Roles of Cys[148] and Asp[179] in Catalysis by Deoxycytidylate Hydroxymethylase from Bacteriophage T4 Examined by Site-Directed Mutagenesis†

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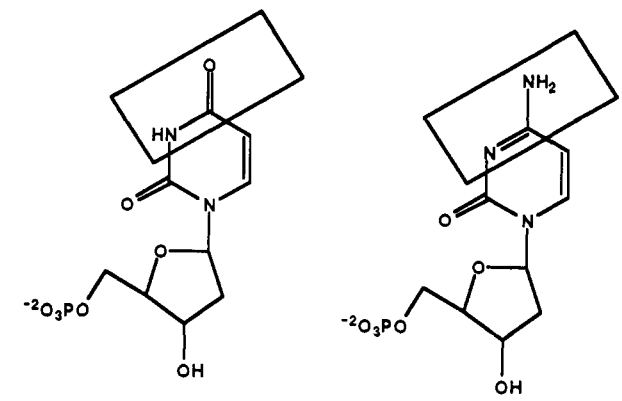
ABSTRACT: The proposed roles of Cys[148] and Asp[179] in deoxycytidylate (dCMP) hydroxymethylase (CH) have been tested using site-directed mutagenesis. CH catalyzes the formation of 5-(hydroxymethyl)-dCMP, essential for DNA synthesis in phage T4, from dCMP and methylenetetrahydrofolate. CH resembles thymidylate synthase (TS), an enzyme of known three-dimensional structure, in both amino acid sequence and the reaction catalyzed. Conversion of Cys[148] to Asp, Gly, or Ser decreases CH activity at least 10^5-fold, consistent with a nucleophilic role for Cys[148] (analogous to the catalytic Cys residue in TS). In crystalline TS, hydrogen bonds connect O4 and N3 of the substrate dUMP to the side-chain amide of an Asn; the corresponding residue in CH is Asp[179]. Conversion of Asp[179] to Asn reduces the value of k_{cat}/K_M for dCMP by (1.5×10^4)-fold and increases the value of k_{cat}/K_M for dUMP by 60-fold; as a result, CH(D179N) has a slight preference for dUMP. Wild-type CH and CH(D179N) are covalently inactivated by 5-fluoro-dUMP, a mechanism-based inactivator of TS. Asp[179] is proposed to stabilize covalent catalytic intermediates, by protonating N3 of the pyrimidine–CH adduct.

The DNAs of T even bacteriophages contain an unusual base, 5-(hydroxymethyl)cytosine, in place of cytosine. The product of phage T4 gene 42, deoxycytidylate (dCMP)[1] hydroxymethylase (EC 2.1.2.8, CH), is responsible for the formation of this unusual DNA component, at the mononucleotide level (Flaks & Cohen, 1957). CH catalyzes (i) the transfer of a methylene group from methylenetetrahydrofolate (CH_2THF) to carbon 5 of dCMP and (ii) the hydration of the transferred methylene group, producing 5-(hydroxymethyl)-dCMP and tetrahydrofolate (THF). Part i of this reaction is analogous to the methylene group transfer catalyzed by thymidylate synthase (TS). TS is a drug-target enzyme and hence has been intensively investigated both structurally (Hardy et al., 1987; Matthews et al., 1990a,b; Montfort et al., 1990) and kinetically (Santi & Danenberg, 1984). Since CH and TS differ in specificity for the pyrimidine moiety of their nucleotide substrates, they may interact differently with N3 and the heteroatom at position 4 of the heterocycle (Chart I). A catalytic mechanism has been proposed for CH, by analogy with that for TS [Subramaniam et al. (1988); see Scheme I]. A key step in this mechanism is the formation of a Michael adduct between an enzymic nucleophile (Cys[148] in TS from *Escherichia coli*) and carbon 6 of the pyrimidine heterocycle (Pogolotti & Santi, 1977).

Many of the mechanistic details for catalysis by TS have been provided by experiments with a substrate analogue, FdUMP [reviewed by Lewis and Dunlap (1981) and Santi and Danenberg (1984)]. TS binds FdUMP covalently in the presence of CH_2THF, but turnover is blocked due to the enzyme's inability to abstract F^+. We report here that CH is also covalently inactivated by FdUMP in the presence of CH_2THF. CH is not inactivated by FdCMP, which is only a weak competitive inhibitor (Subramaniam et al., 1989; Lee et al., 1988).

Chart I: Comparison of 2′-Deoxyuridylate and 2′-Deoxycytidylate



dUMP **dCMP**

In addition to the chemical analogy between the reactions catalyzed by CH and TS, these enzymes are probably structurally homologous. Both are homodimers of (55–60) $\times 10^3$ molecular weight. The amino acid sequence of CH has significant similarity to that of TS (Lamm et al., 1988; Thylén, 1988). In 16 sequences of thymidylate synthases from numerous species, there are 46 invariant residues (Perry et al., 1990); 20 of these are conserved in the sequence of CH. X-ray diffraction studies of crystalline TS have revealed residues which interact with the bound ligands (Finer-Moore et al., 1990; Montfort et al., 1990; Matthews et al., 1990a,b). Fourteen invariant residues in TS contact the bound nucleotide; of these, 12 are conserved in CH. One of the residues conserved in CH is Cys[148], which corresponds to the nucleophile in catalysis by TS, Cys[146].

Two of those 14 residues which contact bound dUMP and are invariant in available TS sequences are different in the

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[1] Abbreviations: CH, dCMP hydroxymethylase; CH_2THF, $^5N,^{10}N$-methylenetetrahydrofolate; dCMP, deoxycytidylate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); dUMP, deoxyuridylate; FdCMP, 5-fluorodeoxycytidylate; FdUMP, 5-fluorodeoxyuridylate; eop, efficiency of plating; su^-, nonsuppressor; su^+, suppressor; THF, tetrahydrofolate; TS, thymidylate synthase.

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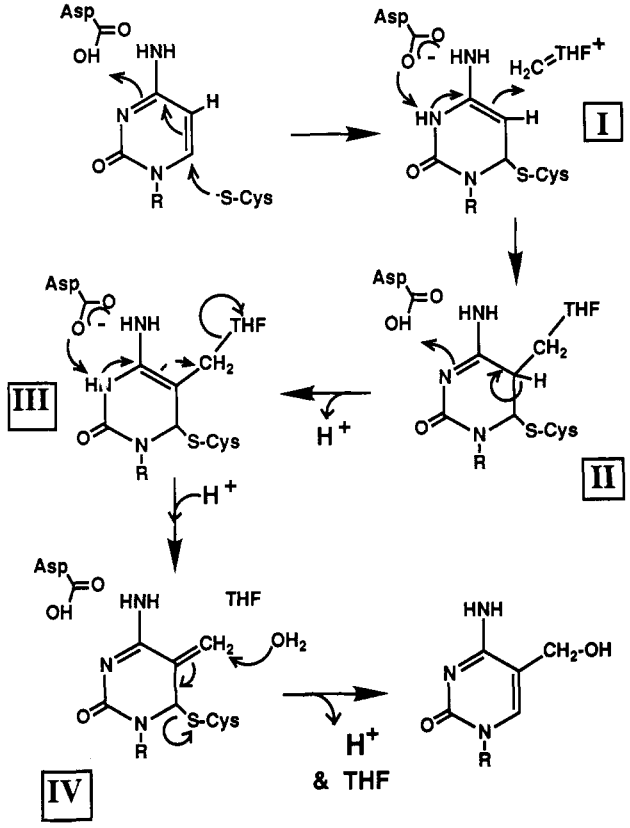
Scheme I: Proposed Mechanism for Catalysis by dCMP Hydroxymethylase



sequence of CH. One of the residues is Asp[179] in CH, which replaces the TS residue Asn[177]. In the structure of the TS ternary complex, the side-chain amide of Asn[177] forms hydrogen bonds with N3 and O4 of the pyrimidine of dUMP. The second residue that is different in CH is Lys[28], which replaces the TS residue Arg[21].

We here report site-directed mutagenesis studies of the roles of two residues in CH, Cys[148] and Asp[179]. We provide evidence of a Michael adduct in catalysis by CH, analogous to those formed during catalysis by TS, formed by addition of the thiol of Cys[148] to C6 of dCMP. Asp[179] is proposed to stabilize the resulting catalytic intermediates and to thereby be a primary determinant of the pyrimidine specificity of CH.

MATERIALS AND METHODS

Materials. Tritium-labeled nucleotides were obtained from Moravek Biochemicals, Brea, CA. CH_2THF was prepared by dissolving THF (Sigma) in a solution of 0.025 M formaldehyde, 0.02 M β-mercaptoethanol, and 10 mM potassium phosphate under nitrogen; the pH was adjusted to 7.5 using 12 M NaOH. The concentration was estimated using the molar absorptivity of 3.2×10^4 at 290–295 nm (Huennekens et al., 1962). Yield of L-(6*R*)-CH_2THF (30–35%) was determined by measuring the complete conversion of CH_2THF to dihydrofolate (DHF) by *Escherichia coli* TS, which is accompanied by a change in molar absorptivity at 340 nm of 6400 (Wahba & Friedkin, 1961). The stated concentrations of CH_2THF have been corrected for the purity of the 6*R* isomer. All other materials were the highest grade commercially available.

E. coli and Bacteriophage Strains. Plasmid pT7-6-42 containing gene *42* and bacteriophage T4 *42*amC87 containing an amber mutation at codon 57 were obtained from Dr. Christopher Mathews (Lamm et al., 1988). Wild-type T4 phage and the nonsuppressor (su⁻) *E. coli* W3110(*L8 lacI*^{q}) were from Dr. A. R. Poteete at University of Massachusetts Medical Center. *E. coli* BL21*thyArecA* (su⁻) was obtained from Barbara Yates (Dev et al., 1988). *E. coli* TG1 used for mutagenesis was from Amersham. *E. coli* JM101Δ*thyA* was from Dr. Marlene Belfort (Bell-Pedersen et al., 1991). Bacteriophage λ CE6 and *E. coli* LE392 (su⁺) and BL21 (su⁻) were obtained from Dr. William Studier (Studier et al., 1988). High-titer λ CE6 stocks were grown in LE392 as described (Patterson & Dean, 1987). The su⁺ strain used for genetic testing and plating of the T4 *42*amC87 phage, *E. coli* Su1-1 [*F lacI*$^{q}_{373}$ *lacZ*$_{U118am}$, *proB*+/*F-ara*, Δ(*lac-pro*)$_{XIII}$, *thi*, *gyrA*, *argAam*, *rif*, *metB supD*], was obtained from Dr. Jeffery Miller at UCLA. R408 helper phage was obtained from Stratagene. When necessary, 100 μg/mL ampicillin was included in liquid or solid media for plasmid selection. JM101Δ*thyA* and BL21*thyArecA* strains were grown in the presence of 50 μg/mL thymidine.

Cloning and Mutagenesis. Conventional recombinant methods were used (Maniatis et al., 1989). Gene *42* from pT7-6-42 was subcloned into pKS+ (Stratagene) as a *Pst*I/*Eco*RI 927 base pair fragment. We refer to these constructs generically as pCH plasmids encoding a specific *42* allele.[2] Gene *42* was also cloned into M13mp18 (Messing et al., 1981) on the same 927 base pair fragment. Single-stranded DNA from either the M13mp18 derivative or pKS+ derivative carrying gene *42* was isolated respectively as recommended by Amersham or by rescue using R408 helper phage as recommended by Stratagene. Oligonucleotide-directed mutagenesis was performed on single-stranded DNA by the phosphorothioate method (Nakamaye & Eckstein, 1986) using the Amersham kit. Mutagenic oligonucleotide primers, 17–22 nucleotides in length containing one or two base changes in the center, and primers for sequencing were obtained from Genetic Designs (now Genosys). Each mutant allele was sequenced in its entirety to ensure that only the desired mutations were present. Mutant alleles generated in M13 were cloned back into pKS+ on the *Pst*I/*Eco*RI fragment. The single-site mutations made are listed in Table I.

Three pCH derivatives containing two mutations in gene *42* were created by in vitro recombination of the D179N mutation with each of the Cys[148] mutations. These double mutants were constructed by ligating the appropriate DNA fragments obtained from cleavages of the requisite pCH plasmids with *Sca*I and *Hpa*I endonucleases. The unique *Hpa*I site of pCH lies within the gene *42* sequence, between the codons for residues 148 and 179. The unique *Sca*I site lies within the β-lactamase gene of the pKS⁺ sequence, ensuring that only the products of ligation which had the desired orientation would confer resistance to ampicillin.

CH Activity in Vivo. CH activity in vivo was estimated from the ratio of the titer of T4 *42*amC87 bacteriophage on an su⁻ host (BL21*thyArecA*) bearing a pCH plasmid encoding a specified *42* allele to the titer on su⁺ (Su1-1) cells; this ratio is the efficiency of plating.

Marker rescue experiments were done by growing the T4 *42*amC87 phage on su⁺ cells harboring the specified pCH plasmids (Table I) and titering progeny on su⁻ (W3110) and su⁺ cells (Thylén, 1988). The reversion rate, measured using a lysate grown in su⁺ cells without a pCH plasmid, is the ratio of phage progeny which grow on su⁻ versus su⁺ cells.

Overproduction and Purification of CH. CH (wild type and variants) were overproduced in strain BL21 (or in

[2] Variants of CH and TS created by mutagenesis of the respective genes 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.

JM101Δ*thyA* or BL21*thyArecA* when necessary to eliminate TS) harboring pCH plasmids by exploitation of the T7 promoter, using λCE6 (Studier et al., 1990). Our purification procedure was a scaled-up version of the method of Mathews et al. (1964), modified as follows. The cells were lysed in a French press under 1100 psi. The pooled fractions containing CH (from chromatography on DEAE cellulose) were concentrated with an Amicon YM10 filter, dialyzed against 50 mM potassium phosphate (pH 6.5), and chromatographed on CM-Sephadex (without purification on calcium phosphate). CH was ca. 95–99% pure after this step. The pooled fractions were concentrated, dialyzed against 0.02 M potassium phosphate (pH 6.7), and further purified on a MonoQ HR 10/10 FPLC column (Pharmacia). Homogeneous CH was stored in 20 mM potassium phosphate (pH 6.7) at 4 or −80 °C. Protein concentrations were routinely determined by the Coomassie binding method (Bradford, 1976), which gave results identical to the Lowry method (Lowry et al., 1951).

As an independent check on the accuracy of the mutagenesis procedure, the molecular weights of CH(wt), CH(D179A), and CH(D179S) were compared by electrospray ionization mass spectrometry, performed on desalted samples by William S. Lane at the Harvard Microchemistry Facility. The molecular masses estimated by this method were 28 486.9, 28 436, and 28 455 for CH(wt), CH(D179A), and CH(D179S), respectively; these values agree reasonably well with those expected from the sequences derived from the DNA sequences (28 487, 28 443, and 28 459).

Enzyme Assays. CH was assayed by monitoring the release of tritium from 5-^{3}H-labeled nucleotides into solvent water (Yeh & Greeberg, 1967). Standard assays contained 80 mM potassium phosphate (pH 7.4), 20 mM β-mercaptoethanol, 0.4 mM dCMP or dUMP (9.25×10^7 Bq of tritium/mmol), and 0.6 mM CH_2THF and were incubated at 30 °C. Initial velocities were measured at less than 10% conversion of substrate to product. The velocities, corrected for nonenzymatic rates of tritium release, were linearly dependent upon added enzyme. For determination of kinetic parameters, initial velocities were fit by nonlinear least squares regression of the data upon the requisite equations. The data were routinely analyzed according to the Michaelis–Menten equation, at fixed saturating concentrations of CH_2THF for determining the values of K_M for nucleotides, and, in some cases, at fixed concentrations of nucleotides for determining the values of K_M for CH_2THF. The determination of the value of K_M for CH_2THF for the CH(D179N)-catalyzed reaction of dUMP required measurements of some initial velocities at concentrations of CH_2THF lower than the enzyme concentration. In this case a modified kinetic analysis was done [as described by Henderson (1973); see also p 74 in Segel (1975)], to ensure that depletion of free substrate by enzyme binding did not invalidate the calculated value of K_M.

Inactivation of CH by FdUMP was done by reacting enzyme with 50 μM FdUMP for timed intervals under conditions identical to those used for activity assays, but lacking nucleotide substrates. Inactivation of CH by 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was done in standard assay buffer (lacking β-mercaptoethanol) at 30 °C for 15 min. Reactive thiol groups on CH were titrated by reacting 0.5 mg/mL enzyme with 1 mM DTNB in 80 mM potassium phosphate at pH 7.4 (Ellman, 1959; Lundblad & Noyes, 1984).

Binding Assays. Filter binding assays were performed under conditions similar to those used for tritium release assays. Each reaction contained (unless stated otherwise) 14.3 μM D179N enzyme monomer and the indicated molar ratio of [6-^{3}H]FdUMP (4.0×10^5 Bq/mmol) in a volume of 0.05 mL. After reaction at 30 °C for 20 min (except as noted), 0.025-mL samples of the reaction mixture were applied to nitrocellulose filters [0.45-μm pore size, from Schleicher and Schuell; prewet with 80 mM phosphate (pH 7.4)]. The samples were left on the filter for 1 min, and the filters were washed five times with 2 mL of 80 mM phosphate. Each filter was dissolved in 1 mL of dimethyl sulfoxide and counted by liquid scintillation in 9 mL of Aquasol (Dupont).

FdUMP complexes for analysis by denaturing gel electrophoresis were prepared as described for filter binding analysis, but in 20 μL. After incubation at 25 °C for 40 min (2 h for the D179A and D179S enzyme variants), samples were heated with an equal volume of SDS sample buffer for 2 min at either 80 or 100 °C. The denatured samples were run on a 1% SDS/12% polyacrylamide gel with a 3% stacking gel in a buffer containing 0.025 M Tris, 0.192 M glycine, and 1% SDS at pH 8.3. A portion of the gel (e.g., lanes M, A, and B, Figure 3) was stained in 2.5% Coomassie. The rest of the gel was treated in 10% trichloroacetic acid for 10 min, rinsed with water, and treated with Amplify (Amersham) for 30 min. The dried gel was exposed to film at room temperature for 36 h, or longer in some cases. Band intensities on the autoradiogram were quantitated using a Hoefer Scientific GS300 scanning densitometer. The intensities were corrected for protein content and normalized to the most intense band on the autoradiogram.

Product Analysis. Products from CH-catalyzed reactions of [6-^{3}H]nucleotides with CH_2THF were fractionated by HPLC and detected by liquid scintillation counting. Prior to HPLC, the reaction mixtures were freed of enzyme and much of the folate using disposable C18 cartridges (Waters Sep-Pak). Portions of the nucleotide products were hydrolyzed to nucleosides with bacterial alkaline phosphatase (Worthington) to allow comparison with an authentic sample of 5-(hydroxymethyl)-dUrd (Sigma). The HPLC analyses were done isocratically on a C18 Lichrosorb column (Alltech) with 5 mM each potassium phosphate and tetrabutylammonium sulfate (pH 7.0) and 10% (v/v) methanol (for nucleotides) or 0.25 M ammonium acetate (pH 6.0) (for nucleosides).

A nonradioactive sample of the product of the reaction between dUMP and CH_2THF catalyzed by homogeneous CH(D179N) was prepared and purified by chromatography on Dowex-1 and HPLC on a Synchropack C18 column (Alltech). The sample was lyophilized and converted to its nucleoside form by phosphatase treatment. The sample was freed of protein, and the nucleoside was purified by HPLC on the Syncropack C18 column. The product was deionized and analyzed by (i) silica gel thin-layer chromatography in 4:1 chloroform/methanol and (ii) fast atom bombardment mass spectrophotometry at the Massachusetts Institute of Technology Mass Spectrometry Facility.

RESULTS

Activity of CH Variants in Vivo. The efficiency of plating (eop) of T4 *42*amC87 on su$^-$ hosts carrying plasmid-borne *42* alleles correlates with the specific activity of the encoded CH variants (Table I). The replacements of Cys[148] or of Asp[179] resulted in a 500-fold decrease in the relative eop. [Replacement of Cys[112], the only other Cys in CH, with Ser does not decrease complemention of T4 *42*amC87, but probably decreases the stability of CH (data not shown).] Identical results were obtained in BL21*thyArecA* and W3110.

The eop on cells containing pCH derivatives with mutant *42* alleles is ca. 10^4 higher than the reversion rate, presumably due to recombination. T4 *42*amC87 grown in su$^+$ cells in the absence of a pCH plasmid exhibit a reversion rate of 10^{-5}.

Table I: Activity of CH (Wild Type and Variants) in Vivo and in Vitro[a]

allele	eop of T4 *42*amC87	initial velocity (μmol min^{-1} mg^{-1})
wild type	1.0	3.0
C148G	2.0×10^{-3}	$<3.8 \times 10^{-6}$
C148D	2.0×10^{-3}	$<2.7 \times 10^{-5}$
C148S	2.0×10^{-3}	$<4.0 \times 10^{-5}$
D179S	2.0×10^{-3}	not detectable[b]
D179A	2.0×10^{-3}	5.7×10^{-5}
D179N	2.0×10^{-3}	5.3×10^{-4}

[a] eop's were determined as described in Materials and Methods. Initial velocities were measured with the purified enzymes by tritium release from [5-^{3}H]dCMP under standard assay conditions. [b] No tritium release could be measured above background levels.

Table II: Kinetic Parameters of CH (Wild Type, D179N, and D179A) with Nucleotide Substrates[a]

allele and substrate	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (mM^{-1} min^{-1})
wild type with			
dCMP[b]	892 ± 88	0.14 ± 0.05	6251 ● 327
dUMP[c]	0.148 ± 0.002	4.4 ± 0.4	0.034 ● 0.006
D179N with[b]			
dCMP	0.248 ± 0.027	0.61 ± 0.07	0.406 ± 0.076
dUMP	0.50 ± 0.04	0.24 ± 0.01	2.08 ± 0.17
D179A with[d]			
dCMP	0.20 ± 0.04	61 ± 13	0.0033 ± 0.0017
dUMP	0.20 ± 0.02	55 ± 23	0.0036 ± 0.0019

[a] The entries are the mean value ● SE (calculated for enzyme dimer). [b] Nucleotide concentrations, 0.05–0.4 mM. [c] Nucleotide concentrations, 0.05–10 mM. [d] Nucleotide concentrations, 1–80 mM.

The fraction of wild-type T4 progeny recovered from an su$^+$ host (Su1-1) was increased 1000-fold when the host contained any of the pCH plasmids listed in Table I.

Activity of CH Variants in Vitro. All of the CH variants listed in Table I could be overproduced in *E. coli* and purified in comparable yields. Approximately 1 mg of homogeneous CH protein per gram of cells is normally obtained. Replacement of Cys^{148} by Ser, Asp, or Gly decreases the specific activity on [5-^{3}H]dCMP at least (7×10^4)-fold. Most, if not all, of the residual activity of the Cys^{148} variants is due to contaminating CH(wt). This was indicated by the inactivation of the variants upon exposure to DTNB. Titration with DTNB indicated 2 equiv. of thiol/CH(wt) dimer, and less than 0.1 equiv. of thiol/dimer with each Cys^{148} variant. [Cys^{112} is apparently inaccessible for reaction with DTNB (Lee et al., 1988).] If the activity of any Cys^{148} variant were due to the mutant enzyme itself, this activity should not have been affected by DTNB.

Many of the CH variants (C148D, C148G, C148S, and D179N) crystallize under conditions identical to those for crystallization of wild-type CH (Hardy, unpublished results). The crystals of CH(wt) and the variants are similar macroscopically, indicating no significant structural changes due to the replacement of Cys^{148} by Asp, Gly, or Ser, or of Asp^{179} with Asn.

Alteration of Kinetic Parameters for dCMP and dUMP by Asp^{179} Substitutions. The major effects of the D179N residue change are decreases in the values of k_{cat} and k_{cat}/K_M for dCMP, which are respectively 10^3- and 10^4-fold lower (Table II). However, the D179N variant also exhibits a 2.5-fold increase in k_{cat} and a 61-fold increase in k_{cat}/K_M for dUMP, compared to the values with CH(wt). Hence the relative value of k_{cat}/K_M for dCMP and dUMP is altered by ca. 5 orders of magnitude by the D179N mutation. The unique values of K_M (Table II) with the Asp^{179} variants indicate that their activities are not due to contaminating CH(wt) or TS.

The D179A substitution decreases the values of k_{cat} and k_{cat}/K_M for dCMP by (4×10^3)- and (2×10^6)-fold, respectively; the values for dUMP are unaffected or slightly decreased. The resulting CH variant has essentially identical activity on dCMP and dUMP (Table II). Replacement of Asp^{179} with Ser results in a total loss of activity on either nucleotide.

The value of K_M for CH_2THF was also slightly affected by the D179N mutation. The value of 3.2 (±0.5) μM determined with CH(wt) at 0.4 mM dCMP is similar to the value reported by Lee et al. (1988). A higher value of 18 (±2) μM for CH(D179N) at 0.4 mM dUMP was calculated from an analysis which is valid at enzyme concentrations higher than that of substrate (Henderson, 1973); this value of K_M for CH_2THF is indistinguishable from a value calculated by a fit of the initial velocity data to a simple hyperbolic (16 ± 4 μM). For every combination of enzyme and nucleotide listed in Table II, the observed initial velocities were independent of the concentration of CH_2THF between 0.3 and 2 mM. Hence each CH variant is saturated with respect to CH_2THF at 0.6 mM, which was the concentration used to obtain the results in Table II.

Product Formation by CH(D179N). Replacement of Asp^{179} with Asn changes the substrate specificity of CH, but does not affect catalytic specificity. The reaction of [6-^{3}H]dUMP with CH_2THF catalyzed by CH(D179N) leads to >90% conversion of the nucleotide to a material which elutes on the HPLC system employed at a retention volume (20.5 mL) intermediate between those of dUMP (15 mL) and dTMP (23 mL), as previously reported for 5-(hydroxymethyl)-dUMP (Kunitani & Santi, 1980). After conversion to the nucleoside, this material coelutes on HPLC with authentic 5-(hydroxymethyl)-dUrd (retention volume 30.5 mL), well-resolved from dUrd (28 mL) and Thd (77 mL).

The nucleoside form of the dUMP-derived product of the CH(D179N) reaction was prepared in low yield (ca. 12%), due to our lengthy purification procedure, and was analyzed by TLC and mass spectrometry. The nucleoside product has the same R_f as authentic 5-(hydroxymethyl)deoxyuridine on silica gel TLC in 4:1 chloroform/methanol (0.62). Mass spectral analysis of the nucleoside product showed an ion of m/z 259, consistent with its identity as the $(M + H)^+$ ion of 5-(hydroxymethyl)-dUrd.

Interaction of CH with FdUMP. Neither CH(wt) nor CH(D179N) is inhibited by 0.5 mM FdCMP. In contrast, both enzymes are inhibited by a 10-fold lower concentration of FdUMP. This inhibition is time-dependent and requires CH_2THF. Treatment of CH(wt) with 0.05 mM FdUMP and 2 mM CH_2THF for 40 min results in 83% inactivation. CH(D179N) only requires 10 min under the same conditions for 100% inactivation. Hence, replacement of Asp^{179} with Asn enhances the enzyme's ability to interact with FdUMP.

Both wild-type CH and the three Asp^{179} enzyme variants form a complex with [6-^{3}H]FdUMP, dependent upon the presence of CH_2THF, which is retained on nitrocellulose filters. Maximum binding for the wild-type enzyme and the D179N variant occurs at approximately the same reaction times required for the maximum inhibition of activity. The D179S and D179A variants of CH, reacted with 0.1 mM [6-^{3}H]-FdUMP for 2 h at 30 °C, exhibit 55% and 22%, respectively, of the binding observed with CH(wt) or CH(D179N). None of the Cys^{148} enzyme variants form a complex with [6-^{3}H]-FdUMP which is detectable on nitrocellulose filters, even after reaction times of up to 2 h. Double mutants possessing both a Cys^{148} residue change and the D179N mutation also do not form a complex detectable on nitrocellulose filters.

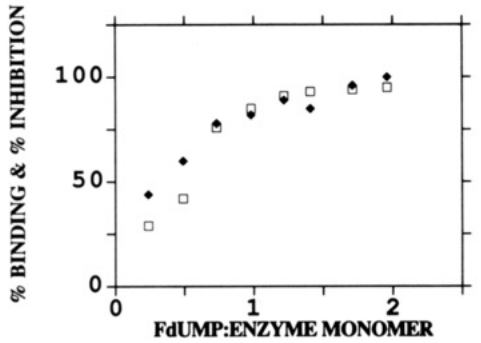


FIGURE 1: Interaction of FdUMP with CH(D179N). Filter binding (diamonds): each reaction contained 1.43 nM CH(D179N) monomer and sufficient [6-^{3}H]FdUMP (74 Bq/nmol) to give the indicated molar ratio. The points are the mean values from triplicate samples, relative to maximum binding at FdUMP:monomer ratios of 1.25–2.00. Inhibition of tritium release from [5-^{3}H]dUMP (squares): each reaction contained 14.4 μM CH(D1790N) monomer and sufficient FdUMP to give the indicated molar ratio.

Titration of CH(D179N) with FdUMP by nitrocellulose filter binding correlates with the inhibition of the enzyme's activity on [5-^{3}H]dUMP (Figure 1). Maximum binding was corrected for binding efficiency of the filters (30%) and normalized to 100% at the molar ratios of 1.25–2.00, where binding was constant within experimental error (20 ± 2 pmol of FdUMP bound per 70 pmol of enzyme placed on the filter). Both maximum binding to nitrocellulose and maximum inhibition of catalytic activity occur at a ratio of 1 mol of FdUMP/1.18 mol of CH monomer. This result is consistent with the idea that the nucleotide analogue binds to the enzyme dimer at both active sites in the presence of CH_2THF.

Covalent Complex Formation with FdUMP. To determine if the complex formed between enzyme, FdUMP, and CH_2-THF is covalent, complexes were analyzed by denaturing gel electrophoresis. Figure 2 compares Coomassie-stained protein standards with an autoradiogram of complexes formed by CH(wt) and CH(D179N) in the presence of [6-^{3}H]FdUMP, denatured with SDS at either 80 or 100 °C. Complex formation under these conditions is dependent on the presence of CH_2THF (lanes D, F, H, and J).

The bands observed on the autoradiogram shown in Figure 2 were quantitated by densitometry; the relative intensities are listed in Table III. Complexes formed with either enzyme are more stable when denatured at 80 °C rather than 100 °C. The yield of covalent complex with CH(wt) is less than that with CH(D179N) at both denaturation temperatures.

Covalent complexes with [6-^{3}H]FdUMP could also be detected by the SDS gel method using the D179A and D179S variants of CH (data not shown). The efficiency of detection of the covalent complexes with CH(D179A) or CH(D179S) was lower than that of CH(wt). Detection of the complex for the D179S variant required a 6-day exposure of an autoradiogram, yielding a faint band, compared to that observed in 16 h with the same amount of CH(wt). None of the Cys[148] or Cys[148]/D179N variant proteins form a complex detectable on the gel, even at film exposure times of up to 6 days.

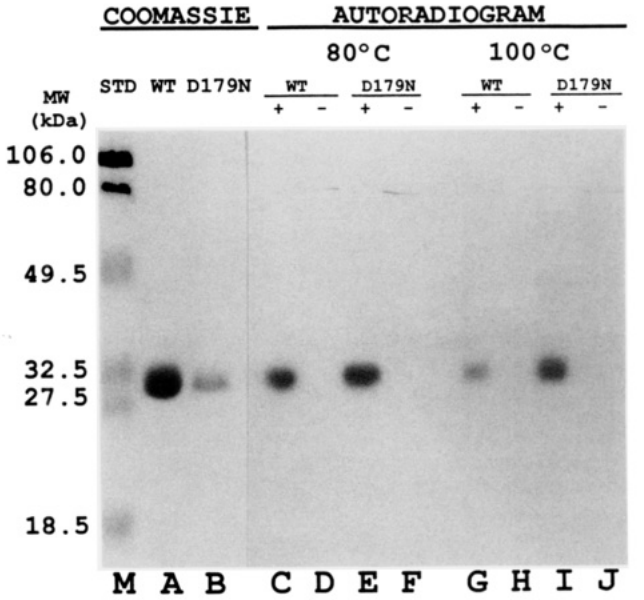


FIGURE 2: Covalent complex formation between [6-^{3}H]FdUMP, CH_2THF, and CH(wt) or CH(D179N) analyzed by SDS–PAGE. The + and – labels at the top of the figure refer to the presence and absence of CH_2THF. Gel samples: lane M, Coomassie-stained molecular weight markers (top to bottom: phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and hen egg lysozyme); lanes A and B, Coomassie-stained protein [A, 53 μmol of CH(D179N); B, 178 μmol of CH(wt)]; lanes C and G, 178 μmol of CH(wt), 2 mM CH_2THF, and 417 pmol of [6-^{3}H]FdUMP (8.9×10^2 Bq/pmol); lanes D and H, same as C and G minus CH_2THF; lanes E and I, 53 μmol of CH(D179N), 2 mM CH_2THF, and 125 pmol of [6-^{3}H]FdUMP; lanes F and J, same as E and I minus CH_2THF. Samples C–F were heated 2 min at 80 °C and samples M, A, B, and G–J at 100 °C before loading. The relative intensities of each band are reported in Table III.

Table III: Covalent Complex Formation by CH (Wild Type and D179N) with FdUMP in the Presence of CH_2THF

enzyme	denaturation temp (°C)	relative intensity[a]
wild type	80	0.23
	100	0.08
D179N	80	1.00
	100	0.26

[a] Band intensities were determined by scanning densitometry of the autoradiogram shown in Figure 2. The values are the intensity of each band relative to the most intense band, normalized for the amount of protein on the gel.

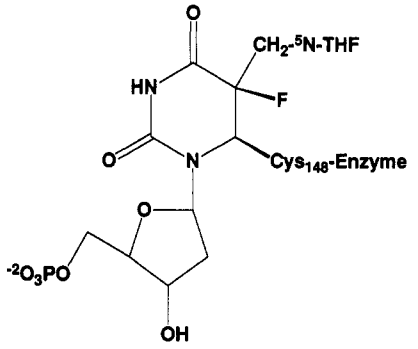
DISCUSSION

Complementation Assay for in Vivo Activity of CH. The ability of the plasmid-encoded *42* gene to increase the plating efficiency of a T4 phage which has a defective *42* gene provides an in vivo assay for CH activity. The increased eop caused by the plasmids listed in Table I may be due to complementation, recombination, or both. However, the frequency of recombination alone is identical for all the plasmid-borne alleles listed in Table I (data not shown). The 500-fold higher eop caused by pCH(wt), compared to pCH carrying mutant *42* alleles (Table I), must therefore be due to the wild-type enzyme's higher specific activity. Our data do not eliminate the possibility that some fraction of the increased eops caused by plasmid-encoded mutant *42* alleles is due to residual activity of the corresponding CH variants.

Similarities between the Mechanisms of CH and TS. The chemical analogy between the reactions catalyzed by TS and CH led to the initial suggestion that the enzymes may utilize similar mechanisms (Pogolotti & Santi, 1977). This idea found strength in the similarities between the amino acid sequences of the two enzymes (Lamm et al., 1988; Thylén, 1988). Based on X-ray structural studies of TS crystallized with ligands bound (Matthews et al, 1990a; Montfort et al., 1990), roles have been proposed for many of the residues in the active site of TS (Finer-Moore et al., 1990; Matthews et al, 1990b). Many of the corresponding residues in CH may have corresponding roles.

CH catalyzes an isotope-exchange reaction between [5-^{3}H]-dCMP and solvent water that requires the presence of THF (Yeh & Greenberg, 1967). This partial reaction is slightly

Chart II: Proposed Structure for Covalent Complex between FdUMP, CH_2THF, and dCMP Hydroxymethylase



faster than the overall turnover reaction,[3] which requires CH_2-THF. The exchange reaction can be explained by the transient formation of a covalent CH–nucleotide adduct, such as I shown in Scheme I. Reversible protonation of I would result in the observed exchange reaction. In this adduct, carbon 5 is poised for the electrophilic addition of CH_2THF. Such Michael adducts are intermediates in reactions catalyzed by TS [reviewed by Santi and Danenberg (1984)]. Michael adducts are probably employed by many enzymes which alkylate C5 of pyrimidines (Santi et al., 1978), such as DNA (^{5}Ctd)-methyltransferase (Osterman et al., 1988), dUMP hydroxymethylase (Kunitani & Santi, 1980), and tRNA (^{5}Urd) methyltransferase (Santi & Hardy, 1987).

Role of Cys^{148} as a Catalytic Nucleophile. The inactivation of CH for either catalysis or FdUMP binding caused by the substitution of Cys^{148} (Table I) indicates this residue's side-chain thiol to be the nucleophile in Michael adduct formation (Scheme I). Cys^{148} is likely to be the essential sulfhydryl group identified by Sander and co-workers (Lee et al., 1988). Cys residues also provide the nucleophile during catalysis by TS from *E. coli* and *Lactobacillus casei* (Bellasario et al., 1979; Belfort et al., 1983), *E. coli* tRNA-(^{5}Urd)methyltransferase (Kealy & Santi, 1991), and DNA (^{5}Ctd)methyltransferase from *Haemophilus aegyptius* (Chen et al., 1991). *E. coli* TS is similarly rendered inactive by replacement of the corresponding nucleophilic residue Cys^{146} with Ala or Gly (Dev et al., 1988). However, *E. coli* TS, in which Cys^{146} is replaced by Ser, can still complete catalytic turnover (Dev et al., 1988), in contrast to the more complete loss of the catalytic activity of CH(C148S).

FdUMP as a Mechanism-Based Inactivator of CH. The inactivation of CH by FdUMP is time-dependent and CH_2-THF-dependent, similar to the inactivation of TS by FdUMP. At least a portion of the complex formed between CH and FdUMP is covalent (Figure 2). By analogy with the known structure of the covalent TS adduct (Matthews et al., 1990a), we propose that CH forms an adduct with FdUMP and CH_2-THF with the structure shown in Chart II. The facts that both the inactivation of CH and the binding between CH and FdUMP require CH_2THF and that THF alone is insufficient argue strongly for the structure shown, despite no direct evidence for the presence of CH_2THF. Further structural and kinetic details of the interaction between CH and FdUMP will be published elsewhere.

Role of Asp^{179} as a Determinant of Substrate Specifity. The most novel result of our studies on CH was the alteration of the specificity of CH toward pyrimidine mononucleotides upon the substitution by Asn of Asp^{179} (Table II). The design of this mutation was based on our studies of a reciprocal mutation in TS. In *E. coli* TS, replacement by Asp of Asn^{177}, which corresponds to Asp^{179} in CH, leads to a reversal of the substrate preference of TS from dUMP to dCMP (Hardy & Nalivaika, 1992). A similar result has been reported by Liu and Santi (1992) for TS from *L. casei*. The putative role of this Asn in TS is to provide stabilization, by hydrogen bonding, of the partial negative charge developed on O4 of covalently bound dUMP during catalysis (Matthews et al., 1990b). Results with the Cys^{148} variants of CH strengthened the hypothesis that the structures and mechanisms of CH and TS are similar and encouraged us to try altering the nucleotide specificity of CH by making the D179N mutation.

The alteration of the enzyme's preference for dCMP and dUMP as substrates was more evident in the values of k_{cat}/K_M than of k_{cat} (Table II). Conversion of Asp^{179} to Asn reduces the value of k_{cat}/K_M for dCMP by (1.5×10^4)-fold and increases the value of k_{cat}/K_M for dUMP by 60-fold; as a result, CH(D179N) has a slight preference for dUMP. The alteration in substrate preference is specific to the D179N mutation, since CH(D179A) lacks nucleotide specificity, and CH(D179S) is completely inactive.

The alteration of Asp^{179} to Asn also increases the yield of the complex between CH(D179N) and FdUMP after denaturation, compared to that obtained with CH(wt) (Figure 2 and Table III). This may be due to either an increased proportion of the native complex which is covalent or an increased stability of the complex once formed. Either way, a more favorable interaction of the uracil heterocycle with CH(D179N) than with CH(wt) is indicated. In contrast, the D179A and D179S mutations interfere with the ability of CH to interact with FdUMP. These observations support the notion that Asp^{179} is a primary determinant of the pyrimidine nucleotide specificity of CH.

In wild-type CH, the 10^5-fold greater value of k_{cat}/K_M for dCMP than for dUMP provides an estimate for the difference in the energies of the transition states for the first irreversible step of the reaction of the two nucleotides. This energy for dCMP is ca. 6.5 kcal/mol lower than the corresponding energy for dUMP. The difference is too great to be due to differences in the hydrogen bonding that might occur with enzyme-bound dCMP and dUMP, to either a charged or uncharged Asp side chain (Fersht et al., 1985). The pyrimidine of either nucleotide, bound noncovalently, could interact with a carboxylic acid, or with a carboxylate group. These interactions could vary somewhat in stability but should be similar for dUMP and dCMP. This idea is not inconsistent with the 30-fold disparity in the values of K_M for dCMP and dUMP with CH(wt). We suggest that the specificity of CH is not based primarily upon noncovalent interactions of the enzyme with dCMP and dUMP, but upon the ability of CH to stabilize covalent catalytic intermediates.

We propose that the role of Asp^{179} during CH catalysis is to protonate N3 of covalently bound dCMP. Asp^{179} thereby acts as a sink for the charge developed on various intermediates (Scheme I). This minimizes the charge on N3. The acidity of this nitrogen is expected to be several orders of magnitude less than the acidity of O4, since amine anions are considerably less stable than oxy anions. Whereas a hydrogen bond from a neutral donor (Asn side chain) might be sufficient to stabilize an oxy anion, stabilizing an amine anion requires an actual proton transfer. This would indicate a perturbed pK_a for the carboxylic acid side chain of Asp^{179}, since the CH is quite active at pH 7 ($k_{cat}/K_{M,dCMP} = 10^5\ M^{-1}\ s^{-1}$). This pH was reported to be the optimum in assays done using fixed substrate concentrations (Pizer & Cohen, 1962; Yeh & Greenberg, 1967).

[3] This indicates that some event after the formation of the Michael adduct is rate-limiting for the overall reaction.

Protonation of N3 may be a general mechanism for stabilizing Michael adducts between an enzymic nucleophile and C6 of cytosine moieties alkylated at C5 by other enzymes. The dCMP methyltransferase of the *Xanthomonas* bacteriophage XP-12, which utilizes CH_2THF as a one-carbon donor and reductant (Feng et al., 1978), is a candidate for such an enzyme. Another is DNA (Ctd)methyltransferase. The sequences of 13 bacterial DNA (Ctd)methyltransferases (Pósfai et al., 1989) have several invariant Asp and Glu residues, one of which might serve as the catalytic acid for protonation of N3 of cytosine in DNA during catalysis. This would require a conformational change of the DNA substrate to expose N3 and the 4-amino group of the cytosine to be methylated. This is consistent with the DNA conformational change required to allow covalent adduct formation to C6 of cytosine during catalysis by DNA (Ctd)methyltransferases (Osterman et al., 1988; Chen et al., 1991).

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Registry No. CH, 9012-68-4; Cys, 52-90-4; Asp, 56-84-8; Asn, 70-47-3; dCMP, 1032-65-1; dUMP, 964-26-1; TS, 9031-61-2; FdUMP, 134-46-3; CH_2THF, 3432-99-3.