{18}O exchange in the absence of added folate is occurring via persistent THF in the enzyme preparation.

Folate Analogues That Lack N-5 Stimulate ^{18}O Exchange. N-5 of CH_2THF is thought to be the nitrogen that forms the methylene bridge to the C-5 of dCMP during catalysis (I and II in Scheme 1). We believe that the bond between N-10 and C-11 of CH_2THF is broken via protonation of N-5 by the invariant Glu 60 (Glu 58) in CH (TS) (Zapf et al., 1993; Graves, Nalivaika, & Hardy, unpublished results), thus yielding a 5-iminium ion form of CH_2THF, which is then available for attack by a C-5 anion of the nucleotide. The evidence for the involvement of N-5 of CH_2THF in a methylene linked to nucleotide is 2-fold. First, studies of the condensation of THF with formaldehyde suggest that an N-5 iminium ion is an intermediate in the condensation reaction (Kallen & Jencks, 1966). Second, crystallographic evidence using the covalent ternary complex with FdUMP, CH_2THF, and TS clearly demonstrates a methylene bridge to FdUMP from N-5 of CH_2THF [Matthews et al., 1990b; see also Perry et al. (1993)]. Therefore, a THF analogue lacking N-5 cannot complete the reverse reaction, as can *bona fide* THF.

Scheme 2: Proposed Mechanism for ^{18}O Exchange by dCMP Hydroxymethylase



5-Deaza-THF and 10-methyl-5,8-dideaza-THF accelerated ^{18}O exchange by 25- and 6-fold, respectively, over the folate-independent exchange rate (Table 1). Note that 5-deaza-THF was nearly as effective as THF at stimulating ^{18}O exchange, indicating that N-5 of THF is not essential for an efficient partial reaction. This result provides strong support for the existence of the 5-exocyclic methylene intermediate that has been proposed for both TS and CH.

The proposed mechanism for the $^{18}O/^{16}O$ exchange reaction is shown in Scheme 2. The alternative, involving direct attack (via an S_N2 process) of a solvent water upon the hydroxymethyl carbon, is unlikely for at least two reasons. First, in the forward reaction, the attacking water molecule would be displacing the N-5 of THF. Indeed, THF accelerates the exchange reaction by 60–70-fold. However, the N-5 of THF is not essential for successful enhancement of the rate of oxygen exchange, since the analogues that lack N-5 increase the rate of exchange by 6–25-fold. The rate enhancements by both 5-deaza-THF and 10-me-5,8-ddaTHF are probably due to their promotion of an enzyme conformation favorable toward Michael addition. Second, the oxygen exchange reaction does not occur if the nucleophilic Cys 148 thiol is removed, indicating that the exchange reaction requires Michael adduct formation. This requirement is more consistent with the mechanism shown in Scheme 2 than with an S_N2 process.

The evidence supporting the intermediacy of exocyclic methylene intermediates in the catalytic mechanisms of TS and CH is mainly derived from a nonenzymatic model reaction: the solvolysis of 1-substituted 5-[(*p*-nitrophenoxy)-methyl]uracils (Pogolotti & Santi, 1977). These ethers are readily hydrolyzed in alkali. The normal values for the secondary α-^2H kinetic isotope effects caused by deuterium substitution at the 5-methylene group indicate sp^3 to sp^2 rehybridization at this carbon during hydrolysis (Pogolotti & Santi, 1977). The evidence from reactions of TS with substrate

analogues is suggestive (Ivanetich & Santi, 1992), but the structures of the enzymic adducts have been inferred, and not directly assessed by structural determinations. The evidence for CH, until the present study, has relied upon analogy with TS. The catalysis by CH of an $^{18}O/^{16}O$ exchange reaction between the hydroxymethyl group of HmdCMP and solvent water in the presence of folates lacking N-5 provides strong support for a 5-exocyclic methylene intermediate (IV in Scheme 1) in CH catalysis.

Conclusions. The penultimate reaction intermediate during the formation of HmdCMP catalyzed by CH is likely to be 5-methylene dCMP covalently linked to the enzyme via a C-6 linkage to Cys 148. This intermediate is quite similar to that proposed for an analogous stage of catalysis by TS, even though the two enzymes must process the corresponding intermediates quite differently.

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