Kinetic and Catalytic Properties of the DNA (cytosine-5)-methyltransferase HhaI are described. With poly(dG-dC) as substrate, the reaction proceeds by an equilibrium (or processive) ordered Bi-Bi mechanism in which DNA binds to the enzyme first, followed by S-adenosylmethionine (AdoMet). After methyl transfer, S-adenosylhomocysteine (AdoHcy) dissociates followed by methylated DNA. AdoHcy is a potent competitive inhibitor with respect to AdoMet (K_i = 2.0 μM) and its disappearance during reaction results in nonlinear kinetics. AdoMet and AdoHcy significantly interact with only the substrate enzyme-DNA complex; they do not bind to free enzyme and bind poorly to the methylated enzyme-DNA complex. In the absence of AdoMet, HhaI methylase catalyzes exchange of the 5-H of substrate cytosines for protons of water at about 7-fold the rate of methylation. The 5-H exchange reaction is inhibited by AdoMet or AdoHcy. In the enzyme-DNA-AdoHcy complex, AdoHcy also suppresses dissociation of DNA and reassociation of the enzyme with other substrate sequences. Our studies reveal that the catalytic mechanism of DNA (cytosine-5)-methyltransferases involves attack of the C6 of substrate cytosines by an enzyme nucleophile and formation of a transient covalent adduct. Based on precedents of other enzymes which catalyze similar reactions and the susceptibility of HhaI to inactivation by N-ethylmaleimide, we propose that the sulfhydryl group of a cysteine residue is the nucleophilic catalyst. Furthermore, we propose that Cys-81 is the active-site catalyst in HhaI. This residue is found in a Pro-Cys doublet which is conserved in all DNA (cytosine-5)-methyltransferases whose sequences have been determined to date and is found in related enzymes. Finally, we discuss the possibility that covalent adducts between C6 of pyrimidines and nucleophiles of proteins may be important general components of protein-nucleic acid interactions.

MATERIALS AND METHODS

RESULTS

Properties of HhaI Methylase—HhaI methylase is a monomer of M, ≈37,000 as determined by gel filtration chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme is rapidly inactivated by 0.4 mM NEM (t_1/2 < 10 s at 22 °C) and is protected by poly(dG-dC) (0.21 μM, t_1/2 ~60 s; at 1.1 μM t_1/2 ~120 s). In contrast, AdoHcy, in mammalian cells, DNA (cytosine-5)-methyltransferases methylate certain CpG sequences which are believed to modulate gene expression and cell differentiation (for review see Ref. 1). Bacterial DNA (cytosine-5)-methyltransferases are a component of restriction-modification systems and serve as valuable tools for the manipulation of DNA structure and the analysis of protein-nucleic acid interactions.

We have undertaken studies to elucidate the mechanisms of catalysis, ligand interactions, and specificity of DNA (cytosine-5)-methyltransferases. Recently we proposed that the catalytic mechanism of DNA (cytosine-5)-methyltransferases involves formation of a transient covalent adduct between the enzyme and the 6-carbon of cytosines, in analogy to other enzymes which catalyze 1-carbon transfer to pyrimidines (2, 3). This hypothesis is supported by observations that DNA containing azacytosine (azaC), a potent inhibitor of DNA (cytosine-5)-methyltransferases, forms covalent complexes with mammalian and bacterial enzymes (4-7). The azaC is presumed to form a stable adduct analogous to the proposed catalytic intermediate of the reaction.

In this paper, we describe the kinetic and catalytic mechanisms of the DNA (cytosine-5)-methyltransferase HhaI methylase, which recognizes the sequence GCGC. Using poly(dG-dC), a synthetic substrate of the enzyme (8), we show that the methylation reaction proceeds by an ordered kinetic scheme in which enzyme first binds to DNA. We also demonstrate a novel enzyme-induced exchange of the 5-H of cytosines in the absence of AdoMet and provide evidence for the formation of a transient covalent intermediate during methylation by HhaI methylase.

4778
AdoMet up to 25 μM (1500 × K_m) does not protect the enzyme from inactivation by NEM. These data suggest that an active site sulfhydryl group may be involved in catalysis by HhaI methylase. One picomole of enzyme is equal to 1.3 units of activity based on estimates of protein concentration in a purified preparation and from inhibition of 5-3H exchange by AdoHcy using the method of Henderson (38).

**Kinetics of HhaI Methylose-catalyzed Methylation of Poly(dG-dC)—**Early in this study we observed that the rate of HhaI-catalyzed methylation of poly(dG-dC) by AdoMet progressively decreased as the reaction proceeded (Fig. 1a). The decrease in rate was inversely related to the concentration of AdoMet and most noticeable at low concentrations of AdoMet (<50 nM). This behavior was not due to decay of enzyme activity under these conditions because <10% loss of activity was lost in 60 min. One probable cause of rapid loss of linearity in this reaction is product inhibition by methylated DNA or AdoHcy. In the reactions of Fig. 1, the concentration of poly(dG-dC) substrate is much greater than the amount of methylated DNA formed (>1000-fold), competitive inhibition by the methylated product would require its K_i to be on the order of 10^{-11}–10^{-12} M (concentration in double-stranded recognition sites), which is unreasonably low. The inhibition appears to be competitive with respect to AdoMet, and the nonlinear kinetics are consistent with competition by the AdoHcy generated in the reaction. The progress curves shown in Fig. 1 fit Equation 1, which describes a reaction wherein a product, i.e. AdoHcy, shows competitive inhibition with respect to the substrate, i.e. AdoMet (9).

\[
P = \frac{V_{max}}{1 - (K_i/K_s)} + \frac{K_m}{S} \frac{1}{S - P} \ln \frac{S}{S - P}
\]

Here, P is the amount of AdoHcy formed at time t, S, is the initial AdoMet concentration, and K_i is the dissociation constant of AdoHcy. The amount of AdoHcy formed corresponds to the amount of methylated DNA, the product which is measured in these experiments. Plots of P/t versus [ln (S_s/ (S_s - P))]t provide a series of lines with positive slopes (Fig. 1b), converging at a negative value on the ordinate. The positive slope indicates that the K_m of AdoMet is much larger than K_i. Replots of the slopes of these lines versus S_s yield kinetic constants according to Equation 2:

\[
\text{Slope} = \frac{K_m}{K_i} + \frac{K_m S_s}{K_m - K_i}
\]

A plot of the slope versus S_s gave a value of 1.0 ± 0.5 nM for the K_i of the generated AdoHcy, in good agreement with the value determined by other methods (see below). In principle, values for K_m and V_max could also be derived from these data, but the large K_i/K_s ratio in this system made it difficult to assess these parameters from progress curves. Therefore, K_m and V_max were calculated from initial velocity experiments.

To determine initial velocities, product formation was measured under conditions such that the maximal final concentrations of AdoHcy generated in the reactions was less than 1 nM, and the highest AdoHcy/AdoMet ratio achieved was below 0.03. These constraints ensured that the overall inhibition of the reaction by AdoHcy was less than 5%.

The K_m for AdoMet is 14.7 ± 1.8 nM. The K_m for poly(dG-dC) is 2.3 ± 0.3 nM. Initial velocity experiments also confirmed that HhaI is a competitive inhibitor with respect to AdoMet (see Fig. 7a). The K_m for AdoHcy is 2.1 ± 0.1 nM, which is in acceptable agreement with the value obtained by progress curve analysis. The V_max was calculated to be 33.8 nmol-min^{-1} mg^{-1} enzyme. Using M_e = 37,000 for HhaI, k_cat is calculated to be 1.3 min^{-1}. The k_cat/K_m for poly(dG-dC) in this reaction is ~9.6 × 10^4 M^{-1} s^{-1}; for AdoMet, k_cat/K_m =1.5 × 10^6 M^{-1} s^{-1}.

**Reverse Reaction—**The ability of HhaI methylase to catalyze the reverse reaction, namely, the transfer of C5 methyl groups from poly(dG-[5-methyl]dC) to AdoHcy, was measured using poly(dG-[5-3H-methyl]dC) (specific activity ~10 μCi/nmol of 5-methylcytosine). Reactions (400 μl) contained 20 nM labeled copolymer (concentration in double-stranded recognition sites), 0, 2, or 200 μM AdoHcy, and 3.3 units of HhaI methylase. Samples (50-μl duplicates) were removed at various time intervals, mixed with 50 μl of poly(dG-[14C]dC) (22,000 dpm total) calibration standard, and measured for 3H remaining on the DNA by adsorption on DE52 as described under "Materials and Methods." No tritium loss was observed in any reaction after 100 min at 37 °C. Using a 5% change in 3H content as the lower limit of detection (equivalent to ~0.1 pmol of methyl group per 50 μl assayed), we calculate that the reverse reaction is less than 0.05 pmol/min, or at least 400-fold slower than the forward reaction based on the enzyme concentration in these reactions. Hence, the methyl transfer reaction is, as far as we're concerned, irreversible.

**Tritium Release from Poly(dG-[5-3H]dC) during Methylation—**HhaI-catalyzed methylation of poly(dG-[5-3H]dC) is accompanied by release of tritium into solvent. In 20 μM [methyl-14C]AdoHcy, tritium release is stoichiometric with and proceeds at a rate identical to the rate of incorporation of [14C]methyl groups into poly(dG-dC) (Fig. 2). In addition to the correspondence between 3H release and 14C incorporation, this experiment confirms that ≥95% of the poly(dG-dC) is methylated by HhaI, as reported by Mann and Smith (8).

**Tritium Exchange from Poly(dG-[5-3H]dC) in the absence of AdoMet—**Incubation of poly(dG-[5-3H]dC) with HhaI methylase in the absence of AdoMet caused a rapid release of tritium from the polymer into water (Fig. 2). The exchange reaction exhibits first-order kinetics and is about 7-fold faster than the 3H release that accompanies methylation. More than 95% of the tritium in the copolymer can be washed out into solvent. Tritium release is not due to chemical modification of cytosine residues, because poly(dG-[5-3H]dC) from which >80% of the label has been exchanged can accept [3H]methyl groups from [methyl-3H]AdoHcy. When determined by adsorption of products to DE52, >99% of the cytosines of poly(dG-[5-3H]dC) was methylated by HhaI after the majority of the 5-3H was exchanged.

The exchange from poly(dG-[5-3H]dC) is specifically catalyzed by HhaI methylase. Other enzymes, for example HpAll methylase and the restriction endonucleases HhaI and BssHII, which also bind to poly(dG-dC), do not cause tritium release from the copolymer.

The K_m for poly(dG-dC) in the exchange reaction is 2.1 nM in recognition sites, in good agreement with the value of 2.3 nM obtained in the methylation assay. The k_cat was 8.85 min^{-1}, some 7-fold greater than that for methylation. From these, k_cat/K_m was calculated to be 6.7 × 10^{-7} M^{-1} s^{-1}.

**Ligand Interactions in the Exchange Reaction—**As shown in Fig. 3, AdoHcy is an extremely potent inhibitor of the exchange reaction with K_i = 1.4 nM, in good agreement with its K_i (2.1 nM) in the methylation reaction. However, we do not know the extent of inhibition of exchange by AdoHcy in the ternary enzyme-DNA-AdoHcy complex (Scheme I). Equation 3 describes the HhaI-catalyzed exchange of poly(dG-[5-3H]dC) from the binary enzyme-DNA and ternary enzyme-DNA-AdoHcy complexes without prior assumptions about the contribution of each complex to the overall rate.

\[
\frac{1}{k_i - k_{cat}} = \frac{1}{k_i} + \frac{K_m}{k_i} = \frac{1}{k_{cat}} = \frac{1}{[\text{AdoHcy}]}
\]
Mechanism of HhaI Methylase

**Scheme I. Kinetics of binding and inhibition of exchange by AdoHcy.**

\[
\begin{align*}
E + {^3H}\text{DNA} & \xrightarrow{k_d} E \cdot {^3H}\text{DNA} \cdot \text{AdoHcy} \\
E + \text{DNA} & \xrightarrow{k_1} E \cdot \text{DNA} \\
E + \text{DNA} + \text{AdoHcy} & \xrightarrow{k_2} E \cdot \text{DNA} \cdot \text{AdoHcy}
\end{align*}
\]

**Scheme II. The order of substrate binding and product release for the reaction catalyzed by HhaI methylase.**

Here, \( k_1 \) is the apparent first-order rate of exchange determined for the binary complex in the absence of inhibitor; \( k_2 \) is the rate of exchange from the ternary enzyme-DNA-AdoHcy complex; \( k_{\text{obs}} \) is the observed rate of exchange, and \( k_0 \) is the dissociation constant for AdoHcy from the ternary complex. A plot of the data from Fig. 3 illustrates the application of Equation 3 (Fig. 4). The plot yields an ordinate intercept \( (1/(k_1 - k_2)) \) of 98 min which, based on \( k_1 = 1.02 \times 10^{-2} \text{ min}^{-1} \), establishes that \( k_0 \), i.e., catalytic turnover of 5-H exchange in the ternary complex is negligible. In a separate experiment containing 0.20 \( \mu \text{M} \) AdoHcy, saturating poly(dG-[5-\text{H}]dC) (3.44 \( \mu \text{M} \)) and HhaI methylase at \( \sim 0.26 \mu \text{M} \), the amount of tritium released after 30 min at \( 33 \degree \text{C} \) was \(<0.3\% \) of the uninhibited reaction. This amount of exchange was indistinguishable from the control-omitting enzyme, and represented \(<3 \text{nol} \% \) of enzyme concentration, showing that single turnover exchange from the ternary enzyme-DNA-AdoHcy complex is also suppressed by AdoHcy.

Tritium exchange was examined by varying poly(dG-[5-\text{H}]dC) concentrations at different fixed concentrations of AdoHcy. Double-reciprocal plots of velocity versus substrate yield an uncompetitive inhibition pattern (Fig. 5), consistent with a mechanism in which free enzyme associates only with DNA. Binary complexes of enzyme and AdoHcy do not form in these conditions. Inhibition of exchange results from the binding of AdoHcy to enzyme-DNA to form a “dead-end” ternary complex.

**Ligand Interactions in the Methylolation Reaction.** Initial velocities were determined for methylation reactions using various concentrations of [methyl-\text{H}]AdoMet and 32P-poly(dG-dC). Double-reciprocal plots of 1/\( v \) versus 1/[AdoMet] gave a series of lines intersecting at the ordinate (Fig. 6a). Plots of 1/\( v \) versus 1/[poly(dG-dC)] gave lines that intersect to the left of the ordinate (Fig. 6b). The data have been fitted to Equation 4, which describes an equilibrium ordered mechanism (Scheme II). DNA (designated A) initially forms a rapidly reversible EA complex, followed by binding of AdoMet (designated B); \( K_a \) is the dissociation constant for the EA complex and \( K_b \) is the Michaelis constant for AdoMet.

\[
\frac{V[A][B]}{K_aK_b + K_a[A] + [A][B]}
\]

Other mechanisms were eliminated by demonstrating unacceptable fits of the data to the appropriate equations using the Fortran programs of Cleland (13). The corresponding expression for a nonequilibrium ordered mechanism has a \( K_b[\text{B}] \) term in the denominator (identical in form to that for a rapid equilibrium random mechanism) and is ruled out by the data. Hence, dissociation of enzyme-DNA is faster than the forward maximal velocity. A competitive mechanism in which enzyme binds DNA by lateral diffusion can also be fitted to Equation 4 and cannot be distinguished from the conventional rapid equilibrium mechanism by the present work. AdoHcy inhibited the reaction competitively with respect to AdoMet and uncompetitively with respect to DNA (Fig. 7). Kinetics of inhibition by AdoHcy were best fitted to a modification of Equation 4, in which the term \( K_a[A] \) was replaced by the term \( K_a[A](1 + [P]/K_p) \), where \( P \) is AdoHcy and \( K_p \) its inhibition constant.

AdoMet did not show substrate inhibition at concentrations \( \geq 1 \text{ mM} \) (60,000-fold \( K_a \)). Thus, it does not bind significantly to the enzyme-DNA product. Since AdoHcy is a competitive inhibitor with respect to AdoMet, AdoHcy and AdoMet must bind to the same enzyme form, which we have shown to be the substrate enzyme-DNA complex. This conclusion is in accord with results of AdoHcy binding in the 5-H exchange reaction. Since AdoHcy does not bind to free enzyme, the order of product release must be AdoHcy first, followed by methylated DNA. Furthermore, dissociation of AdoHcy from the methylated enzyme-DNA-AdoHcy product complex is thermodynamically favored as indicated by the poor binding of AdoHcy to the methylated DNA-enzyme product (in contrast to its affinity for substrate enzyme-DNA). Otherwise, AdoHcy would show noncompetitive inhibition kinetics with respect to AdoMet.

**Discussion**

In this work we describe kinetic and catalytic properties of the DNA (cytosine-5)-methyltransferase HhaI, which methylates the internal cytosine residue of the tetranucleotide sequence GCGC. The enzyme is a monomer with native \( M \), \( \sim 37,000 \).

**Enzyme Assays and Kinetic Parameters.** Poly(dG-dC) was chosen as the substrate in these studies because it offers several practical advantages. (a) The alternating copolymer provides recognition sites that are not flanked by heterogeneous sequences. In other DNAs, such heterogeneity could complicate kinetic interpretations. (b) As shown here and elsewhere (8), over 95% of the cytosine residues of poly(dG-dC) can be methylated. Therefore, use of the copolymer allows high concentrations of substrate sites. The nearly complete methylation of poly(dG-dC) demonstrates that the enzyme also methylates internal cytosines in the partially methylated sequence GCGMeC. We recognize that partially methylated sequences could alter kinetic properties of the enzyme, but this is not apparent in our data. (c) For future studies of processivity, alternating sequences of poly(dG-dC) are ideal because the substrate sites are separated by a frame of only two nucleotides. (d) The polymer contains repeating units of the CG substrate sites of mammalian DNA (cytosine-5)-methyltransferase; we anticipate that many results obtained with the bacterial enzyme can be directly correlated with those which will be obtained with the mammalian enzyme.

In addition to the standard assay which measures incorporation of labeled methyl groups into DNA from AdoMet, we use two other assays for HhaI methylase. In the first, we monitor the AdoMet-dependent release of tritium from [5-\text{H}]cytosine of poly(dG-[5-\text{H}]dC) into water. In the second, we monitor the enzyme-catalyzed release of tritium from...
poly(dG-[^3]H[dC]) in the absence of AdoMet. The latter exchange reaction has not been reported and has important implications for the catalytic mechanism of the methylase (see below). AdoMet inhibits the 5-[^3]H exchange with protons of water. At saturating concentrations of AdoMet, the loss of velocity determinations, with consequent errors in derived analysis (9) or stringent initial velocity conditions. Our results suggest that the practice of monitoring single time points during methylation could result in low and imprecise initial velocity determinations, with consequent errors in derived kinetic parameters.

The 5-[^3]H exchange for HhaI-catalyzed methylation of poly(dG-dC) was about 2.3 nM in double-strand recognition sites. This number agrees well with the Km values for other DNA methyltransferases (18, 19). However, the Km for AdoMet (-15 nM) and the Km for AdoHcy (2.0 nM) were substantially lower (about 50- and 100-fold, respectively) than values reported for other DNA methyltransferases.

We can estimate the rate of DNA association to HhaI methylase based on its Km and the turnover numbers. The kcat/Km for DNA (6.5 × 10^7 M^-1 s^-1) in the exchange reaction represents a minimum estimate of the rate constant for the association of DNA to enzyme, and approximates the diffusion-controlled limit (10^9-10^10 M^-1 s^-1) for enzyme-ligand interactions (20). Based on this value and the dissociation constant Km for the enzyme-DNA complex derived for the methylation reaction, the lower limit for the dissociation rate constant is calculated to be 0.15 s^-1. Thus, dissociation of the binary complex is significantly faster than kcat of methylation (0.02 s^-1) and verifies other kinetic data (see below) which indicate that the rate-determining step for the methylation reaction occurs after formation of the enzyme-DNA complex. Our data do not allow assignment of the rate-determining step.

Kinetic Mechanism—The HhaI methylase-catalyzed reaction proceeds by an ordered bi-bi mechanism as shown in Scheme I. DNA first binds to enzyme, followed by AdoMet. Kinetic data indicate the rapid equilibrium binding of DNA; interaction of enzyme with this substrate is more rapid than catalysis. Methyl transfer in the ternary enzyme-DNA-AdoMet complex yields the product complex of enzyme-DNA-AdoHcy. AdoHcy is subsequently released to give the enzyme-DNA complex. Whereas AdoHcy and AdoMet are tightly bound to the substrate enzyme-DNA complex, they are poorly bound to the product enzyme-DNA complex, which possesses a methylated cytosine residue in the substrate site. Consequently, release of AdoHcy from the product ternary complex is favorable, and high concentrations of AdoMet do not cause significant substrate inhibition. We cannot distinguish whether the enzyme fully dissociates from the product DNA after each methylation event, or processively moves to an adjacent substrate site on the same molecule of DNA. The two mechanisms are kinetically equivalent.

The arguments for the assigned kinetic mechanism are as follows: (a) Double-reciprocal plots of initial velocity data with AdoMet as the variable substrate and poly(dG-dC) as the fixed substrate give a pattern of lines intersecting at the reciprocal velocity axis. With poly(dG-dC) as the varied substrate, plots give lines that intersect to the left of the reciprocal velocity axis. The data are inconsistent with an equilibrium ordered mechanism and indicate rapid association and dissociation of DNA to the enzyme. These results rule out a random mechanism as well as a ping-pong mechanism which might have indicated a methylated enzyme intermediate. They do not address whether the enzyme is processive or distributive, nor do they demonstrate the order of product release; the latter is established by data described below. (b) The fact that HhaI methylase catalyzes 5-[^3]H exchange from poly(dG-[^5]H[dC]) in the absence of AdoMet with a Km for DNA similar to that in the methylation reaction confirms the formation of a catalytically competent binary enzyme-DNA complex. Evidence for a binary enzyme-DNA complex is also provided by the protection which poly(dG-dC) affords HhaI methylase against inactivation by NEM. (c) Studies of inhibition demonstrate that AdoHcy binds to the enzyme-DNA binary complex, but not to free enzyme. AdoHcy is an uncompetitive inhibitor with respect to DNA in both 5-[^3]H exchange (K_i = 1.4 nM) and methylation (K_i = 2.0 nM). Uncompetitive inhibition demonstrates the formation of an enzyme-DNA-AdoHcy complex and rules out the formation of a binary enzyme-AdoHcy complex. Since AdoHcy does not associate with free enzyme, dissociation of products in the methylation reaction must also be ordered. AdoHcy must first dissociate from the enzyme, followed by methylated DNA.

From the kinetics of methylation, AdoMet does not appreciably bind to the binary product complex enzyme-DNA-AdoHcy. Formation of such a ternary dead-end complex would result in substrate inhibition at high concentrations of AdoMet. Based on the competitive kinetics of inhibition by AdoHcy with respect to AdoMet, we also conclude that AdoHcy does not bind tightly to the enzyme-DNA-AdoMet product complex. Formation of a tight enzyme-DNA-AdoHcy complex would have resulted in non-competitive inhibition by AdoHcy with respect to AdoMet in the methylation reaction. Thus, although AdoMet and AdoHcy bind tightly to substrate enzyme-DNA complexes, their affinity for the product enzyme-DNA complex is low.

Catalytic Mechanism—The methylation of DNA by DNA (cytosine-5)-methyltransferases is analogous to other enzyme-catalyzed transfers of 1-carbon units to the C5 of pyrimidine nucleotides. Examples of enzymes that catalyze this type of reaction include thymidylate synthase, dUMP and dCMP hydroxymethylases, certain RNA-modifying enzymes, and DNA (cytosine-5)-methyltransferases (see Ref. 3 for review). The mechanism of this class of enzymes has been established most thoroughly for thymidylate synthase, and several salient features of catalysis have emerged. The primary consideration is that the carbon at the 5-position of the pyrimidine is not sufficiently nucleophilic to react with biological donors of 1-carbon units. However, the heterocycle is susceptible to addition/elimination reactions which activate the 5-position for electrophilic substitution reactions. In thymidylate synthase, a nucleophile of the enzyme adds to C6 of dUMP to generate a 5,6-dihydropyrimidine intermediate with anionic character at the 5-position. This carbanion equivalent is sufficiently nucleophilic to condense with 1-carbon units; subsequent β-elimination of the 5-H and enzyme nucleophile generates the 5-substituted pyrimidine and catalytically active protein. Most aspects of this mechanism have been verified with studies of model chemical counterparts (21, 22). Thus, the hallmark of biological electrophilic substitution reactions at the 5-position of pyrimidines appears to be covalent catalysis involving addition of a nucleophile to the 6-carbon of the heterocycle.

Two approaches to detect covalent intermediates are relevant to DNA (cytosine-5)-methyltransferases. The first is
Mechanism of HhaI Methylase

Scheme III. The mechanism of cytosine methylation by HhaI methylase.

---

demonstration of an exchange of the 5-H of the substrate pyrimidine with solvent protons. Insofar as we know, this reaction can only occur via nucleophilic attack at the 6-position and the formation and reversal of 5,6-dihydropyrimidine intermediates (21,23-25). A second approach employs mechanism-based inhibitors to trap the covalent intermediate. These analogs form stable covalent adducts with the enzyme as a consequence of catalysis. The adducts represent analogs of a steady-state intermediate of the reaction and provide important tools for studying aspects of catalysis. 5-Fluoro-2'-deoxyuridine 5'-monophosphate inhibition of thymidylate synthase represents a paradigm for this approach (3).

Previous studies demonstrated covalent binding of several DNA (cytosine-5)-methyltransferases to DNA containing azaC (5, 6, 26). The mechanism of inhibition by azaC has been proposed to involve covalent addition at C6 of azaC residues in substrate sites by an enzyme nucleophile (2, 5).

We show here that HhaI methylase catalyzes 5-3H release from poly(dG-[5-3H]dC) in the absence of AdoMet. Other enzymes which catalyze 5-H exchange of their pyrimidine nucleotide substrates do so at rates much slower than those of the normal methylation reactions (23-25). In contrast, 5-H exchange catalyzed by HhaI methylase is about 7-fold faster than methylation and provides direct support for the formation of covalent intermediates during catalysis by HhaI methylase. The most reasonable mechanism is shown in Scheme III. Upon binding of substrate(s), an enzyme nucleophile adds to the C6 of cytosine to generate the dihydropyrimidine which possesses a carbanion equivalent at C-5. This reactive intermediate has two alternative paths in the forward reaction. In path a, accepts a methyl group from AdoMet to form the 5-methyl covalent adduct and AdoHcy; this represents a commitment to catalysis since the reverse step is energetically unfavorable. Abstraction of the 5-H from and , elimination of the enzyme nucleophile at C-6 generates the methylated cytosine and free enzyme. In path b, accepts a proton from water (or general acid of the protein) to form the 5,6-dihydropyrimidine adduct . Removal of tritium from the opposite face of the pyrimidine (see below) and elimination (reverse of formation of ) results in 5-H exchange.

Stereospecificity—The stereochemical configurations at C5 and C6 are determined by which face of the pyrimidine accepts the substituents. We expect addition of the enzyme nucleophile at C6 and subsequent methyl transfer from AdoMet to C5 to proceed in a stereospecific manner. 5-H exchange, however, does not require stereospecific addition/elimination of protons at C5 of intermediates and , as discussed below.

DNA structure could influence the stereochemical course of nucleophilic addition to C6. We have used the molecular graphics computer program Insight (27) to examine the local environment of cytosine residues in DNA. In B-DNA, covalent addition to cytosine C6 is most favorable via the si face of the heterocycle (the facial assignment as defined at C6), because this face is exposed in the major groove of DNA. The C6 appears inaccessible from the re face, which is bounded.

*L. Hardy and D. V. Santi, unpublished results.
closely by atoms of neighboring base residues (average inter-
nuclear distance <4 Å). Therefore, unless there is a large
disruption of the native double helix upon association of the
enzyme, covalent addition must occur from the 5’ face. In Z-
DNA, the C6 positions of cytosines are sterically blocked on
both faces. This factor may account for the inability of HhaI
to methylate Z-DNA (28, 29), although reduced binding in-
teractions between the enzyme and Z-DNA could also explain
the phenomenon. Access to C5 also is hindered on the 3’ face,
such that methyl transfer to give 2 may be required to proceed
from the same face as nucleophilic addition. Thus the overall
reaction may involve cis addition/trans elimination, which
contrasts with the trans- addition/cis- elimination reaction es-
tablished for thymidylate synthase (30).

Whatever the stereochemical course of addition of the
enzyme nucleophile and methyl group across the 5,6-double
bond of cytosine to give 2, subsequent elimination of the 5-H
must occur from the face opposite that of methyl addition.
Thus, trans addition would be followed by cis elimination or
vice versa. However, the stereochemistry of the enzyme-cat-
alyzed 5-H exchange is enigmatic. If the reaction is stereose-
ppecific and exchange simply involves a reversal of steps leading
to formation of 3, then the same proton added to 1 to give 3
would be reversed during reversal. The result is that formation
and reversal of 3 would not be accompanied by 5-H exchange.
Two alternative explanations may account for the observed
5-H exchange. The first is that proton addition to 1 and
removal from 3 might occur in a non-stereospecific fashion
from either face of the pyrimidine (cis or trans to the enzyme
at C6). In this case, the rate of formation and reversal of 3
could actually be greater than the observed rate of 5-H ex-
change. The second explanation is that proton addition to 1
and removal from 3 follows the same stereochemical course
as methylation and is completely stereospecific. Thus, like
the methyl group of AdoMet, the proton from water would
add to a single face to provide 3 having an asymmetric C5.
The tritium that was originally at C5 would be abstracted
from the opposite face of the dihydroxypyrimidine 3, and sub-
sequent β-elimination yields the 5-proto-cytosine and free
enzyme. As with the methylation reaction, this mechanism
for 5-H exchange requires trans addition/cis elimination (or
vice versa) across the 5,6-double bond of cytosine. We favor
this hypothesis because it utilizes catalytic features of the
normal enzymic reaction of methyl-group transfer.

- **5-H Exchange from Ternary Complexes**—We have shown
  that AdoHcy inhibits the HhaI-catalyzed 5-H exchange of
  poly(dG-5-[3H]dC). This inhibition is most simply explained by
  the ordered association of ligands with the enzyme: at a
  saturating concentration of the second substrate, dissociation
  of the first is suppressed. In this situation, 5-H exchange from
  multiple substrate sites requires the enzyme to release
  AdoHcy from the ternary complex before it can bind to a new
  DNA site. In the presence of saturating AdoHcy, the enzyme-
  DNA complex preferentially partitions to the enzyme-DNA
  AdoHcy complex; dissociation of the binary complex is sup-
  pressed. Thus, catalysis of 5-H exchange is inhibited simply
  because the enzyme is locked onto the same tetranucleotide
  sequence which it initially bound (the consequences to pro-
  cessivity in this system will be addressed in a future report).
  In addition, binding of AdoHcy to the enzyme-DNA complex
  in itself causes complete suppression of 5-H exchange from the
  ternary complex (see below).

Unlike AdoHcy, AdoMet could not inhibit 5-H exchange
by simply preventing dissociation and reassociation of the
enzyme-DNA complex. Catalysis of methylation requires that
dissociation of products and association of substrates occur
at a rate commensurate with turnover. However, if the binary
enzyme-DNA complex is the species responsible for 5-H ex-
change, then high concentrations of AdoMet will partition it
toward the ternary complex. This effect would reduce the
steady-state concentration of the binary complex and conse-
quently the apparent rate of 5-H exchange. For example,
using a lower limit of 1.5 × 10⁶ M⁻¹ s⁻¹ for the association rate
constant of AdoMet and the kₚ for exchange, at 1 μM AdoMet
the rate of 5-H exchange would be at most one-eleventh of
exchange in the absence of AdoMet. This explanation does
not address the issue of whether 5-H exchange occurs within
the enzyme-DNA-AdoMet central complex. At saturating
AdoMet concentrations, we could not detect tritium release
beyond that accountable by methylation (see Fig. 2). Assum-
ing a 10% difference in the rates of H⁻ release and methylation
as the minimum detectable level, we estimate from the relative
kₚ values that 5-H exchange from the ternary complex is at
least 70-fold slower than from the binary complex. Hence,
the presence of AdoMet within the enzyme-DNA complex also
appears to suppress 5-H exchange.

There are several possible mechanisms by which AdoHcy
or AdoMet could inhibit single-turnover exchange from the
respective ternary complex. First, AdoHcy may simply pre-
vent covalent addition of the enzyme to C6 of cytosine, so
that the essential intermediate 1 is not formed. However, this
cannot be the mechanism for AdoMet-induced suppression of
exchange because the covalent adduct 1 is formed during
catalysis of methylation. Second, if exchange results from
non-stereospecific formation and reversal of 3, then the pre-
ence of bound AdoMet or AdoHcy could simply enhance the
stereospecificity of the reaction. In this case, 3 would be
formed, but the proton released from C5 would be the same
as the one received; hence no net exchange would be observed.
A third possibility is that the presence of bound AdoMet or
AdoHcy precludes accessibility of C5 to protons of solvent.
In the presence of AdoHcy, there may be transient formation of
1, but it could only undergo reversal. In the presence of
AdoMet, 1 could accept a methyl group from the co-factor but
not a proton from solvent.

The **Active-site Nucleophile and Its Role in Protein-Nucleic
Acid Interactions**—We have demonstrated that DNA (cyto-
sine-5)-methyltransferase shares several catalytic features
with other enzymes that catalyze 1-carbon transfer reactions
at the 5-position of pyrimidines, and it is reasonable to expect
that elements important in catalysis would be conserved in
such enzymes. In thymidylate synthase, the paradigm for such
enzymes, the nucleophile that initiates catalysis by addition
to the 6-position of dUMP is a sulffhydryl of cysteine, which
in all sources thus far examined is preceded by a proline (31–
33). Our observations that HhaI methylase is inactivated by
NEM and that the enzyme is protected from such inactivation
by substrate DNA suggest that it may also utilize cysteine as
the essential active-site nucleophile. In addition, we have
noted that a Pro-Cys doublet found at residues 89–81 in HhaI
methylase is also found in the deduced amino acid sequences
of BsuRI, BspRI, and Bacillus subtilis phage SPR methylases
(14, 34–36). This observation is significant for the following
reasons: (i) the Pro-Cys doublets are embedded in a highly
conserved region of the methylases (comprising over 200
amino acids), (ii) the doublets are aligned among all the
sequences, and (iii) this alignment contains the only cysteine
which is invariant in these DNA (cytosine-5)-methyltransfer-
ases. Based on the above considerations, we propose that the
common sulffhydryl found in the conserved Pro-Cys sequence
of DNA (cytosine-5)-methyltransferases (residue 81 of HhaI
methylase) is the nucleophilic catalyst in the methylation and 5-H exchange reactions.

Finally, formation of reversible covalent adducts between C6 of pyrimidines and nucleophiles of proteins may be an important component of protein-nucleic acid interactions. This phenomenon is clearly not limited to small molecules and it need not be relegated solely to a role in catalysis. In principle, covalent adducts could modulate the affinity and rate of dissociation between proteins and their ligands over a wide range, influence recognition and specificity, and participate in catalysis. Thus far, covalent protein-pyrimidine adducts have been documented in the interactions of DNA (cytosine-5)-methyltransferase with DNA, tRNA-uridine methyltransferase with tRNA, and aminoacyl-tRNA synthetase with cognate tRNAs (37). A number of approaches to detect such intermediates are available (3) and it should be possible to determine the extent of their involvement in protein-nucleic acid interactions.

Acknowledgements—We thank Dr. Thomas Meek for providing computer analyses of the kinetic data, Dr. Larry Hardy for providing his observations of tRNA uridine methyltransferase, and Dr. David Osterman for helpful suggestions on the manuscript.

REFERENCES

1. Doerfler, W. (1983) Annu. Rev. Biochem. 52, 93–124
2. Santi, D. V., Garrett, C. E., and Barr, P. J. (1983) Cell 33, 9–10
3. Jones, P. A., and Taylor, S. M. (1988) Cell 20, 85–89
4. Taylor, S. M., and Jones, P. A. (1982) J. Mol. Biol. 162, 679–692
5. Santi, D. V., Norment, A., and Garrett, C. E. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 6993–6997
6. Friedman, S. (1985) J. Biol. Chem. 260, 5698–5705
7. Santi, D. V., and Danenberg, P. V. (1984) in Folic Acids and Pterins (Blakley, R. L., and Benkovic, S. J., eds) pp. 345–398, John Wiley & Sons, New York
8. Mann, M. B., and Smith, H. O. (1979) in Transmethylation (Usdin, E., Borchardt, R. T., and Creveling, C. R., eds) pp. 483–492, Elsevier/North-Holland, New York
9. Orsi, B. A., and Tipton, K. F. (1979) Methods Enzymol. 63, 159–183
10. Wagner, J., Danzin, C., and Mamont, P. (1982) J. Chromatogr. 227, 349–368
11. Wells, R. D., Larson, J. E., Grant, R. C., Shortle, B. E., and Cantor, C. R. (1970) J. Mol. Biol. 54, 465–497
12. Yoo, O. J., and Agarwal, K. L. (1980) J. Biol. Chem. 255, 6445–6449
13. Cleland, W. W. (1967) Adv. Enzymol. Relat. Areas Mol. Biol. 29, 1–32
14. Caserta, M., Zacharias, W., Nwankwo, D., Wilson, Geoffrey, G., and Wells, R. D. (1987) J. Biol. Chem. 262, 4770–4777
15. Herman, G. E., and Modrich, P. (1982) J. Biol. Chem. 257, 2605–2612
16. Günthert, U., Freund, M., and Trautner, T. A. (1981) J. Biol. Chem. 256, 9340–9345
17. Modrich, P., and Roberts, R. J. (1982) in Nucleases (Linn, S. M., and Roberts, R. J., eds) pp. 59–154, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
18. Rubin, R. A., and Modrich, P. (1977) J. Biol. Chem. 252, 7265–7272
19. Günthert, U., Jentisch, S., and Freund, M. (1981) J. Biol. Chem. 256, 9346–9351
20. Fersht, A. (1985) Enzyme Structure and Mechanism, W. H. Freeman & Co, New York
21. Santi, D. V., and Brewer, C. F. (1973) Biochemistry 12, 2416–2424
22. Pogolotti, A. L., and Santi, D. V. (1974) Biochemistry 13, 456–466
23. Pogolotti, A. L., Weill, C., and Santi, D. V. (1979) Biochemistry 18, 2794–2798
24. Kunitani, M., and Santi, D. V. (1980) Biochemistry 7, 1271–1275
25. Ye, Y.-C., and Greenberg, G. R. (1967) J. Biol. Chem. 242, 1307–1313
26. Christian, J. K., Schneiderman, N., and Acs, G. (1985) J. Biol. Chem. 260, 4059–4068
27. DerNinger, H. E., Tramontano, A., Sprang, S. R., and Fletterick, R. J. (1986) J. Mol. Graphics 4, 82–87
28. Zacharias, W., Larson, J. E., Kilpatrick, M. W., and Wells, R. D. (1984) Nucleic Acids Res. 12, 7677–7692
29. Vardimon, L., and Rich, A. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 3268–3272
30. James, T. L., Pogolotti, A. L., Ivanetich, K. M., Wataya, Y., Lam, S. S. M., and Santi, D. V. (1976) Biochem. Biophys. Res. Commun. 72, 404–410
31. Chu, F. K., Maley, G. F., Maley, F., and Belfort, M. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 3049–3053
32. Belfort, M., Maley, G., Pedersen-Lane, J., and Maley, F. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 4914–4918
33. Takeishi, K., Kaneda, S., Ayusawa, D., Shimizu, K.,Gotoh, O., and Seno, T. (1985) Nucleic Acids Res. 13, 2035–2043
34. Kiss, A., Posfai, G., Keller, C. C., Venetianer, P., and Roberts, R. J. (1985) Nucleic Acids Res. 13, 6403–6421
35. Posfai, G., Kiss, A., Erdei, S., Posfai, J., and Venetianer, P. (1983) J. Mol. Biol. 170, 597–610
36. Posfai, G., Balazs, F., Erdei, S., Posfai, J., Venetianer, P., and Kiss, A. (1984) Nucleic Acids Res. 12, 9039–9049
37. Starzyk, R. M., Koontz, S. W., and Schimmel, P. (1982) Nature 298, 136–140
38. Henderson, P. J. F. (1972) Biochem. J. 127, 321–333
Mechanism of HhaI Methylase

John C. Wu and Saima S. Haq

Materials and Methods

HhaI methylase and AdoHcy were purchased from Promega Corp. Stock solutions of HhaI methylase (10-12 M) and [3H]AdoHcy (10-12 M) were prepared for each reaction by diluting the stock solutions with 10 mM Tris-HCl (pH 7.5) (buffer A) to a final concentration of 10-12 M HhaI methylase and 10-12 M [3H]AdoHcy. Reactions were conducted at 37°C in 10 mM Tris-HCl buffer (pH 7.5) and contained 10-12 M HhaI methylase and 10-12 M [3H]AdoHcy and had a final volume of 50 μL. The reactions were performed in microcentrifuge tubes and were subjected to the desired conditions. The buffer used for each reaction was Tris-HCl (pH 7.5) and contained 10-12 M HhaI methylase and 10-12 M [3H]AdoHcy. Reactions were conducted at 37°C in 10 mM Tris-HCl buffer (pH 7.5) and contained 10-12 M HhaI methylase and 10-12 M [3H]AdoHcy and had a final volume of 50 μL. The reactions were performed in microcentrifuge tubes and were subjected to the desired conditions.

Results

The results obtained from the experiments are shown in Figure 1. The data show that the enzyme activity increases with increasing concentrations of AdoHcy. The activity also increases with increasing temperature, but this effect is more pronounced at higher concentrations of AdoHcy. The results also indicate that the enzyme activity is inhibited by the presence of a specific inhibitor, and that the inhibition is reversed by the addition of a specific activator.

Discussion

The results of this study support the hypothesis that the enzyme activity is dependent on the concentration of AdoHcy. The data also suggest that the enzyme activity is modulated by temperature and that the activity is inhibited by the presence of a specific inhibitor. The inhibition is reversed by the addition of a specific activator, which indicates that the enzyme activity is regulated by a feedback mechanism.

Conclusion

The results of this study provide evidence that the enzyme activity is dependent on the concentration of AdoHcy. The data also suggest that the enzyme activity is modulated by temperature and that the activity is inhibited by the presence of a specific inhibitor. The inhibition is reversed by the addition of a specific activator, which indicates that the enzyme activity is regulated by a feedback mechanism.
Figure 6. Kinetics of methylation by HhaI methylase. Reactions contained the indicated concentrations of [125I]-5'pCp[32p]dGp-61 (1.33 pmol/microliter) and methyl-32HAdoMet (500pmol/ml) in standard buffer at 25°C. HhaI methylase was added in a final concentration of 0.055units/ml (free) volume/ml (ml). The reactions were stopped after 5 minutes by addition of 20μl 25% glutaric acid and assayed as described in methods.

Figure 7. Inhibition of methylation by AdoMet. Reciprocal plots of the rates of methylation versus AdoMet concentration at different fixed concentrations of poly(dC-dG).

Figure 8. Inhibition of methylation by poly[dC-dG]. Reciprocal plots of the rates of methylation versus poly[dC-dG] concentration at different fixed concentrations of AdoMet.