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Deoxycytidylate Hydroxymethylase: Purification, Properties, and the Role of a Thiol Group in Catalysis†

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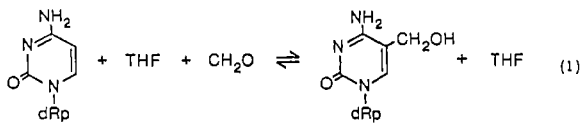
ABSTRACT: Deoxycytidylate (dCMP) hydroxymethylase from *Escherichia coli* infected with a T-4 bacteriophage amber mutant has been purified to homogeneity. It is a dimer with a subunit molecular weight of 28 000. Chemical modification of the homogeneous enzyme with *N*-ethylmaleimide (NEM) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) leads to complete loss of enzyme activity. dCMP can protect the enzyme against NEM inactivation, but the dihydrofolate analogues methotrexate and aminopterin alone do not afford similar protection. Compared to dCMP alone, dCMP plus either methotrexate or aminopterin greatly enhances protection against NEM inactivation. DTNB inactivation is reversed by dithiothreitol. For both reagents, inactivation kinetics obey second-order kinetics. NEM inactivation is pH dependent with a pK_a for a required thiol group of 9.15 ± 0.11. Complete enzyme inactivation by both reagents involves the modification of one thiol group per mole of dimeric enzyme. There are two thiol groups in the totally denatured enzyme modified by either NEM or DTNB. Kinetic analysis of NEM inactivation cannot distinguish between these two groups; however, with DTNB kinetic analysis of 2-nitro-5-thiobenzoate release shows that enzyme inactivation is due to the modification of one fast-reacting thiol followed by the modification of a second group that reacts about 5–6-fold more slowly. In the presence of methotrexate, the stoichiometry of dCMP binding to the dimeric enzyme is 1:1 and depends upon a reduced thiol group. It appears that the two equally sized subunits are arranged asymmetrically, resulting in one thiol-containing active site per mole of dimeric enzyme.

Deoxycytidylate hydroxymethylase (EC 2.1.2.8) catalyzes the reversible hydroxymethylation of dCMP[1] (eq 1). The



(1)

enzyme was discovered in T-even phage infected *Escherichia coli* (Flaks & Cohen, 1957). In 1964, it was semipurified with a low yield (Mathews et al., 1964). THF and CH_2O are required; however, the active cofactor is likely methylenetetrahydrofolate, formed by the rapid condensation of THF and CH_2O (Kallen & Jencks, 1966a,b). The enzyme can be assayed by the measurement of formaldehyde incorporation into product (Flaks & Cohen, 1959) and by ^3H exchange from C-5 of dCMP to solvent (Yeh & Greenberg, 1967). Steady-state kinetic constants have been determined for dCMP, THF, and CH_2O; however, unlike other enzymes (Pogolotti & Santi, 1977) proposed to be mechanistically similar, 5-F-dCMP does not appear to inhibit the crude enzyme (Pizer & Cohen, 1963; Flaks & Cohen, 1959). The objectives of this paper are to show methods for the large-scale production and purification of the enzyme from *E. coli* infected with a T-4 bacteriophage mutant lacking the genes for bacterial cell lysis and to provide chemical modification data implicating the requirement of a single thiol group for catalysis.

Materials and Methods

Materials. Bacteriophage, T-4, 45 am, E-51x5, reg A1x3, and the amber supressor host *E. coli* Cr 63 were generously provided by Dr. John Wiberg of the University of Rochester. Unless specified, all materials were from Sigma Chemical Co. Blue Sepharose CL-6B and Sephadex G-100 and G-25 were from Pharmacia. DEAE-Bio-Gel A and all electrophoresis reagents were from Bio-Rad. Scinti Verse E liquid scintillation cocktail and formaldehyde were from Fisher. *N*-Ethyl[2,3-^{14}C]maleimide was purchased from Amersham. [5-^3H]dCMP and [2-^{14}C]dCMP were obtained from Moravek Biochemicals.

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[1] Abbreviations: dCMP, deoxycytidylate; THF, tetrahydrofolate; MTX, methotrexate (4-amino-10-methylfolic acid); NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; Gdn-HCl, guanidine hydrochloride; Tris, tris(hydroxymethyl)-aminomethane; dCMP hydroxymethylase, deoxycytidylate hydroxymethylase; DEAE, diethylaminoethyl; HPLC, high-pressure liquid chromatography; SDS, sodium dodecyl sulfate; dUMP, deoxyuridine 5′-phosphate.

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Components of the bacterial culture media were from Difco.

Growth of Bacteriophage-Infected E. coli. Stocks of the bacteriophage amber mutant were grown in *E. coli* Cr 63 by standard methods (Adams, 1959) using GCA (glycerol casein hydrolysate) medium containing 30 g of glycerol, 15 g of casamino acids, 10.5 g of Na_2HPO_4, 4.5 g of KH_2PO_4, 1.0 g of NH_4Cl, 0.3 g of $MgSO_4$, and 0.3 mL of 1.0 M $CaCl_2$ per liter. Following cell lysis, bacteriophage were concentrated to approximately 10^{12} cells/mL by membrane filtration.

E. coli B infected with the mutant phage were grown in a 250-L New Brunswick fermentor (200-L culture) with the same media. The cells were grown with aeration to a concentration of 3.5×10^9 cells/mL (200 Klett units) followed by addition of 10 mg/L L-tryptophan as adsorption factor. Concentrated bacteriophage were then added to a multiplicity of 4–5 plaque-forming units per *E. coli* cell. On the basis of data showing that enzyme specific activity was about maximal after about 100 min of infection, the infected cells were usually harvested after 90 min of infection with vigorous aeration using a Sharples continuous flow centrifuge. Yields of infected cells from a typical fermentation were about 7 g of wet cell paste/L of media. The cell paste was stored at −70 °C until used.

Enzyme Purification. The enzyme was purified to homogeneity from *E. coli* B infected with the amber, T-4 bacteriophage mutant by using a modification of the method of Mathews et al. (1964). Changes to these previously used procedures include use of lysozyme to prepare the crude extract, use of DEAE-Bio-Gel A rather than DEAE-cellulose in the ion-exchange step, and affinity chromatography on Blue Sepharose CL-6B rather than calcium phosphate gel chromatography.

To each gram of infected *E. coli* B was added 2.0 mL of 0.05 M KH_2PO_4, pH 9.0, followed by the addition of 5.0 M NaOH to adjust the pH to 8.5. Lysozyme was added to a final concentration of 2.0 mg/mL followed by incubation at room temperature for 1 h and addition of 4% sodium deoxycholate to a final concentration of 0.05%. This crude preparation was then subjected to streptomycin sulfate treatment, $(NH_4)_2SO_4$ fractionation, and DEAE ion-exchange chromatography as described by Mathews et al. (1964). The dialyzed DEAE chromatography effluent was then applied to a Blue Sepharose CL-6B affinity column (1.0 mL of swollen gel/7.0 mg of protein) that had been equilibrated with 0.02 M KH_2PO_4, pH 6.8. The column was then washed with a minimum of 10 bed volumes of equilibrating buffer to elute nonspecifically bound protein. The enzyme was specifically eluted with 0.25 mM dCMP and 0.30 mM THF in 0.02 M KH_2PO_4, pH 6.8. One-milliliter fractions containing enzyme activity were pooled and exhaustively dialyzed against four 4.0-L changes of 0.02 M KH_2PO_4, pH 6.8 at 4 °C. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was used as a criterion of purity. The enzyme was stored at −20 °C.

Enzyme Assays. Enzyme activity was routinely assayed by measuring the exchange of tritium from [5-^{3}H]dCMP to solvent by using a modification of the assay previously used by Tomich et al. (1974). Reaction mixtures contained 80 mM KH_2PO_4, pH 7.6, 20 mM 2-mercaptoethanol, 0.40 mM [5-^{3}H]dCMP, 0.60 mM *dl*, L-THF in a total volume of 0.50 mL. Reaction mixtures were incubated in microcentrifuge tubes for 10 min at 30 °C and terminated by the addition of 0.80 mL of a stirred suspension containing 200 mg of activated charcoal/mL in a 10 mM phosphate and 10 mM pyrophosphate, pH 7.0, buffer. Charcoal adsorption continued with frequent stirring at room temperature for 2–3 min followed by a 3-min centrifugation in a Model 235 B Fisher microcentrifuge. Tritium exchanged to solvent was measured in 0.20 mL of the supernatant solution in 5.0 mL of Scinti Verse E cocktail with a Beckman LS 7000 liquid scintillation counter.

High-pressure liquid chromatography was used to develop an assay that measured the 5-hydroxymethyl-dCMP product. Vydac reverse-phase, C_{18} columns (0.46 × 25 cm) were used to separate dCMP from 5-hydroxymethyl-dCMP by using a Gilson instrument with sample detection at 254 nm. Reaction mixtures contained 30 mM KH_2PO_4, pH 7.6, 3.0 mM 2-mercaptoethanol, and 10–20 μg of enzyme in a final volume of 0.20 mL. Following initiation by the addition of enzyme, reaction mixtures were incubated 10 min at 37 °C and terminated by transfer of 0.10 mL to a glass tube preheated in a heating block. Samples were then injected on the column and eluted isocratically with 5.0 mM tetrabutylammonium hydrogen sulfate in 5.0 mM KH_2PO_4, pH 5.0, at a flow rate of 1.0 mL/min.

Protein concentration was routinely measured by the method developed by Lowry et al. (1951).

Enzyme Inactivation with NEM. NEM concentration was spectrophotometrically measured at 302 nm by using an extinction coefficient of 620 M^{-1} cm^{-1} (Lundblad & Noyes, 1984). The enzyme and NEM were incubated at 30 °C under various conditions. Aliquots were removed with time and assayed for enzyme activity by using the titrium exchange assay. Pseudo-first-order rate constants for inactivation were determined from semilogarthmic plots of extent inactivation versus time. Enzyme thiol groups were titrated by using *N*-ethyl[2,3-^{14}C]maleimide and liquid scintillation counting.

Enzyme Inactivation with DTNB. Enzyme thiol groups were modified by DTNB according to the method of Ellman (1959). The kinetics of enzyme inactivation were measured as previously described for NEM inactivation, except experimental conditions were the same as those used when TNB formation was measured as an index of thiol group modification. The kinetics of thiol group modification were followed spectrophotometrically by measuring the increase in absorbance at 412 nm associated with the formation of TNB as a reaction product. Assuming stoichiometry between the formation of modified thiol groups and the formation of TNB, the number of modified thiols was calculated by using an extinction coefficient of 13 600 M^{-1} cm^{-1} (Lundblad & Noyes, 1984). Since the kinetics of enzyme inactivation with DTNB were biphasic, the kinetics of chemical modification as measured by TNB release were analyzed with the chemical kinetics program KINSIM (Barshop et al., 1983). The following reaction pathway (eq 2a and 2b) for the fast and slower modification

$$E \xrightarrow{k_1} M \tag{2a}$$

$$E' \xrightarrow{k_2} M' \tag{2b}$$

of two different thiol groups was used in this computer simulation for independent thiol modification (E and E′ represent enzyme thiol groups and M and M′ represent those groups modified by DTNB). The first-order rate constants k_1 and k_2 are the experimentally determined rate constants for DTNB enzyme inactivation that should reflect the rate of M and M′ formation. A sequential thiol modification pathway was also simulated (eq 3a and 3b). In this model E represents two

$$E \xrightarrow{k_1} M \tag{3a}$$

$$M \xrightarrow{k_2} M' \tag{3b}$$

identical enzyme thiol groups, M represents the enzyme with one of these thiols modified, and M′ represents the enzyme with both thiols modified. Values of the rate constants k_1 and

Table I: Purification of Deoxycytidylate Hydroxymethylase[a]

	protein (g)	enzyme[b] act. (10^{-7} cpm)	sp act. (10^{-5} cpm g^{-1})	recovery (%)	purification (x-fold)
crude preparation	203	8.0	3.9	100	1.0
streptomycin sulfate	109	7.5	6.9	94	1.8
45% saturated $(NH_4)_2SO_4$	91	6.6	7.3	83	1.9
75% saturated $(NH_4)_2SO_4$	19	4.0	21.1	50	5.4
dialysate	15	5.8	38.7	73	9.9
DEAE-Bio-Gel A	0.25	5.4	2160	68	554
Blue Sepharose CL-6B	0.08	3.2	4000	40	1026

[a] Starting material was 730 g of wet *E. coli* cell paste. [b] Tritium-exchange enzyme assay.

k_2 are the same as described for the analysis of the independent pathway.

The number of thiol groups modified on both partially and totally inactivated enzyme was determined by spectrophotometrically measuring at 412 nm the TNB released from the modified enzyme by treatment with KCN (Vanamen et al., 1970; Birchmeir et al., 1973). Total enzyme thiol groups were measured in the same way on DTNB-modified enzyme denatured with either 1% sodium dodecyl sulfate or 4.6 M Gdn-HCl heated for 2.0 min in a boiling water bath.

Stoichiometry of dCMP and MTX Binding. The binding of dCMP and MTX to the enzyme was measured by using the gel separation technique developed by Hirose and Kano (1971). The method used is illustrated for dCMP binding. To 50 mg of dry Sephadex G-50–80 were added varying concentrations of enzyme in 0.35 mL of 20 mM KH_2PO_4, pH 6.8. The gel particle suspension was allowed to swell overnight in a refrigerator. A solution (0.15 mL) containing specific concentrations of [2-^{14}C]dCMP and MTX was added at room temperature and stirred vigorously for 2–3 min. The supernatant (0.05 mL) was withdrawn and added to 2 mL of Ready-Solv EP liquid scintillation cocktail. Radioactivity was measured by using a Beckman LS 7000 liquid scintillation counter. For each experimental sample, a control tube that lacked enzyme was prepared and treated in exactly the same fashion to construct a standard curve for ligand concentration in the supernatant.

The total volume in the gel accessible to free ligands (V_t) was spectrophotometrically determined at 271 nm by using free dCMP. The volume excluded from the gel (V_0) was measured at 280 nm with bovine serum albumin. The volume inside the gel (V_i) was calculated by the difference, $V_i = V_t - V_0$.

Results

Enzyme Purification. The modifications to the methods of Mathews et al. (1964) result in an effective way to purify dCMP hydroxymethylase. Starting with 700–800 g of *E. coli* B cell paste, these methods routinely allow purification of 50–100 mg of homogeneous enzyme with a 35–65% recovery and a 1000-fold increase in specific activity. Critical to this purification is affinity chromatography on Blue Sepharose CL-6B and elution with dCMP and THF followed by exhaustive dialysis; however, a problem may be incomplete removal of the THF used to elute the enzyme from the affinity column. Table I shows the results of a typical enzyme purification. Figure 1 illustrates both the final purity of the enzyme preparation and an estimate of subunit molecular weight of 28 000. The enzyme molecular weight determined with a calibrated Sephadex G-100 column is 51 400 ± 850. Thus, the enzyme is a dimer with subunits of equal size. The enzyme is stable at room temperature for at least 24 h and can be stored frozen in 20 mM KH_2PO_4, pH 6.8, for at least 6 months. It is subject to oxidation, but low specific activity samples can be activated by a 30-min room temperature incubation in 50 mM DTT.

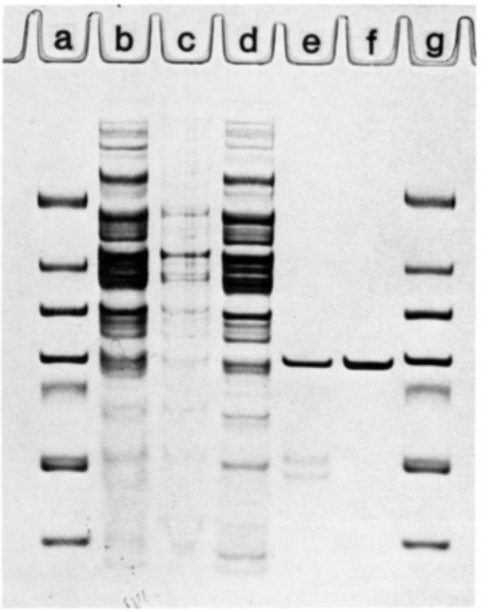

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of dCMP hydroxymethylase fractions and appropriate molecular weight standards. Samples applied to the lanes are (a) protein standards, (b) crude fraction (c) after 45% $(NH_4)_2SO_4$ fractionation, (d) after 75% $(NH_4)_2SO_4$ fractionation, (e) after DEAE ion-exchange chromatography, and (f) after Blue Sepharose CL-6B affinity chromatography, and (g) protein standards.

Steady-State Kinetics. The commonly used assay for dCMP hydroxymethylase measures the enzyme's ability to catalyze the exchange of tritium from C-5 of dCMP to water in the presence of THF. Since CH_2O is not required, the assay is indicative of only part of the catalytic pathway. Thus, in preparation for more detailed studies, an HPLC assay that measures the 5-hydroxymethyl-dCMP product was developed. Figure 2 shows the double-reciprocal relationships used to kinetically determine binding constants for dCMP in the presence of fixed but increasing THF concentration and for THF in the presence of fixed but increasing dCMP concentration by using the tritium-exchange assay. These linear, intersecting plots are consistent with a sequential order of substrate binding. Although the apparent K_m values for dCMP become larger as a function of increasing THF concentration, direct binding studies of dCMP to enzyme in the presence of MTX show that K_s for dCMP actually decreases with increasing MTX concentration. This indicates that K_m does not represent K_s for dCMP binding. Rather, it contains kinetically important terms and $K_m > K_s$ (Fersht, 1977). Values of the kinetically determined dissociation constants for the enzyme–dCMP and enzyme–THF complexes were calculated to be 0.16 and 0.01 mM, respectively, from the intersecting points of the data shown in Figure 2. Under the experimental

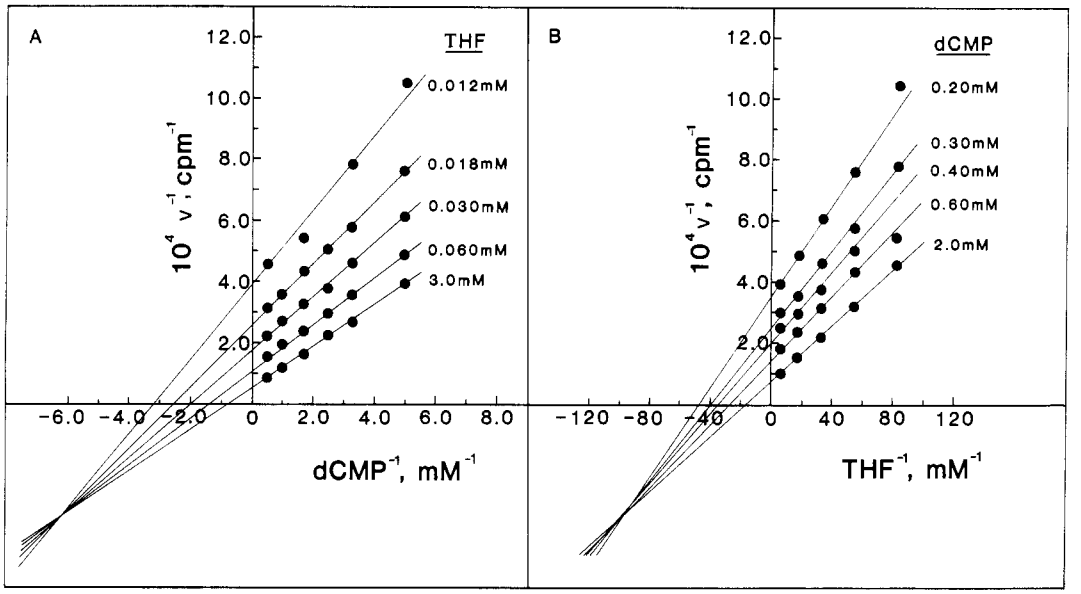

FIGURE 2: Double-reciprocal relationships between initial velocity and dCMP concentration at fixed concentrations of THF (A) and between initial velocity and THF concentration at fixed concentrations of dCMP (B). Initial velocitities were measured with the tritium-exchange assay described under Materials and Methods.

conditions given under Materials and Methods, dCMP and 5-hydroxymethyl-dCMP are well separated on reverse-phase, C_{18}, HPLC columns with retention times of 6.9 and 7.9 min, respectively. The method's utility was further illustrated by obtaining linear double-reciprocal plots for both dCMP and THF dependencies at single concentrations of the second substrate. With respect to THF, dihydrofolate and its analogues, methotrexate and aminopterin, are competitive inhibitors of dCMP hydroxymethylase. When a fixed dCMP concentration (0.40 mM) is used, the apparent K_m for THF is 0.04 mM. Relative to this value for THF, K_i values for dihydrofolate, MTX, and aminopterin are 0.021, 0.016, and 0.001 mM, respectively.

Inactivation by NEM. NEM causes a concentration-dependent, first-order inactivation of dCMP hydroxymethylase. The kinetics of NEM inactivation shows that dCMP alone and in combination with THF analogues can greatly diminish the rate of inactivation. In the absence of dCMP, MTX and aminopterin, inhibitors competitive with THF, do not protect against NEM inactivation. Interestingly, both MTX (0.08 mM) and aminopterin (0.02 mM), when added to 0.50 mM dCMP, afford about 6- and 9-fold greater protection against NEM inactivation, respectively, than 0.50 mM dCMP alone. Incubation of NEM-inactivated enzyme with either 50 mM DTT or 200 mM 2-mercaptoethanol in 0.10 M KH_2PO_4, pH 8.5, does not result in enzyme reactivation. In 40 mM KH_2PO_4, pH 7.6, 30 °C, the pseudo-first-order rate constants for enzyme inactivation are linearly dependent on NEM concentration. Under these conditions, the second-order rate constant for NEM inactivation of dCMP hydroxymethylase is 18 M^{-1} s^{-1}.

The pH dependence of the pseudo-first-order rate constants for NEM inactivation of enzyme was studied between pH 6.5 and 9.5 (Figure 3). Above pH 8.0, due to the rapid degradation of NEM, values of k_{inact} were measured from the first, linear parts (0.50–1.0 half-lives) of semilogarithmic plots of percent of activity versus time. The experimental data fit well with Cleland's WAVL program (Cleland, 1979), yielding a single ionizable group with $pK_a = 9.15 \pm 0.11$.

The enzyme thiol groups were titrated with [^{14}C]NEM and compared with enzyme activity. Figure 4 illustrates the results of extrapolating plots of percent of enzyme activity against

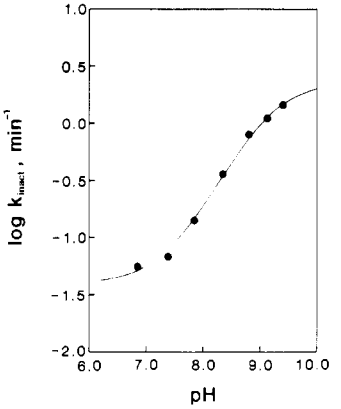

FIGURE 3: Relationship between pH and the pseudo-first-order rate constants for dCMP hydroxymethylase inactivation by NEM. The data are fitted by using Cleland's (1979) WAVL program. Reaction mixtures containing 1.62 μM enzyme, 40.8 μM NEM, and either 0.20 M Tris-HCl (pH 6.5–9.0) or glycine NaOH (pH 9.1–9.5) buffer, ionic strength 0.12 M, were incubated at 30 °C. Activity was measured by using the tritium-exchange assay. Pseudo-first-order rate constants were determined from semilogarithmic plots of residual activity versus time.

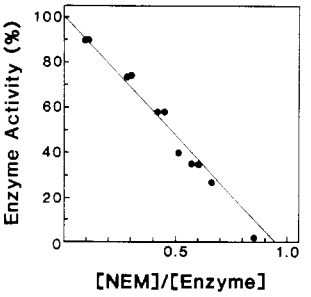

FIGURE 4: Titration of dCMP hydroxymethylase with [^{14}C]NEM. Reaction mixtures containing 26 μM enzyme, 0.12 mM NEM, and 60 mM KH_2PO_4 buffer, pH 7.6, were incubated at 30 °C. At 1.5, 5.0, 9.0, 13.5, 21, and 33 min, samples (0.40 mL) were withdrawn and applied to a 1 × 25 cm, Sephadex G-25 column to remove the excess NEM. Enzyme activity was measured by the tritium-exchange reaction and NEM incorporation by liquid scintillation counting.

moles of [^{14}C]NEM incorporated per mole of enzyme. These data indicate that one thiol group per dimeric enzyme molecule is required for activity.

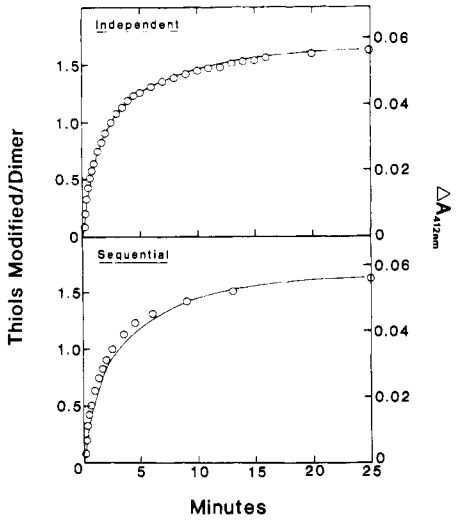

FIGURE 5: Kinetics of DTNB modification of dCMP hydroxymethylase. The points are experimental absorbance values. The lines were calculated as described under Materials and Methods by using a model calling for either independent or sequential thiol group modification. For these calculations, the first-order rate constants for thiol group modification and the total thiol groups modified per dimer are $k_1 = 0.82$ min^{-1}, $k_2 = 0.15$ min^{-1}, and 1.65 thiol groups per dimer, respectively. Enzyme (2.59 μM) was incubated at 30 °C in 0.10 M Tris-HCl buffer, pH 7.5. The 412-nm absorbance associated with the release of the TNB product was recorded following addition of DTNB to a final concentration of 0.50 mM at zero time. Absorbance values were corrected for DTNB autoxidation by subtraction of absorbance changes observed in a blank containing no enzyme.

In several separate experiments using trichloroacetic acid precipitation methods (Moore et al., 1986), it was shown that the moles of [^{14}C]NEM incorporated depend upon the oxidation state of the enzyme thiol groups. Enzyme not pretreated with DTT prior to NEM inactivation incorporated between 0.7 and 1.0 mol of NEM/mol of enzyme. Denaturation by incubation for 2 min in 4.6 M Gdn-HCl in boiling water, followed by NEM treatment, allowed 1.4–2.0 mol of NEM incorporation/mol of enzyme. DTT-activated enzyme (see Materials and Methods) that was passed over a 1 × 25 cm Sephadex G-25 column incorporated 0.9–1.1 mol of [^{14}C]NEM/mol of enzyme in the native form and 1.9–2.0 mol/mol of enzyme after Gdn-HCl denaturation. These results suggest that enzyme activity is associated with one active-site thiol group which can react with NEM. In the native enzyme, the second enzyme thiol group does not react with NEM and probably is not related to the catalytic activity.

Inactivation by DTNB. The modification of enzyme thiol groups was easily monitored at 412 nm by spectrophotometrically following the formation of TNB that occurs as a function of the enzyme's thiol groups reacting with DTNB. The kinetics of TNB formation, observed by reacting the native enzyme with 0.50 mM DTNB, are complex (Figure 5) and apparently involve two reactions proceeding at different rates. The experimental points in Figure 5 are observed changes at 412-nm absorbance corrected by reagent blanks that contained no enzyme. By use of an extinction coefficient of 13 600 M^{-1} cm^{-1} for TNB at 412 nm, values of ΔA_{412nm} were converted to the number of thiol groups modified per dimeric molecule of enzyme. The data were fitted as described under Materials and Methods with either an independent or sequential reaction model using rate constants for the two reactions of $k_1 = 0.82$ min^{-1} and $k_2 = 0.15$ min^{-1}. In this calculation, complete modification of all available enzyme thiol groups involved reaction of 1.65 thiol groups per dimer, a reasonable figure since the specific activity of this non-DTT-treated enzyme sample was about 83% of similar samples reduced with DTT. The results of fitting the data in Figure 5 support the modification of two thiol groups that react independently of each other at about 6-fold different rates. The faster reacting thiol group is responsible for enzyme activity because, under the same conditions, the kinetics of enzyme inactivation show the reaction of a single group with a first-order rate constant of $k_{inact} = 0.82$ min^{-1}, which is identical with the larger rate constant used in the simulation shown in Figure 5. The rate of DTNB inactivation of native enzyme is concentration dependent with a second-order rate constant of 59 M^{-1} s^{-1}. Thus, unlike NEM that can react with only one thiol in the native enzyme, DTNB can react with both enzyme thiol groups. The second group, not responsible for activity, reacts at a 6-fold slower rate. Supporting this conclusion is the further slow reaction of NEM-inactivated native enzyme with DTNB.

Table II: Relationship between the Number of Modified Thiol Groups and Residual Enzyme Activity[a]

time (min)	temp (°C)	denaturant	TNB released (mol/mol of enzyme)	residual act. (%)
2.5	30	0	0.51	52
9.5	30	0	0.96	9
70	30	0	1.4	6
300	30	0	2.1	3
900	30	0	1.9	2
2.0	100	1% SDS	2.0	0
2.0	100	4.6 M Gdn-HCl	2.2	0

[a] Enzyme (31.2 μM) was incubated in 0.10 M Tris-HCl, pH 7.5, with 0.50 mM DTNB at 30 °C. At the indicated times, 0.50-mL aliquots were applied to 1 × 25 cm Sephadex G-25 columns to remove excess DTNB and TNB. In the experiments using denaturants 4.1 μM enzyme was incubated for 2.0 min in a boiling water bath in the same Tris-HCl buffer. Enzyme activity was measured with the tritium-exchange reaction. The number of modified thiol groups was determined spectrophotometrically (412 nm) by measuring the moles of TNB released by treating the modified enzyme with 50 mM KCN in 0.075 M Tris-HCl, pH 8.15, at room temperature.

To better correlate the relationship between the enzyme thiol groups modified and enzyme activity, fully reduced enzyme was incubated with DTNB. Samples withdrawn as a function of time were assayed for enzyme activity and for the number of modified thiols. The latter data were obtained by treating the TNB-modified enzyme thiol groups with KCN, which results in the release of TNB that can be measured at 412 nm and the formation of enzyme cyanocysteine residues. These results, presented in Table II, show that after 2.5 min of incubation with DTNB, there exists a 1:1 correlation between remaining enzyme activity and the modification of one enzyme thiol group. After 9.5 min, TNB released by KCN treatment is equivalent to about 1.0 mol of thiol modified with 91% enzyme inactivation. Extensive (300 and 900 min) DTNB treatment causes the modification of 1.9–2.1 mol of thiol group/mol of enzyme with essentially complete enzyme inactivation. Enzyme denatured with either 1% sodium dodecyl sulfate or 4.6 M Gdn-HCl has between 2.0 and 2.2 DTNB-modifiable thiol groups/mol of enzyme. Also of interest is the selective chemical cleavage of the amino peptide bond of the cyanocysteine residue that results after KCN treatment of enzyme incubated for 900 min with DTNB (Jacobson et al., 1973; Castimpoolas & Wood, 1966). This completely DTNB-modified enzyme sample was left at room temperature for 24 h in 75 mM Tris-HCl, pH 8.15, and 50 mM KCN. Sodium dodecyl sulfate–polyacryamide gel electrophoresis indicates that under these conditions peptide bond cleavage was about 60% complete, generating two new distinctive

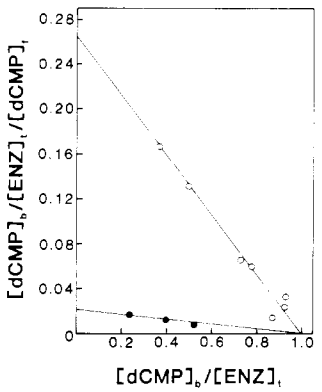

FIGURE 6: Scatchard plot for dCMP binding to dCMP hydroxymethylase in the presence of 10 μM (●) and 150 μM (○) MTX. The binding of [2-^{14}C]dCMP was measured as described under Materials and Methods. Samples contained 60 μM enzyme, 5.0 mM DTT, and 20 mM KH_2PO_4, pH 6.8. The concentration of dCMP was varied between 20 and 90 μM.

peptide bands with molecular weights of about approximately 17 000 and 11 000.

Substrate Binding to the Enzyme. The binding of dCMP to fully reduced enzyme in the presence of MTX, a cofactor analogue, was measured directly by using a gel separation technique (Hirose & Kano, 1971). The results are shown in Figure 6. The data show that dCMP binds to fully reduced dimeric enzyme with 1:1 stoichiometry. Other experiments with partially oxidized enzyme indicated fractional stoichiometry for dCMP binding. In the presence of 150 μM and 10 μM MTX, apparent K_s values for dCMP–enzyme are 4 and 47 μM, respectively. While the absolute values of these dissociation constants may be influenced by trace levels of residual THF, they clearly illustrate that a THF cofactor analogue can enhance dCMP binding to the enzyme. Unmodified thiol groups are required for dCMP binding, as NEM-treated enzyme in the presence of 150 μM MTX does not bind dCMP. Similar experiments were conducted for MTX binding to dCMP hydroxymethylase with fractional stoichiometry being obtained. MTX binding to the enzyme does not require either dCMP or reduced enzyme thiol groups. Neither enzyme reduction with DTT nor alkylation with NEM substantially effects the degree of MTX binding to the enzyme. Thus, it can be concluded that dCMP and MTX, a competitive inhibitor with respect to THF, bind separate sites on the enzyme. The fractional stoichiometry for MTX binding is not easily explained and may be due to incomplete removal, even after exhaustive dialysis, of the THF used to elute the enzyme from the Sepharose CL-6B affinity column. Under these conditions, values of K_d for MTX dissociation are 25–26 μM.

Discussion

Previously, dCMP hydroxymethylase has been difficult to purify in amounts sufficient for detailed study, principally because of bacterial cell lysis following bacteriophage infection of *E. coli*. Use of the T-4 bacteriophage mutant that lacks the genes for cell lysis eliminates this problem. The modified purification method features a terminal affinity chromatography step on Blue Sepharose CL-6B, resulting routinely in about 100 mg of homogeneous enzyme/kg of infected *E. coli* cell paste. This is approximately 4-fold greater purification and 2-fold greater recovery than the methods previously used (Mathews et al., 1964).

dCMP hydroxymethylase is a dimer with equal-sized subunits [28 000, molecular weight 51 400, a value lower than the 68 500 obtained by Pizer and Cohen (1962) using sedimentation methods]. Apparent values of K_m for dCMP, CH_2O, and THF are essentially the same as those previously obtained by Pizer and Cohen (1962). K_m values for CH_2O are for comparative purposes only, as the actual substrate is most likely 5-methylene THF, the condensation product of CH_2O and THF (Kallen & Jencks, 1966b).

Thymidylate synthase and uridylate hydroxymethylase share important catalytic characteristics. These include the ability to catalyze C-5 proton exchange from the pyrimidine, the requirement for a reduced thiol group at the active site, and covalent inhibition by 5-fluorodeoxyuridylate (Pogolotti & Santi, 1977). These characteristics coupled with data on the mechanism of nucleophile addition to C-6 of pyrimidines (Sander, 1978) have allowed the conclusion that these enzymes proceed via nucleophilic attack of an active-site thiol group on C-6 of the pyrimidine ring, thus explaining C-5 proton exchange and irreversible inhibition by the 5-fluoropyrimidine analogues. dCMP hydroxymethylase shares the ability to catalyze C-5 proton exchange, but, importantly, it is not inhibited by 5-fluorodeoxycytidylate (Pizer & Cohen, 1963), a negative result that has been exhaustively confirmed by using the homogeneous enzyme. Thus, isolation and characterization of a stable enzyme/5-F-dCMP/methylene-THF ternary complex does not appear to be a viable strategy to show thiol group participation in dCMP hydroxymethylase catalysis.

Other reagents such as NEM and DTNB, although perhaps less directed to the catalytic site than 5-fluoropyrimidines, allow similar conclusions to be drawn about the necessity for an active-site thiol group in dCMP hydroxymethylase catalysis. Both NEM and DTNB are highly specific for thiol group modification, and both inhibit dCMP hydroxymethylase by second-order reactions. Excess DTT reverses the inactivation with DTNB but not with NEM, as would be expected for reagents that form mixed disulfides and alkylate enzyme cysteine residues, respectively. In the case of NEM inactivation, dCMP and mixtures of dCMP and MTX strongly protect against enzyme inactivation. MTX alone does not protect the enzyme against NEM inactivation. The kinetics of NEM inactivation measured as a function of pH allow an estimate of the pK_a of the modified group to be 9.15 ± 0.11, a value comparable to that of free cysteine in solution. Direct binding studies of dCMP and MTX to the enzyme indicate that the states of both enzyme thiol group oxidation and NEM modification influence dCMP but not MTX binding to the enzyme. Thus, it can be concluded that dCMP and MTX, a THF analogue, bind to separate sites on the enzyme and that the dCMP binding site contains a reduced cysteine residue. Consequently, although unlike thymidylate synthase and dUMP hydroxymethylase in which the active-site thiol group reacts directly with a 5-fluoropyrimidine substrate analogue, on the basis of C-5 proton exchange reaction and the need for a reduced thiol group at the dCMP binding site, it is likely that dCMP hydroxymethylase proceeds via a catalytic pathway similar to these other THF-requiring enzymes.

The reason for the failure of dCMP hydroxymethylase to react with 5-F-dCMP is not clear; however, the mechanism for the nucleophilic addition of SO_3^{2-} to uracils and cytosines is substantially different (Gautam-Basak et al., 1985). Thus, it may not be completely valid to consider that the enzymes which methylate and hydroxymethylate dUMP are mechanistically identical with dCMP hydroxymethylase. Steric reasons for the failure of 5-fluoropyrimidine analogues to not inhibit the enzyme are unlikely because preliminary evidence indicates that, like thymidylate synthase, dCMP hydroxymethylase can catalyze the dehalogenation of the more bulky

5-halopyrimidine substrate analogues.

The stoichiometry of both NEM and DTNB thiol modification of dCMP hydroxymethylase clearly shows that the reaction of one thiol per enzyme dimer results in complete enzyme inactivation. However, total thiol groups modified with DTNB in native enzyme and with both NEM and DTNB in denatured enzyme are equal to two per mole of dimer. As measured by TNB formation, the kinetics of DTNB modification show that two thiol groups are being independently modified at 6-fold different rates. The pseudo-first-order rate constant for the faster reacting thiol is identical with the rate constant for DTNB enzyme inactivation. In the case of NEM inactivation, only one thiol group reacts in the native enzyme. Treatment of the native enzyme with a large molar excess of NEM does not result in further radioactive NEM incorporation, suggesting that the second thiol group is not accessible for reaction with NEM. This result, coupled with the slow rate of DTNB modification of the second thiol group, indicates that the two thiol residues per mole of enzyme dimer have distinctly different environments. The first group, responsible for the observed enzyme activity, is easily accessible to both NEM and DTNB, while the second group, which reacts only slowly with DTNB, is either sterically or electronically unable to react with the double bond of NEM (Brubacher & Glick, 1974). These data support the conclusion that dCMP hydroxymethylase has one cysteine-containing, dCMP binding active site, although the enzyme appears to have two identical subunits. Further supporting evidence is the fact that KCN cleaves the fully TNB-modified enzyme into only two peptides of molecular weight of 11 000 and 17 000. Thus, the subunits are either asymmetrically oriented, making one thiol-containing active site inaccessible to both thiol group modification and dCMP binding, or ligand binding induces this asymmetry. On the basis of the equality of the rates of DTNB enzyme inactivation and DTNB thiol modification, the former is the more likely explanation. Thymidylate synthase has also been shown to have one active site per dimer (Galivan et al., 1976; Leary et al., 1975) with asymmetrically linked, identical subunits.

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