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# 5-Fluorocytosine in DNA Is a Mechanism-Based Inhibitor of *Hha*I Methylase†

David G. Osterman,‡ Gia D. DePillis,‡ John C. Wu,‡ Akira Matsuda,§ and Daniel V. Santi*,‡,‖

*Department of Biochemistry and Biophysics and Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94143, and Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan*

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ABSTRACT: 5-Fluorodeoxycytidine (FdCyd) was incorporated into a synthetic DNA polymer containing the GCGC recognition sequence of *Hha*I methylase to give a polymer with about 80% FdCyd. In the absence of AdoMet, poly(FdC-dG) bound competitively with respect to poly(dG-dC) ($K_i$ = 3 nM). In the presence of AdoMet, the analogue caused a time-dependent, first-order ($k$ = 0.05 min$^{-1}$) inactivation of the enzyme. There is an ordered mechanism of binding in which enzyme first binds to poly(FdC-dG), then binds to AdoMet, and subsequently forms stable, inactive complexes. The complexes did not dissociate over the course of 3 days and were stable to heat (95 °C) in the presence of 1% SDS. Gel filtration of a complex formed with *Hha*I methylase, poly(FdC-dG), and [*methyl*-$^3$H]AdoMet gave a peak of radioactivity eluting near the void volume. Digestion of the DNA in the complex resulted in a reduction of the molecular weight to the size of the methylase, and the radioactivity in this peak was shown to be associated with protein. These data indicate that the complexes contain covalently bound *Hha*I methylase, poly(FdC-dG), and methyl groups and that 5-fluorodeoxycytidine is a mechanism-based inactivator of the methylase. By analogy with other pyrimidine-modifying enzymes and recent studies on the mechanism of *Hha*I methylase (Wu & Santi, 1987), these results suggest that an enzyme nucleophile attacks FdCyd residues at C-6, activating the 5-position for one-carbon transfer. Subsequent transfer of the methyl group of AdoMet to the activated FdCyd forms a stable complex in which the enzyme is covalently bound to the 6-position of FdCyd in the polymer and a methyl group is attached to C-5. The effect of 5-fluorodeoxycytidine on the inhibition of DNA-cytosine methyltransferases is thus due to irreversible, covalent inactivation.

DNA-cytosine methyltransferases (DCMT)[1] (EC 2.1.1.37) catalyze the transfer of the activated methyl group of *S*-adenosylmethionine (AdoMet) to the C-5 position of cytosine. In bacteria, modification by methylation prevents the digestion of host DNA by the host restriction endonucleases. Foreign DNA lacking this pattern of methylation is degraded (Arber, 1974). In mammals, this postreplicative modification is an important element in the control of gene expression. Changes

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* To whom correspondence should be addressed.
‡ University of California.
§ Hokkaido University.
‖ D.V.S. is a Burroughs Wellcome Scholar in Molecular Parasitology.

[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; azaC, 5-azacytidine; BSA, bovine serum albumin; DCMT, DNA-cytosine methyltransferase; DNase I, deoxyribonuclease I; DTT, dithiothreitol; FdCyd, 5-fluorodeoxycytidine; FdUrd, 5-fluorodeoxyuridine; FdUMP, 5-fluorodeoxyuridylic acid; poly(FdC-dG), poly(5-fluoro-dC-dG).

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in the state of methylation of certain cytosines are involved in gene expression, cell differentiation, and perhaps oncogenesis (Doerfler, 1983; Jones, 1986).

The important role of methylation in cellular function is highlighted by the results of studies employing 5-azacytidine (azaC), an inhibitor of the mammalian DCMT (Jones, 1985). When incorporated into DNA, this cytidine analogue causes the inactivation of the methylase (Taylor & Jones, 1982; Creusot et al., 1982). The proposed mechanism of action of azaC involves the formation of a covalent, inactive enzyme–DNA complex arising from attack of the enzyme at C-6 of the azaC ring (Santi, et al., 1984; Friedman, 1986). The changes in the methylation pattern induced by azaC result in the enhanced expression of certain genes (Harris, 1983), cause the differentiation of cultured cells (Constantinides, et al., 1977), and can be tumorigenic (Carr et al., 1984). Several other 5-substituted pyrimidines, including 5-fluorodeoxycytidine (FdCyd), have been reported to cause a decrease in the levels of active DCMT (Taylor & Jones, 1982).

Enzymes that catalyze one-carbon transfer to the 5-position of pyrimidines, such as thymidylate synthase and tRNA (Ura-5-)methyltransferases, have been shown to activate the C-5 position of pyrimidines via attack of an enzyme nucleophile at the C-6 position (Santi & Danenberg, 1984; Santi & Hardy, 1987). Addition of the nucleophile results in the formation of a carbanion at C-5 of a dihydropyrimidine intermediate. Following reaction with the one-carbon donor at the 5-position, the proton at C-5 is liberated, and the enzyme is eliminated. Substrate analogues that contain fluorine at the C-5 position of the Ura heterocycle can act as mechanism-based inhibitors of such enzymes (McHenry & Santi, 1972). In FdUMP inhibition of thymidylate synthase and FUra-tRNA inhibition of tRNA (Ura-5)methyltransferase, the carbon–fluorine bond of the covalent adducts is not broken, resulting in formation of stable enzyme–inhibitor complexes. We have surmised that since the DCMTs proceed by a similar mechanism (Wu & Santi, 1987), FdCyd incorporated into DNA may act as an inactivator of these enzymes.

We previously reported studies on the catalytic mechanism of the type II methyltransferase *Hha*I from *Haemophilus haemolyticus* (Wu & Santi, 1987). In the present work, we synthesized DNA containing FdCyd (FdC-DNA) as part of the GCGC recognition sequence of *Hha*I methylase. We show that the kinetics of inactivation and formation of stable covalent enzyme–DNA complexes are consistent with our hypothesis that FdC-DNA acts as a mechanism-based inhibitor of *Hha*I methylase. Our results provide an explanation of how FdCyd can have such dramatic effects on methylation of DNA.

## Materials and Methods

*Materials.* *S*-Adenosylmethionine *p*-toluenesulfonate salt was purchased from Sigma. DE-81 filters (24 mm) were from Whatman. Nitrocellulose BA85 filters (24 mm) were obtained from Schleicher & Schuell. [*methyl*-$^3$H]AdoMet (65 Ci/mmol), [*methyl*-$^{14}$C]AdoMet (59.8 mCi/mmol), Aquasol II, and Omnifluor were obtained from New England Nuclear. Poly(dG-[5-$^3$H]dC) (1.2 Ci/mmol) was prepared as described (Wu & Santi, 1987). [1′,2′-$^3$H]dGTP was purchased from New England Nuclear. *Hha*I restriction endonuclease, *Hha*I methylase, and DNA polymerase Klenow fragment were purchased from New England Biolabs. Phosphodiesterase I and alkaline phosphatase were from Boehringer Mannheim. Nucleosidemonophosphate kinase, nucleosidediphosphate kinase, pyruvate kinase, and DNase I were purchased from Sigma. The nick translation kit was from BRL.

*HhaI Methylase Assay.* *Hha*I methylase activity was measured by use of the exchange of tritium from C-5 of poly(dG-[5-$^3$H]dC) in the presence of a saturating concentration (100 μM) of AdoMet (Wu & Santi, 1987). Reactions were performed in a standard assay buffer consisting of 50 mM Tris (pH 7.5), 10 mM EDTA, 10 mM DTT, and 200 μg/mL BSA. Tritiated water was measured following charcoal adsorption of reactions as described (Wu & Santi, 1987). One unit of *Hha*I methylase catalyzes the transfer of 1 pmol of methyl groups from AdoMet to poly(dG-dC) in 1 min at 37 °C. We estimate that 1.3 units of enzyme activity equal 1 pmol of enzyme (Wu & Santi, 1987).

*DNA Preparations.* FdCMP was synthesized by a modification of the procedure of Tanaka et al. (1981). Ten milligrams (40 μmol) of FdCyd was dissolved in 200 μL of triethyl phosphate at 0 °C. Four microliters (43 μmol) of freshly distilled phosphoryl chloride was added with stirring. After being stirred for 2.5 h at 0 °C, the reaction was quenched by addition of 500 μL of ice-cold 2 M triethylammonium bicarbonate. The product was purified by reverse-phase HPLC using a 25 × 1 cm Hibar Prep C18 column. Products were eluted isocratically with a solvent system consisting of 2% acetonitrile in 0.1 M triethylammonium bicarbonate (pH 7.3) at a flow rate of 3 mL/min. Elution volumes were as follows: FdCMP, 96 mL; 5-fluorodeoxycytidine 3′,5′-diphosphate, 120 mL; FdCyd, 160 mL. After lyophilization, 14 μmol of FdCMP was obtained (35% yield).

The purified FdCMP was enzymatically converted to the triphosphate with nucleosidemonophosphate kinase and nucleosidediphosphate kinase (Imazawa & Eckstein, 1979). The reaction was performed at 25 °C in 4 mL of 100 mM Tris buffer (pH 8.0), 0.25 mM EDTA, and 12 mM $MgCl_2$ containing the following: 10 mM ATP, 5 mM FdCMP, 25 mM phosphoenolpyruvate, 9 units of pyruvate kinase, 1 mM DTT, 0.5 unit of nucleosidemonophosphate kinase, and 25 units of nucleosidediphosphate kinase. After 6.5 h, the reaction was deproteinized by addition of 4 mL of 0.5 M trichloroacetic acid. After neutralization with 8 mL of a 0.5 M solution of tri-*n*-octylamine in 1,1,2-trichlorotrifluoroethane (Pogolotti & Santi, 1982), FdCTP was purified by reversed-phase HPLC using 1% acetonitrile in 0.1 M triethylammonium bicarbonate (pH 7.3) at a flow rate of 2.25 mL/min. Elution volumes were as follows: FdCTP, 68 mL; ATP, 125 mL. The final yield of the nucleoside triphosphate relative to the FdCMP starting material was >90%.

Poly(FdC-dG) was prepared with poly(dG-dC) [instead of poly(dI-dC) (Wells et al., 1970)] as a primer for DNA polymerase I Klenow fragment. Primer (0.1 mM in nucleotides) was incubated with 0.5 mM dGTP, 0.5 mM FdCTP, 50 ng/mL DNase I, and 40 units of Klenow fragment in 2 mL of 50 mM Tris (pH 8.3), 5 mM $MgCl_2$, and 1 mM 2-mercaptoethanol at 42 °C. The reaction was monitored by following the decrease in UV absorbance at 260 nm. After 6 h, proteins were precipitated with chloroform/phenol/isoamyl alcohol (25:24:1) (Maniatis et al., 1982). The aqueous phase was extracted 5 times with diethyl ether, excess ether was removed by a stream of nitrogen, and the solution was dialyzed once against 10 mM Tris (pH 8.0), 1 mM EDTA, and 0.5 M NaCl and twice against 10 mM Tris and 1 mM EDTA. The final concentration of DNA (in nucleotides) was 570 μM in 1.5 mL (~40% yield based on initial amounts of nucleotides and primer) as determined with the value of 8400 $M^{-1}$ $cm^{-1}$ for poly(dG-dC) at 254 nm (Wells et al., 1970).

The percentage of FdCyd in the polymer was determined by reverse-phase HPLC following enzymatic hydrolysis of the

DNA to nucleosides. Fifteen nanomoles of poly(FdC-dG) (based on nucleotides) was hydrolyzed in 30 μL of 10 mM Tris (pH 8.0) and 10 mM $MgCl_2$ containing 8 units of alkaline phosphatase, 2 μg of phosphodiesterase I, and 3 μg of DNase I for 12 h at 37 °C. Nucleosides were separated on an Altex Ultrasphere $C_{18}$ analytical column. The solvent program delivered 1% MeOH/50 mM ammonium phosphate (pH 4.0) for 10 min at a flow rate of 1.5 mL/min followed by a gradient to 3.5% MeOH/50 mM ammonium phosphate (pH 4.0) over 25 min. Elution volumes were as follows: dCyd, 9 mL; FdCyd, 14 mL; dGuo, 30 mL. The relative composition of the DNA was determined to be as follows: FdCyd, 79; dCyd, 21; dGuo, 100.

Radioactively labeled poly(FdC-[1′,2′-$^3$H]dG) was prepared by nick translation with [1′,2′-$^3$H]dGTP. Twenty microCuries (20 μL) of [$^3$H]dGTP was dried of solvent by evaporation with nitrogen gas. The dried [$^3$H]dGTP was redissolved in 225 μL of solution containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 10 μg/ml BSA, 20 μM FdCTP, and 5 μg of poly(FdC-dG). The reaction was initiated by addition of 25 μL of nick translation enzyme mix (New England Nuclear) containing 10 units of DNA polymerase I and 1 μg of DNase I. After 1-h incubation at 15 °C, the reaction was stopped by addition of 15 μL of 0.50 M EDTA. The reaction (265 μL) was loaded onto a 5-mL Sephadex G-50 column and eluted with 10 mM Tris (pH 7.4) and 1 mM EDTA. Fractions of 700 μL were collected, radioactivity was measured by scintillation counting, and peak fractions were pooled. The specific activity of the isolated DNA was 1.8 Ci/mmol of nucleotides.

*Filter Binding Assays.* Protein–DNA complexes were isolated by binding to nitrocellulose filters (Woodbury & von Hippel, 1983). Filters were presoaked in 20 mM sodium phosphate (pH 7.4). Samples were spotted on the filter and allowed to bind for 1 min before washing. The filters were washed with 5 × 1-mL portions of buffer, and bound radioactivity was determined by scintillation counting following dissolution of the filter in 10 mL of Aquasol 2.

Binding of DNA to DE-81 filters was measured following washing with 5 × 1-mL portions of 200 mM ammonium bicarbonate, 1 mL of water, and 2 mL of absolute ethanol. The filters were dried by suction and counted in Omnifluor.

*Stability of the Covalent Complex toward Denaturants.* Reactions were performed in buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 10 mM DTT, 0.2 mg/mL BSA, 1 μM poly(FdC-[$^3$H]dG), 100 μM AdoMet, and 10 nM (for urea experiment) or 20 nM *Hha*I methylase (for SDS experiment). The reactions were incubated at 37 °C for 1 h, at which time poly(dG-dC) solution was added to the reactions to give a final concentration of 100–150 μM. Duplicate 20-μL samples (~80 000 dpm) of each reaction were measured for radioactivity bound on nitrocellulose filters as described above. For SDS denaturation, 10 μL of 10% SDS was added to 100-μL samples; the samples were heated at 95 °C for 10 min and passed through Sephadex LH20 to remove SDS (Santi et al., 1984), and duplicate 20-μL samples were analyzed by the nitrocellulose filter assay. For urea denaturation, 3 volumes of 8 M urea was added to samples, and after 60 min, 80-μL samples were analyzed on nitrocellulose filters. Control reactions omitted enzyme.

*Gel Permeation Chromatography.* Labeled complexes were prepared by incubation of 1.1 μM poly(FdC-dG) and 5 μCi of [$^3$H]AdoMet with either 0 or 50 nM *Hha*I methylase in 200 μL of assay buffer containing 1 mM EDTA. After 5 h at 37 °C, 2 μL of a solution containing 500 mM $MgCl_2$ with 150 units of *Hha*I restriction endonuclease and 1 μg of phosphodiesterase I was added to each reaction. The DNA was hydrolyzed for 12 h at 37 °C. The specific activity of the labeled complex was determined to be 600 mCi/mmol by its binding to DE-81 filters. Complexes were also prepared without digestion by incubation of 20 nM *Hha*I methylase with 1.1 μM poly(FdC-dG) and 15 μCi of [$^3$H]AdoMet in 500 μL of assay buffer.

HPLC was performed with a 1 × 30 cm Superose 12 column (Pharmacia) equilibrated with buffer containing 50 mM 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid, 300 mM KCl, 1 mM EDTA, and 5 mM DTT at a flow rate of 0.4 mL/min. A 50-μL aliquot of the nondigested complex was injected, and 400-μL fractions were collected. Aliquots of 300 μL were counted in 10 mL of Aquasol II. The digested reactions (100 μL) were collected in fractions of 1 mL, of which 100 μL was counted.

## Results

*Preparation of FdCyd-Containing Oligonucleotide Analogues.* Our studies required the preparation of DCMT recognition sequences containing FdCyd. Our initial approach was to prepare synthetic oligonucleotides that contained the analogue in appropriate positions corresponding to methylation sites of DCMTs. This approach was discontinued when we found that $N^4$-benzoyl-FdCyd (Wempen et al., 1961) was unstable in the acidic conditions commonly used for removal of the dimethoxytrityl blocking group in oligonucleotide synthesis. When $N^4$-benzoyl-FdCyd (0.7 mg) was treated with dichloroacetic acid/$CH_2Cl_2$ (5 mL; 5% w/v), analysis on silica gel TLC ($CHCl_3$/EtOH, 5:1) showed decomposition of $N^4$-benzoyl-FdCyd ($R_f$ 0.47) to another product ($R_f$ 0.29) with $t_{1/2} \sim 20$ min. The product was not identified but by comparison to authentic standards was shown not to be FC, FdCyd, FU, or FdUrd. As an alternative approach, we undertook the enzymatic synthesis of poly(FdC-dG) as a potential inhibitor of *Hha*I methylase which recognizes and methylates the internal C of the sequence GCGC. As previously described (Wu & Santi, 1987), the advantage of this system is that the alternating copolymers provide substrates or inhibitors of homogeneous sequences in which every C or FC represents a potential site for reaction. FdCMP, prepared by chemical phosphorylation, was converted in >90% yield to FdCTP with nucleotide mono- and diphospho kinases. Poly(FdC-dG) was prepared (about 40% yield) with FdCTP, dGTP, poly(dG-dC) as primer, Klenow fragment of DNA Pol I, and DNase I; inclusion of the latter enzyme improved the polymerization reaction, probably by introducing nicks on the primer to increase the number of polymerase initiation sites. HPLC analysis of the hydrolyzed product indicated the polymer contained 40% FdCyd, 10% dCyd, and 50% dGuo.

*Kinetics.* In the *absence* of AdoMet, poly(FdC-dG) was a competitive inhibitor ($K_i$ = 3 nM) of the *Hha*I methylase catalyzed 5-$^3$H exchange from poly(dG-[5-$^3$H]dC) (data not shown); over the period examined, poly(FdC-dG) by itself did not cause time-dependent loss of the enzyme, and the total enzyme activity (measured at $V_{max}$) was not changed. However, in the *presence* of AdoMet a first-order loss of enzyme activity was observed, indicating the involvement of a ternary enzyme–poly(FdC-dG)–AdoMet complex (Figure 1A). We have previously determined that the mechanism of *Hha*I methylase is ordered, with the DNA binding first (Wu & Santi, 1987); AdoMet does not bind to free enzyme. Hence, the mechanism of inactivation is in accord with the general pathway depicted in Scheme I; this depicts an ordered mechanism of reversible binding, followed by AdoMet-de-

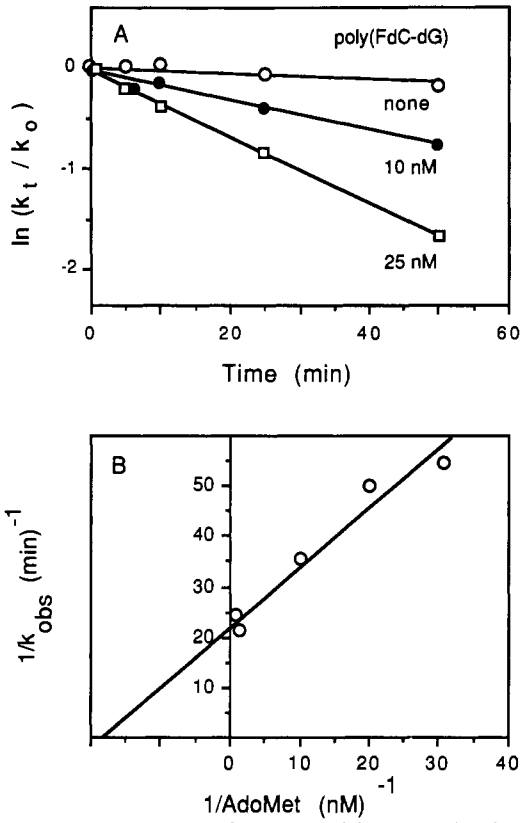



FIGURE 1: (A) Plot of the log of enzyme activity versus time incubated with poly(FdC-dG). Reactions were performed as described under Materials and Methods with 1 nM *Hha*I methylase, 100 $\mu$M AdoMet, and the indicated concentrations of poly(FdC-dG). Enzyme activity was measured by assay for $^3H_2O$ as described under Materials and Methods; $k_t$ is the enzyme activity at time $t$, and $k_0$ is initial enzyme activity (at time = 0 min). (B) Double-reciprocal plot of the rate of inactivation of *Hha*I methylase versus AdoMet concentration at a fixed concentration of poly(FdC-dG). Reactions containing 0.2 nM *Hha*I in assay buffer with 25 nM poly(FdC-dG) and either 0, 0.033, 0.050, 0.1, 1, 10, or 100 $\mu$M AdoMet were incubated at 37 °C. Aliquots (400 $\mu$L) were removed at various times and added to 40 $\mu$L of a 13.2 $\mu$M solution of poly(dG-[5-$^3$H]dC) with 100 $\mu$M AdoMet. After incubation for 60 min, the reactions were assayed for tritium exchange as described under Materials and Methods.

Scheme I

$$\text{Enz} + \text{FC-DNA} \underset{}{\overset{K_A}{\rightleftharpoons}} \text{Enz}\cdot\text{FC-DNA} \overset{K_B}{\rightleftharpoons}$$

$$\text{Enz}\cdot\text{FC-DNA}\cdot\text{AdoMet} \xrightarrow{k_{inact}} \text{Enz—FC(Me)-DNA}$$

pendent inactivation of the enzyme. The kinetics of inactivation according to this mechanism are described by eq 1,

$$\frac{1}{k_{obsd}} = \frac{K_A K_B}{[A][B]k_{inact}} + \frac{K_B}{[B]k_{inact}} + \frac{1}{k_{inact}} \quad (1)$$

where $k_{obsd}$ is the apparent first-order rate constant for loss of activity, A is poly(FdC-dG), B is AdoMet, $K_A$ and $K_B$ are the dissociation constants for poly(FdC-dG) and AdoMet, respectively, and $k_{inact}$ is the first-order rate constant of inactivation. Figure 1B shows a plot of $1/k_{obsd}$ versus $1/[\text{AdoMet}]$. The reciprocal of the vertical intercept provides $k_{inact} = 0.05$ min$^{-1}$ ($t_{1/2} = 14$ min). With use of $K_A = 3$ nM, the aforementioned $K_i$, the value of $K_B$ is calculated to be 50 nM from the slope term.

*Nitrocellulose Filter Binding.* Radioactive complexes formed between enzyme and poly(FdC-[$^3$H]dG) could be trapped on nitrocellulose filters under conditions where protein–ligand complexes are retained but poly(FdC-[$^3$H]dG) by itself does not bind (Figure 2).[2] The complexes were formed rapidly (complete in less than 1 min) and were isolable regardless of whether AdoMet was present in the reaction mixture. To measure the rates of dissociation, complexes were formed by incubating 0.01 $\mu$M enzyme and 1 $\mu$M poly(FdC-[$^3$H]dG) in the presence and absence of 100 $\mu$M AdoMet, conditions where a limiting amount (1000 dpm/$\mu$L; about 30%) of the total radioactivity was bound. A 100-fold excess of unlabeled poly(dC-dG) was then added to the preformed complexes, and at intervals, 20-$\mu$L aliquots were analyzed for bound radioactivity. Once the bound radioactive ligand dissociates and equilibrates with the pool of unlabeled competitor, it will not reassociate. If AdoMet was not included in the preincubation, there was complete loss of bound radioactivity within 3 min ($t_{1/2} \leq 30$ s) (Figure 2), reflecting the rapid rate of dissociation of the binary enzyme–poly(FdC-[$^3$H]dG) complex. When AdoMet was included in the preincubation for various times *before* the chase was added, there was an initial rapid dissociation of a fraction of the complex to a given level which remained constant thereafter. The fraction which initially dissociated in the presence of AdoMet proceeded with a half-life estimated to be 2 min, compared to a 30-s half-life in the absence of AdoMet. The fraction of the complex which dissociated decreased with increasing time of preincubation with AdoMet: following an incubation of 5 min, 70% of the initially bound radioactivity rapidly dissociated whereas 30% was stable; after 45-min incubation, 30% dissociated, and 70% was stable. We presume that the dissociable complexes are reversible enzyme–poly-

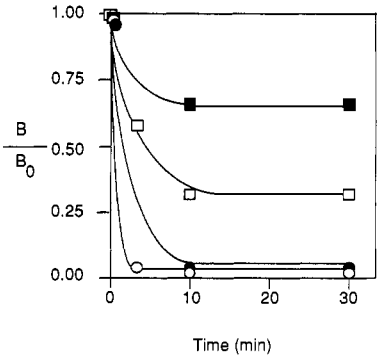



FIGURE 2: Plot of fractional amount of dpm bound to nitrocellulose filters at various times following addition of a 100-fold excess of poly(dG-dC), where $B_0$ is dpm bound before addition of poly(dG-dC) and $B$ is dpm bound at the indicated times. Reactions contained 10 nM *Hha*I and 1.0 $\mu$M poly(FdC-dG). Reactions proceeded for either 5 or 45 min in the presence and absence of 100 $\mu$M AdoMet. Bound counts were measured at the indicated times by application of 20-$\mu$L aliquots to nitrocellulose filters: (○) minus AdoMet, 5 min; (●) minus AdoMet, 45 min; (□) plus AdoMet, 5 min; (■) plus AdoMet, 45 min.

Table I: Stability of *Hha*I Methylase–Poly(FdC-[$^3$H]dG)–AdoMet Complex toward Protein Denaturation[a]

| reaction | dpm bound[b] |
|---|---|
| native complex | 21 000 |
| native complex + 6 M urea | 22 000 |
| control, minus enzyme + 6 M urea | 530 |
| native complex | 16 800 |
| native + 1% SDS | 22 560 |
| control, minus enzyme + 1% SDS | 350 |

[a] As measured by the nitrocellulose binding assay described under Materials and Methods. [b] Total dpm applied to each filter was 78 000.

[2] The nitrocellulose binding assay was not used with [$^3$H]AdoMet because of unacceptably high background binding.

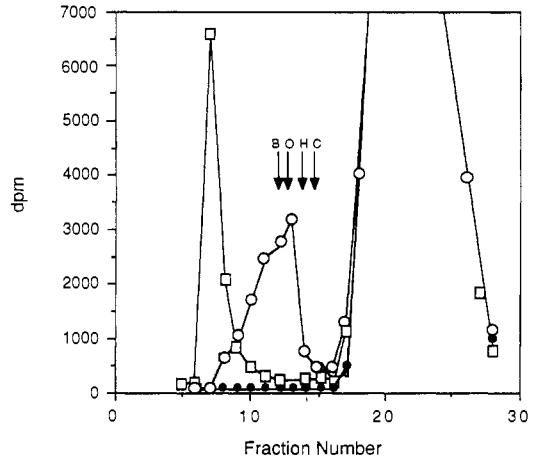



FIGURE 3: Elution profile from Superose 12 chromatography. Reactions were prepared and chromatographed as described under Materials and Methods. (□) *Hha*I methylase–poly(FdC-dG)–[3H]AdoMet complex; (○) *Hha*I methylase–poly(FdC-dG)–[$^3$H]-AdoMet complex after digestion; (●) poly(FdC-dG) + [$^3$H]AdoMet after digestion. The column was calibrated by injection of a standard mixture containing (B) BSA ($M_r$ 60000), (O) ovalbumin ($M_r$ 43000), (H) *Hha*I methylase ($M_r$ 37000), and (C) chymotrypsinogen ($M_r$ 26000).

(FdC-dG)–AdoMet complexes that have not yet undergone covalent bond modification. As described below, the nondissociable forms are methylated complexes covalently bound to the enzyme.

*Stability of the Complex to Protein Denaturation.* The ternary *Hha*I methylase–poly(FdCdG)–AdoMet complex is stable toward protein denaturation by 6 M urea and 1% SDS (Table I). Native *Hha*I methylase–poly(FdC-[$^3$H]dG)–AdoMet complexes could be isolated on nitrocellulose filters. Since filtration efficiencies could not be determined, exact quantitation of complexes could not be made; however, at least 30% of the total DNA was measured as bound. A sample of each reaction (binary and ternary complexes) was made (a) 6 M in urea or (b) 1% in SDS; SDS samples were heated to 95 °C for 10 min. Urea solutions were directly filtered, and SDS was removed by Sephadex LH20 prior to filtration on nitrocellulose (Santi et al., 1984). The complex denatured with urea contained 95% of the counts found in the native complex, whereas the SDS/heat-treated sample had 135%. The higher counts in the SDS-treated complex compared to those in the native one are believed to result from a higher filtration efficiency of the denatured complex. In any case, the binding of the enzyme to poly(FdC-dG) in the presence of AdoMet is clearly stable after protein denaturation.

*Gel Permeation Chromatography.* The complexes were also characterized by FPLC gel permeation chromatography following incubation of *Hha*I methylase, poly(FdC-dG), and [*methyl*-$^3$H]AdoMet. Using this method, the radioactive [*methyl*-$^3$H]AdoMet was well resolved from tritiated methyl groups associated with macromolecules (Figure 3). Chromatography of a preincubated mixture of all components gave a peak of radioactivity eluting near the void volume, as well as the major included peak corresponding to [*methyl*-$^3$H]-AdoMet. Thus the high molecular weight radioactivity corresponds to tritiated methyl groups associated with DNA. When the complex was digested with *Hha*I endonuclease and phosphodiesterase, subsequent gel permeation chromatography showed the macromolecular radioactivity converging to about 50 kDa, close to the size of the methylase (Wu & Santi, 1987). Nitrocellulose filtration of the peak fractions showed that 80 ± 10% of the total radioactivity was bound to the filter, demonstrating that radioactivity was bound to protein. Thus, the [$^3$H]methyl groups are associated with *both Hha*I methylase and DNA.

To characterize the complex further, tritium-labeled complexes were formed, digested by nucleases, and isolated as described above. The digested complex (30 nM) was treated in the absence or presence of a large excess of poly(dG-dC) (10 μM), and the radioactivity retained on nitrocellulose was monitored as a function of time. Over a period of 3 days, there was no change in bound radioactivity (1200 dpm/25 μL), demonstrating that there was no detectable dissociation of the complex over this time.

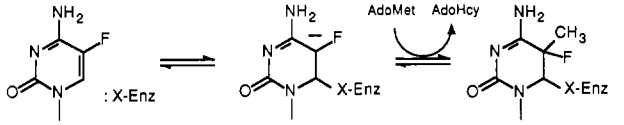
## Discussion

We have investigated the interaction of poly(FdC-dG) with *Hha*I methylase, a bacterial DNA-cytosine methyltransferase that methylates the internal cytosine residue of GCGC sequences in DNA. We have shown that, in the presence of AdoMet, poly(FdC-dG) causes an irreversible inactivation of the enzyme due to formation of a stable covalent complex containing the enzyme, DNA, and methyl groups of the cofactor. The evidence for this is as follows.

*First*, in the presence of all components there is a time-dependent inactivation of the enzyme. In the absence of AdoMet, poly(FdC-dG) behaves as a simple competitive inhibitor ($K_i \sim 3$ nM), and there is no time-dependent inactivation. However, in the presence of AdoMet there is a time-dependent, first-order loss of enzyme activity ($k = 0.05$ min$^{-1}$). The kinetics of inactivation are consistent with an ordered mechanism of binding in which enzyme binds to poly(FdC-dG) first, followed by binding of AdoMet and subsequent first-order inactivation; this order is in accord with the order of binding of the normal enzymic reaction (Wu & Santi, 1987). *Second*, using a nitrocellulose filter binding assay, we have isolated and studied the properties of *Hha*I methylase–poly(FdC-dG) complexes. Isolable complexes are formed between *Hha*I methylase and poly(FdC-dG) both in the absence and in the presence of AdoMet. However, in the absence of cofactor, the binary complex rapidly dissociates, showing a half-life of less than 30 s. In contrast, addition of AdoMet causes the formation of complexes that do not undergo detectable dissociation for as long as 3 days and are stable upon denaturation in SDS. *Third*, the stable complexes formed contain the enzyme, DNA, and the methyl group from AdoMet. Gel filtration of complexes formed with *Hha*I methylase, poly(FdC-dG), and [*methyl*-$^3$H]AdoMet gives a peak of radioactivity eluting near the void volume, consistent with the association of the radioactive methyl group with DNA. Digestion of the DNA in this complex results in a reduction of the molecular weight to the limit size of the methylase. The radioactivity in the peak was shown to be associated with protein by nitrocellulose filter binding assays, and this complex does not appreciably dissociate for days. Thus, the stable complex consists of *Hha*I methylase, poly-(FdC-dG), and methyl groups of the cofactor.

The molecular mechanism of inactivation may be deduced by considering the present results together with knowledge of the mechanism of catalysis of DCMTs (Wu & Santi, 1987) and the mechanism of inhibition of the related enzyme thymidylate synthase by FdUMP (Santi & Danenberg, 1984). First, there is rapid formation of a tight, rapidly reversible enzyme–poly(FdC-dG) complex (Scheme II). (We do not currently know whether reversible covalent bonds between the enzyme and 6-position of FdCyd residues are present in this complex.) Next, AdoMet binds to the reversible complex to form a reversible ternary enzyme–poly(FdC-dG)–AdoMet complex. Within the ternary complex, a nucleophilic catalytic

Scheme II




residue of the enzyme adds to the 6-position of an FdCyd within a potential methylation site. This addition activates the otherwise inert 5-carbon of FdCyd for reaction with the methyl group of AdoMet. Following methyl transfer, a 5,6-dihydropyrimidine adduct is formed in which a covalent bond joins a nucleophile of the enzyme to the 6-position of the heterocycle, and the methyl group is bonded to the 5-carbon of FdCyd. Unlike the analogous complex of thymidylate synthase with FdUMP and methylenetetrahydrofolate, there is no simple manner in which this complex can be envisioned to be chemically reversible.

With thymidylate synthase—the paradigm for enzymes catalyzing one-carbon transfer at the 5-position of pyrimidines—the nucleophile which initiates catalysis by addition to the 6-carbon of the heterocycle is the sulfhydryl of a conserved cysteine residue, which in all enzymes thus far sequenced is preceded by a proline [see Hardy et al. (1987)]. We have previously described studies (Wu & Santi, 1987) which suggested that the nucleophilic catalyst in the *Hha*I methylase catalyzed reaction is also a sulfhydryl group, probably the one found in the cysteine of the Pro-Cys sequence at positions 80–81 (Caserta et al., 1987). The Pro-Cys residues are invariable in sequences of the other DCMTs thus far determined and imbedded in a highly conserved region (Tran-Betcke et al., 1986; Kiss et al., 1985; Posfai et al., 1983, 1984). It is likely that a nucleophilic cysteine, probably that within the Pro-Cys doublet, is a common feature necessary for catalysis in all DCMTs and involved in covalent bond formation of FdCyd-containing DNA. Studies are in progress to isolate and identify the peptide fragment of *Hha*I methylase which is covalently linked to the FC of poly(FdC-dG).

Both azaC and FdCyd have been shown to inhibit methylation of DNA with resultant effects on gene expression and cell differentiation (Jones & Taylor, 1980; Taylor & Jones, 1982). The biochemical mechanisms of inhibition by both drugs have now been shown to be, in general, similar: incorporation into DNA and formation of specific covalent complexes with DCMTs. Although azaC has been more widely used than FdCyd, FdCyd possesses different, and in some cases advantageous, properties as a biochemical tool. One of the major difficulties in working with azaC is its instability. AzaC hydrolyzes rapidly, placing severe limitations on its storage life and on long-term experiments (Chan et al., 1979). Incorporation into DNA appears to increase its stability to some extent, but it is still difficult to isolate azaC-containing DNA intact (Santi et al., 1984). In contrast, FdCyd and DNA containing FdCyd are quite stable to chemical hydrolysis. A major shortcoming of FdCyd is that it is susceptible to enzymatic deamination to toxic 5-fluorodeoxyuridine metabolites (Newman & Santi, 1982); however, the effects of such metabolites are predictable and in many cases can be circumvented with other enzyme inhibitors, metabolites, or mutant cell lines. Finally, there are notable differences in the formation and stability of covalent inhibitory complexes formed between DCMTs and DNA containing azaC or FdCyd. FdCyd requires AdoMet to form such complexes whereas azaC inactivation does not (Santi et al., 1984). As a result, the extent of inhibition of DCMTs by FdCyd could be modulated by changing the levels of intracellular AdoMet. Also, the complexes formed by FdCyd and AdoMet are chemically irreversible, while the unstable ring of azaC is susceptible to decomposition (Jones, 1985). It would not be surprising if these analogues showed interesting different biological properties.

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# The Dimer of the β Subunit of *Escherichia coli* DNA Polymerase III Holoenzyme Is Dissociated into Monomers upon Binding Magnesium(II)†

Mark A. Griep‡ and Charles S. McHenry*

*Department of Biochemistry, Biophysics, and Genetics, University of Colorado Health Sciences Center, Denver, Colorado 80262*

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ABSTRACT: The β subunit of *Escherichia coli* DNA polymerase III holoenzyme binds $Mg^{2+}$. Reacting β with fluoresceinmaleimide (FM) resulted in one label per β monomer with full retention of activity. Titration of FM–β with $Mg^{2+}$ resulted in a saturable 11% fluorescence enhancement. Analysis indicated that there was one noncooperative magnesium binding site per β monomer with a dissociation constant of 1.7 mM. Saturable fluorescence enhancement was also observed when titration was with $Ca^{2+}$ or spermidine(3+) but not with the monovalent cations $Na^+$ and $K^+$. The $Mg^{2+}$-induced fluorescence enhancement was specific for FM–β and was not observed with FM–glutathione, dimethoxystilbenemaleimide–β, or pyrenylmaleimide–β. Gel filtration studies indicated that the β dimer–monomer dissociation occurred at physiologically significant β concentrations and that the presence of 10 mM $Mg^{2+}$ shifted the dimer–monomer equilibrium to favor monomers. Both the gel-filtered dimers and the gel-filtered monomers were active in the replication assay. These and other results suggested that the fluorescence increase which accompanies β dissociation is due to a relief from homoquenching of FM when the β dimer dissociates into monomers.

DNA polymerase III holoenzyme is the multisubunit enzyme that is responsible for the majority of replicative DNA synthesis in *Escherichia coli* [for reviews, see Kornberg (1982) and McHenry (1985, 1988)]. The β subunit can be chromatographically separated from holoenzyme,[1] to yield pol III*, a subassembly of the remaining subunits (Wickner & Kornberg, 1973; McHenry & Kornberg, 1977).

β is the *dnaN* gene product (Burgers et al., 1981). From the DNA sequence (Ohmori et al., 1984) and the amino-terminal sequence of β (Johanson et al., 1986), it is known that β has 366 amino acid residues for a molecular mass of 40.6 kDa. While all other holoenzyme subunits are present at only 10–20 copies per cell (Kornberg, 1980), there are about 90–100 β molecules per *E. coli* cell. This value was calculated from β purification data (Johanson & McHenry, 1980): 12 900 β units/g of cell paste in the first fraction and a final specific activity of $12.6 \times 10^6$ units/mg. For this calculation, it was assumed that there are $10^{12}$ *E. coli* cells/g of cell paste. If the volume of an *E. coli* cell is estimated to be 4 fL (~0.8 × 0.8 × 2.0 μm) (DeRobertis & DeRobertis, 1980), then the concentration of β in the cell is about 40 nM monomers.

The ability of β to increase the processivity of holoenzyme subassemblies has become the best understood enzymatic process of DNA polymerase III. DNA polymerase III holoenzyme can replicate an entire 5000-nucleotide G4 circle without dissociating (Fay et al., 1981). β has a significant role in this processivity since pol III*, which lacks β, adds only 200 nucleotides to a primer before it dissociates from the DNA (Fay et al., 1982). β is also required in the formation of initiation complex, the step preceding elongation in which holoenzyme binds to oligonucleotide-primed single-stranded DNA coated with single-stranded DNA binding protein.

There appear to be two pathways to initiation complex formation, involving either the presence or absence of ATP. The complex formed in the presence of ATP is stable; it remains a functional complex even after gel filtration (Wickner & Kornberg, 1973; Wickner, 1976; Burgers & Kornberg, 1982a,b; Johanson & McHenry, 1980, 1982). When a deoxynucleoside triphosphate mixture is added to this complex, highly processive replication occurs. Studies with anti-β IgG (Johanson & McHenry, 1980, 1982) have shown that β becomes immersed within the complex such that it is resistant to the antibody's inhibitory action and remains with the replicative complex during elongation. The stability of the complex is thought to be related to the hydrolysis of ATP (Burgers & Kornberg, 1982b; Johanson & McHenry, 1984; Oberfelder & McHenry, 1987). Once formed, it continues to exhibit ATPase activity (Wickner & Kornberg, 1973; Burgers & Kornberg, 1982a,b; Johanson & McHenry, 1984). However, the full role of the DNA-dependent ATPase has not been elaborated.

Excess β is required to form initiation complex in the absence of ATP. Even though this complex is not as stable to

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* Author to whom correspondence should be addressed.
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[1] Abbreviations: DMSM, 2,5-dimethoxystilbene-4-maleimide; FM, fluorescein-5-maleimide; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; holoenzyme, DNA polymerase III holoenzyme; PM, *N*-1-pyrenylmaleimide; pol III*, DNA polymerase III holoenzyme subassembly lacking the β subunit.

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