Initial velocity determinations were conducted with human DNA (cytosine-5) methyltransferase (DNMT1) on unmethylated and hemimethylated DNA templates in order to assess the mechanism of the reaction. Initial velocity data with DNA and S-adenosylmethionine (AdoMet) as variable substrates and product inhibition studies with methylated DNA and S-adenosylhomocysteine (AdoHcy) were obtained and evaluated as double-reciprocal plots. These relationships were linear for plasmid DNA, exon-1 from the implanted small nuclear ribonucleoprotein-associated polypeptide N, (CGG-CGG)_{12}, (m5CGG-CGG)_{12}, and (CGG-CGG)_{33} but were not linear for (CGG-Cm5CG)_{12}. Inhibition by AdoHcy was apparently competitive versus AdoMet and uncompetitive/noncompetitive versus DNA at \( \leq 20 \mu M \) AdoMet. Addition of the product (methylated DNA) to unmethylated plasmid DNA increased \( V_{\text{max app}} \) resulting in mixed stimulation and inhibition. Velocity equations indicated a two-step mechanism as follows: first, activation of DNMT1 by methylated DNA that bound to an allosteric site, and second, the addition of AdoMet and DNA to the catalytic site. The preference of DNMT1 for hemimethylated DNA may be the result of positive cooperativity of AdoMet binding mediated by allosteric activation by the methylated CG steps. We propose that this activation plays a role \textit{in vitro} in the regulation of maintenance methylation.

The genome of most organisms contains modified nucleotides including N\(^6\)-methyladenine, N\(^4\)-methylcytosine, and C\(^5\)-methylcytosine (m\(^5\)C)\(^1\)–\(^3\). However, both the biological significance and the types of DNA methylation differ greatly between prokaryotes and eukaryotes. In prokaryotes, most modified bases participate in restriction-modification, a defense mechanism that protects the host from heterologous phage infection\(^4\). In addition, N\(^6\)-methyladenine plays a role in the initiation of DNA replication\(^5\) and in post-replicative methyl-directed mismatch repair\(^6\).

In higher eukaryotes, DNA methylation is confined to m\(^5\)C and is implicated in the regulation of development, genomic imprinting\(^7,8\), X chromosome inactivation, gene expression\(^9\), and retrotransposon inactivation\(^10–12\). In mammals, the patterns of methylation are inherited from both parental genomes but are erased and reconstructed \(\textit{de novo}\) in somatic cells following implantation\(^13,14\). Such patterns are then copied and maintained by hemimethylation of the daughter strands during semiconservative DNA synthesis in S phase (maintenance methylation)\(^15,16\). However, the mechanisms by which both \textit{de novo} and maintenance methylation occur remain to be elucidated.

Several DNA methyltransferases have been isolated from human and mouse \(^17–20\), but it is not clear whether the \textit{de novo} and maintenance methylations are carried out by separate proteins \textit{in vivo} or whether both activities are shared by one or more enzymes\(^21–26\). Nevertheless, a role \textit{in vivo} methylation has been established for isoforms of the human DNMT\(^1\) gene\(^27,28\) whose product, DNMT1, methylates C at a CG dinucleotide step, both in single-stranded and double-stranded, unmethylated or hemimethylated, templates\(^29–34\). Hemimethylated templates are the most effective for the reaction, and methylation rates increase in the neighborhood of pre-existing m\(^5\)C residues\(^35–38\). However, little is known about the mechanisms responsible for this preference.

DNMT1 has a bipartite structure with the C-terminal 570 amino acids containing the catalytic domain. This region shares sequence homologies with all prokaryotic type II cytosine-5 methyltransferases\(^39,40\), including a PC dipeptide motif that is part of the catalytic center in the crystal structures of \(\text{M.HhaI}^41\) and \(\text{M.HaeIII}^42\), and the binding site for S-adenosylmethionine (AdoMet), the methyl donor for methyltransferases\(^43,44\). The remaining \(\sim 1000\) N-terminal amino acids, which are not present in the prokaryotic enzymes, contain a nuclear localization signal, a replication foci targeting sequence\(^15\), and are important in the discrimination between unmethylated and hemimethylated substrates\(^33\).

Herein, we report kinetic analyses with the human full-length, recombinant, DNMT1 on a variety of DNA substrates with the aim of learning about the mechanism of the methyl transfer reaction and the role of DNA in regulating the enzyme activity. The results confirm the preference of DNMT1 for pre-methylated DNA; however, the kinetics reveal a complex behavior with DNA substrates that are bound more tightly. The reaction follows a sequential mechanism whereby both substrates (DNA and AdoMet) must bind to the enzyme before
Fig. 1. Conditions for linearity of the methyl transfer reaction with DNMT1 and supercoiled pRW3602. A, 40 nM DNMT1 was incubated at 37 °C with 25 μM CG steps and 10 μM AdoMet in a 300-μl reaction volume in buffer A. At 30 s and subsequently at 5-min intervals, 25 μl were withdrawn and processed, and the results were analyzed as described under “Experimental Procedures.” The means and standard deviations for the data points were derived from two independent experiments. B, a concentration range of 2–60 nM DNMT1 was used in reactions containing 10 μM CG steps and 10 μM AdoMet at 37 °C for 30 min. The means and standard deviations for the data points were derived from two independent experiments. The value at 60 nM DNMT1 was excluded from the interpolation. Inset, the concentration ranged from 2 to 200 nM DNMT1.

any product (methylated DNA and AdoHcy) is released and is consistent with a two-step process. First, DNMT1 binds DNA at an allosteric site (probably in the N-terminal domain) and activates the catalytic center, and second, AdoMet and the DNA (which may either be the same molecule bound to the regulatory site or a new DNA molecule) occupy the catalytic site. Allosteric binding of pre-methylated CG is proposed to increase the accessibility of AdoMet to the catalytic center, thus blocking the enzymatic turnover. In the experiments with methylated DNA, the concentration range of the reactants was 4.0–25.0 μM CG, 2.5–25.0 μM AdoMet, and 2.0–40.0 μM AdoMet. Two methylated DNAs were used as products. The first was a 40-bp duplex oligonucleotide (45–nmol) containing canonical Watson-Crick pairs but with 5-methylcytosines substituting for all cytosines. The second was a 36-bp duplex oligonucleotide with the sequence CGG(F5CGG)11C(G5CG), named (F/MeCG)12, where F/C designates 5-fluorocytosine. The rationale for using this fluorinated oligonucleotide was that, contrary to (5-MeCG)12, (5-F-MeCG)12 may form an irreversible complex with DNMT1, thus blocking the enzymatic turnover. In the experiments with methylated DNA, the concentration range of the reactants was 4.0–25.0 μM CG for pRW3602, 2.5–60 μM CG for (MeCG)12, or 11 nM to 40.0 μM CG for (5-F-MeCG)12, and 6.67 and 30.0 μM AdoMet.

Enzyme Assay for Initial Velocities in the Presence of Products—Addition of known concentrations of the products to an enzymatic reaction (product inhibition) is a powerful strategy for deciphering an enzyme mechanism, i.e. whether the substrates bind (and the products dissociate) in an ordered or random fashion. Methylation reactions on supercoiled pRW3602 were performed in the presence of added S(-adenosyl)-L-homocysteine (AdoHcy) or methylated DNA as product in conjunction with DNMT1 and bacterial SssI methylase (New England Biolabs); unincorporated AdoMet was removed by Sephadex G-50 fine (Amersham Pharmacia Biotech) column chromatography. The eluate was constituted in buffer A to a concentration of ~6000 cpm/25 μl (IC solution). The nanomolar [3H]CH3 (N) present in the DNA at the end of the reaction was calculated as n = (C − B/RF, where C was the counts/min of a sample, B the blank (cpm in the absence of DNMT1), RF the ratio of IC filter/IC solution, and B the cpm/nl free AdoMet. The initial velocity (v) was obtained as v/ ||ε,TN and F the cpm/nl free AdoMet. The initial velocity (v) was obtained as v/ ||ε,TN and F the cpm/nl free AdoMet.
Kinetics of Human DNA (Cytosine-5) Methyltransferase

The velocity equation for the methyltransferase reaction by DNMT1, A, free DNMT1 (E) binds the activator DNA (D) at a regulatory site and subsequently AdoMet (Am) and a second molecule of DNA to the catalytic site. The sequence of addition of AdoMet and the DNA to the catalytic site is arbitrary. The kinetic data do not distinguish whether the reaction may be ordered with DNA binding before AdoMet or the reverse. The velocity equation shows that the first step involves the binding of DNA (D) to a regulatory site in DNMT1 (E), distinct from the catalytic site, to give a DNA-DNMT1 initial complex. The second step consists in the binding of a CG-CG (or CGm5CG or m5CG-CG) and AdoMet (Am) to the catalytic site to give the ternary complex competent for catalysis.

Based on the initial velocity patterns that were obtained, the reaction is treated at steady state (the turnover rate of the enzyme is not limited by the binding of substrate but by the rate at which the chemical reaction takes place) except for the interconversion between free DNMT1 and DNA-DNMT1, which must occur at equilibrium within the steady state. Addition of the substrates is shown to be ordered, with AdoMet preceding the DNA; however, this sequence of addition is not proven by the present data, as discussed later. Indeed, the reaction may be ordered with DNA binding before AdoMet or random, where either substrate can bind first. The velocity equations for an ordered or random sequential bimolecular system (such as this) are identical, and both $V_{max}$ and the Michaelis constant for the substrates ($K_{M}$) can be derived, even though the mechanism is unknown. However, the dissociation constant $K_D$ for the first substrate that adds to the reaction cannot be assigned unless it is identified. Our derivation of the following velocity Equation 1 was based on the formulation by King-Altman and Cha, which are described in detail by Segel (49). The method requires that the section of the reaction at equilibrium be grouped into a single corner of the scheme, as illustrated in Fig. 2B. The rate $k_1$ is then corrected for $f_1$, the ratio of DNA-DNMT1 to DNA-DNMT1 plus free DNMT1. In the following equations, [CG] is given the same meaning as [D] (DNA) in Fig. 2.

The velocity Equation 1, in the absence of products, is as follows.

$$v = \frac{V_{max}[AdoMet][CG]}{K_{m(app)}[AdoMet] + K_{m(app)}[CG] + [AdoMet][CG]}$$

(1)

where $K_{m(app)} = K_m(1 + K_{CG}[CG])$, $K_m = h_i/k_r$, $K_{m(app)} = K_{m(app)}(1 + K_{CG}[CG])$, and $K_{CG} = K_{CG}'$.

When [AdoMet] is the variable substrate (see Equation 2)

$$\frac{1}{v} = \frac{1}{V_{max}[CG]} + \frac{K_{m(app)}}{V_{max}[AdoMet]} + \frac{K_{CG}}{V_{max}[CG]}$$

(2)

for small values of $K_{CG}[CG]$ and $K_{CG} < K_{m(app)}$.

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**Table I**

| DNA Substrates | Relevant properties |
|---------------|-------------------|
| pRW3602 supercoiled | A supercoiled plasmid (average negative superhelical density −0.045) containing a total of 2705 bp with a random distribution of 338 CG steps (1 every 8 bp on average) |
| pRW3602 relaxed | Plasmid treated with topoisomerase I to provide a circular substrate with the 338 CG steps in a topologically relaxed DNA conformation (average negative superhelical density = 0) |
| pRW3602 linear | Plasmid cleaved with BglI to obtain two fragments of 1.57 and 1.14 kbp that contain 206 and 132 CG steps, respectively |
| SNRPN | Chemically synthesized duplex oligonucleotide (75 bp) corresponding to exon 1 of the small nucleolar protein N gene, which is part of an imprinted transcriptional domain on human chromosome 15q11–13 |
| SNRPN-US | Chemically synthesized oligonucleotide (75 bp) containing 5-methylcytosine at all CG steps (DNA hemimethylated on the upper strand) |
| SNRPN-LS | Chemically synthesized oligonucleotide (75 bp) where the lower strand of SNRPN contains 5-methylcytosine at all CG steps (DNA hemimethylated on the lower strand) |
| (CGG · CCG)_{12} | Chemically synthesized oligonucleotide (36 bp) containing the CCG repeat sequence whose expansion in the 5'UTR of the FMR1 gene (Xq27.3) causes fragile-X syndrome |
| (m^5CGG · CCG)_{12} | Chemically synthesized oligonucleotide (36 bp) where the upper strand of the CCG repeat contains 5-methylcytosine at all CG steps (DNA hemimethylated on the upper strand) |
| (CGG · Cm^5CG)_{12} | Chemically synthesized oligonucleotide (36 bp) where the lower strand of the CCG repeat contains 5-methylcytosine at all CG steps (DNA hemimethylated on the lower strand) |
| (CGG · CCG)_{73} | Restriction fragment of ~256 bp from pRW3691 (47) containing 146 CG steps from 73 consecutive copies of CCG · CCG and an additional 8 CG steps from the flanking DNA sequences |
| poly d(I-C) | Synthetic polymer routinely used to assay for DNA methyltransferase activity |
| DNA Inhibitors | |
| (m^5CGG)_{10} | Chemically synthesized 40-bp duplex oligonucleotide (“Experimental Procedures”) containing 5-methylcytosine at all 20 CG steps but containing no CNG steps |
| (F/MeCG)_{12} | Chemically synthesized 36-bp duplex oligonucleotide containing the FMR1-associated triplet repeat (CGG · CCG) substituted with 5-fluorocytosines on the upper strand and 5-methylcytosines on the lower strand at the CG steps |

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**Fig. 2.** Scheme of an ordered Bi Bi mechanism with allostERIC activation for the methyltransferase reaction by DNMT1. A, free DNMT1 (E) binds the activator DNA (D) at a regulatory site and subsequently AdoMet (Am) and a second molecule of DNA to the catalytic site. The sequence of addition of AdoMet and the DNA to the catalytic site is arbitrary. The kinetic data do not distinguish whether AdoMet, or the DNA, or both are the first to bind to this site. E, D, and DE are grouped together since their interconversion is at equilibrium relative to the other enzyme species. This assumption is made in order to develop a velocity equation that yields linear responses; $f_1$ is DE/E + DE; M and Ah signify the products of the reaction, methylated DNA and AdoHey, respectively.

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A stock solution of 500 μM CG steps was treated by optical absorption on a Beckman DU 640 spectrophotometer using the extinction coefficients calculated from the known coefficients of the component nucleotides. Since this synthetic 40-mer contains only mC at all CG steps and contains no CNG sequences, it gives a substrate for DNMT1 and was used as a product inhibitor. A list of all the substrates and inhibitors used and their relevant properties is given in Table I.
Plots obtained at changing fixed concentrations of [CG] intersect at 1/$K_m$ and 1/$v = (1 - K_m[CG]/V_{max})/V_{max}$.  

When [CG] is the varied substrate (see Equation 3), for small values of $K_{cG}[CG]$, the velocity is as follows:

$$\frac{1}{v} = \frac{1}{V_{max}} \left( 1 + \frac{K_{\text{AdoMet}}}{[\text{AdoMet}]} \right) + \frac{K_{CG}}{V_{max}} \left( 1 + \frac{K_m}{[\text{AdoMet}]} \right) \frac{1}{[CG]}$$

(Eq. 3)

and the family of double-reciprocal plots intersects at 1/$[CG] = -K_m[CG]/K_{\text{AdoMet}}[CG]$, and 1/$v = (1 - K_m[CG]/V_{max})/V_{max}$.

**RESULTS**

Initial velocity experiments enable the evaluation of kinetic constants and provide insights into the mechanism of a reaction. A comprehension of the mechanism of this key enzyme, human DNMT1, is critical for understanding its role in developmental processes and in the etiology of fragile X syndrome and other diseases (7-10, 50). A variety of DNA templates (Table I) were methylated to less than 5–8% of the total CG steps by purified, recombinant DNMT1 with the aim of assessing the effects of sequences flanking the substrate CG on the kinetic constants, the role of negative supercoiling, and the mechanism of the reaction. Experimental data were analyzed by graphing the extent of methylation as a function of concentration of DNA or AdoMet, as variable substrates, on double-reciprocal Lineweaver-Burk plots. This report is the second of a series of three papers describing the purification and characterization of DNMT1 (45) and focuses on the mechanism of the methyltransferase reaction. The third paper will describe the effect of DNA topology on the reaction rates at CG sites in random as well as CGG-CGG repeat tracts and compare the kinetic properties of DNMT1 with the bacterial M.SsaI.

**Linear Velocity Responses**—For biradent enzymes (such as DNMT1), double-reciprocal plots generally give linear responses where 1/$v$ is graphed as a function of 1/$s$. For most of the DNAs used, which included supercoiled pRW3602 as purified from *Escherichia coli* ($-\sigma = 0.045$), relaxed circular ($-\sigma = 0$), or linear, as well as the SNRPN oligonucleotide (unmethylated or hemimethylated), (CGG-CGG)$_{12}$, (m$^3$CGG-CGG)$_{12}$, and (CGG-CGG)$_{72}$, the velocity curves were linear with respect to the variable substrate, whether this was the DNA or AdoMet. Fig. 3 shows the data with supercoiled pRW3602. Fig. 3A shows the concentration of $[^3]$HCH$_3$ groups incorporated when the DNA was the variable substrate (on the x axis) and AdoMet the fixed substrate. Conversely, Fig. 3B shows the results with AdoMet as the variable substrate and DNA as the fixed substrate. For all of the DNA templates listed above, the velocity patterns were as in Fig. 3, i.e. they converged to the left of the y axis and above or below the x axis for both substrates. Fig. 4 shows the double-reciprocal plots for the triplet repeat sequences (CGG-CGG)$_{12}$ (Fig. 4, A and B), (m$^3$CGG-CGG)$_{12}$ (Fig. 4, C and D), and (CGG-CGG)$_{72}$ (Fig. 4, E and F). These patterns contrast with those obtained with M.HhaI (51) and M.SsaI$^2$ methylases, where the families of lines converge on the y axis when AdoMet is the variable substrate. This result shows that AdoMet and DNA do not bind by an ordered and rapid equilibrium mechanism to DNMT1, as in the case with M.HhaI and M.SsaI.

Fig. 5 shows the replots of the slope and y axis intercept ($1/V_{\text{max(app)}}$) for each of the lines in Fig. 3 that were used to derive the kinetic constants. As shown in Equations 2 and 3, four replots are possible that give the following constants: (a) $1/V_{\text{max}}$ on the y axis intercept (Fig. 5A) and 1/$K_m$ on the x axis intercept by replotting the intercepts of Fig. 3A as a function of 1/AdoMet (Equation 3); (b) $K_m^{0.5}/V_{\text{max}}$ on the y axis intercept (Fig. 5A) and 1/$K_m$ on the x axis intercept by replotting the slopes of Fig. 3A as a function of 1/AdoMet; (c) $1/V_{\text{max}}$ on the y axis intercept (Fig. 5B) and 1/$K_m^{5}$ on the x axis intercept by replotting the intercepts from Fig. 3B as a function of 1/CG (Equation 2); and (d) $K_m^{0.5}$/AdoMet/V$_{\text{max}}$ on the y axis intercept (Fig. 5B) and $K_m$/AdoMet/1/[CG] on the x axis intercept by replotting the slopes from Fig. 3B as a function of 1/CG. Therefore, these four replots yield the maximum velocity and the Michaelis constants for AdoMet (at DNA = 0) and DNA (at AdoMet = 0). As pointed out previously, the dissociation constant for AdoMet cannot be assigned to the DNA or AdoMet because the method does not distinguish the order of addition. The experimental values for the constants are reported in the companion papers (45).

**Curved Velocity Responses**—Unexpectedly, two template DNAs (d(I-C-C)).$_{7000}$ and (CGG-Cm$^3$CG)$_{12}$ gave non-linear initial velocity curves. The results for d(I-C-C)).$_{7000}$ are described in the accompanying paper (45), whereas the double-reciprocal plots for (CGG-Cm$^3$CG)$_{12}$ are reported in Fig. 6. Fig. 6A shows that the methylation rate was linear when DNA was the variable substrate. The data also indicate that, contrary to Figs. 3 and 4, velocities were maximal at 1.74 $\mu$M AdoMet, such that further increases (up to 10.0 $\mu$M) did not result in a decrease in slope or intercept. Fig. 6B shows that plots were not linear when AdoMet was the variable substrate. Velocities

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1. Bacolla, S. Pradhan, J. E. Larson, R. J. Roberts, and R. D. Wells, manuscript in preparation.
were unchanged, and plots were parallel to the x axis when AdoMet concentrations rose above 2 μM. The responses were still dependent on DNA concentration, since 1/V_{max(app)} (y axis intercepts) decreased with increasing CG content. A replot of 1/V_{max(app)} versus 1/CG was linear (Fig. 7A), whereas both intercept and slope replots from the data at fixed AdoMet were curved (Fig. 7B).

This result indicates that when this particular DNA was bound to DNMT1, the velocity of the reaction was already maximal at a very low (2 μM) AdoMet concentration, contrary to the expectation that maximum velocity requires infinite amounts of AdoMet. The plots in Figs. 6 and 7 still enable the calculation of 1/V_{max}, 1/K^m_{CG}, and 1/K^m_{AdoMet}; however, K^m cannot be derived.

The most significant conclusion that can be drawn from these results is that the kinetic behavior of DNMT1 may be dramatically altered by both sequence of the DNA template and its pre-methylation status. Obviously, the scheme in Fig. 2 and its velocity equations are not adequate to describe the results with (CGG\_zCCG)_{12} which implies that DNMT1 is capable of complex kinetics. Overall, this combination of linear plus non-linear responses indicates a steady-state mechanism, where the DNA can act simultaneously both as a substrate and as an activator for the reaction. The activation is proposed to occur through binding of a DNA molecule at a site distinct from the catalytic center, i.e. an allosteric, or regulatory, site. The precise sequence of the chemical steps that lead to such non-linear responses, however, is unknown. Nevertheless, it seems clear that the role of the DNA bound to the allosteric site is to...
increase the affinity of the DNA-DNMT1 complex for AdoMet since low levels of AdoMet are sufficient to maximally drive the reaction.

It is noteworthy that the complex enzymatic behavior is associated with a DNA sequence, the (CGG-CCG)$_m$ triplet repeat, whose expansion in the chromosomal FRAX locus leads to aberrant methylation and to disease in humans.

In summary, these studies indicate that the methylation reaction by DNMT1 may follow complex mechanisms, and both the sequence composition as well as the methylation status of the DNA substrate contribute to this complexity.

**Product Inhibition with AdoHcy**—Product inhibition studies of bireactant enzymes provide a means to distinguish random from ordered sequential Bi Bi mechanisms. Ordered systems give competitive patterns with the first substrate that binds to the enzyme versus the last product that leaves the enzyme (the plots converge on the y axis) and non-competitive patterns with the other combinations (the lines converge to the left of the y axis). On the contrary, random mechanisms give competitive patterns with the first substrate that binds to the enzyme and product), and CG the fixed co-substrate (4.0 to 25.0 M). The pattern of inhibition was non-competitive from 2.0 to 10.0 M AdoMet, rather uncompetitive at 15.0 and 20.0 M AdoMet, and non-competitive again from 26.0 to 40.0 M AdoMet (not shown).

In double-reciprocal plots where AdoMet was the variable substrate, AdoHcy the changing-fixed inhibitor (like substrate and product), and CG the fixed co-substrate (4.0 to 25.0 M), velocities increased, as expected, up to 20.0 M AdoMet; however, higher concentrations caused strong inhibition by AdoHcy, a result that was not anticipated. In fact, the expectation was that AdoMet would progressively overcome the inhibition by AdoHcy, linearly increasing the reaction rates as its concentration rose. The slopes obtained from the 1/v versus 1/CG plots (which were linear) at various AdoHcy concentrations were as follows: open diamonds, 0.10 µM; filled diamonds, 0.12 µM; open squares, 0.16 µM; filled squares, 0.25 µM; open circles, 0.50 µM; filled circles, 1.00 µM. DNMT1 concentration was 40 nM.

In separate studies, the fixed concentration of AdoMet ranged from 2.0 to 40.0 µM. The pattern of inhibition was non-competitive from 2.0 to 10.0 µM AdoMet, rather uncompetitive at 15.0 and 20.0 µM AdoMet, and non-competitive again from 26.0 to 40.0 µM AdoMet (not shown).

FIG. 5. Replots of intercepts and slopes of initial velocities for the methylation of supercoiled pRW3602. A, replot of slopes and y axis intercepts of the 1/v versus 1/CG data shown in Fig. 3A. B, replot of slopes and y axis intercepts from the 1/v versus 1/AdoMet data shown in Fig. 3B. Error bars are the standard error associated with the double-reciprocal plots before constraint to the convergence point was applied. Lines drawn through the experimental data points are from fitting of the data to the slopes and intercepts of Equation 3 for A and Equation 2 for B.

FIG. 6. Double-reciprocal plots for the methylation of (CGG-Cm’CG)$_n$. A, [3H]CH$_3$ concentration as a function of DNA at fixed AdoMet concentrations were as follows: open triangles, 1.02 µM; filled triangles, 1.23 µM; open diamonds, 1.43 µM; filled diamonds, 1.74 µM; open squares, 2.56 µM; filled squares, 4.17 µM; open circles, 5.02 µM; and filled circles, 10.0 µM. B, nm [3H]CH$_3$ incorporated as a function of AdoHcy at fixed DNA. CG concentrations were as follows: open diamonds, 0.10 µM; filled diamonds, 0.12 µM; open squares, 0.16 µM; filled squares, 0.25 µM; open circles, 0.50 µM; filled circles, 1.00 µM. DNMT1 concentration was 40 nM.
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for AdoMet ≤ 20.0 μM, at each fixed value of AdoHcy. The plots were linear, and their slopes (Slope\(^{\text{CG/AdoMet}}\)) were finally graphed as a function of AdoHcy (Fig. 8B). The result was a linear, rather than a parabolic, curve indicating that only one AdoMet-binding site per DNMT1 molecule was detected. The x axis intercept gives the K\(_i\) for AdoHcy, which is ~14 μM.

Product Inhibition with Methylated and Fluorinated DNA—

The second part of the inhibition studies consisted of the use of the other product, fully methylated DNA. In conjunction with the data with AdoHcy, fully methylated DNA was expected to give non-competitive inhibition patterns versus both substrates for an ordered steady-state mechanism or competitive inhibition versus DNA for a random mechanism. A concentration range of 2.5 to 60.0 μM of m^5CG steps containing 5-methylcytosine in a 40-bp duplex synthetic oligonucleotide (CmCG)\(_{20}\) was added to 4.00 to 25.0 μM CG from supercoiled pRW3602 (like substrate and product) and 6.67 or 30.0 μM AdoMet. Fig. 9A shows the replots of intercepts from double-reciprocal plots where pRW3602 was the variable substrate and (CmCG)\(_{20}\) the changing-fixed inhibitor, with AdoMet fixed at 6.67 μM. Intercepts were expected not to change for a competitive system or increase with increasing m^5CG for a non-competitive mechanism. On the contrary, whereas some scatter was observed, 1/V\(_{\text{max(app)}}\), decreased as more inhibitor was added, indicating enzyme activation rather than inhibition. Slope effects were more complex but not substantial. At 30.0 μM AdoMet, velocities with 2.5, 5.0, and 10.0 μM (CmCG)\(_{20}\) were indistinguishable from those in the absence of inhibitor, whereas 20, 30, and 40 μM (CmCG)\(_{20}\) caused inhibition. These latter three concentrations gave parabolic curves, whereas, in all cases, intercepts were unchanged (Fig. 9B). Overall, these data indicate that fully methylated DNA acted in two ways as follows: first, it inhibited the reaction by competing with unmethylated DNA for the catalytic center, and second, it accelerated the turnover number of the enzyme by binding to an allosteric site.

To verify this dual role of methylated DNA further, experiments were carried out with an oligonucleotide (CGG(F/MeCG)\(_{12}\)) that contained m^5CG on one strand and F\(^5\)CG (5-fluorocytosine) steps on the complementary strand ([F/MeCG]\(_{12}\)). [F/MeCG]\(_{12}\) has two characteristics as follows: on the one hand, it acts as a dead-end inhibitor since F\(^5\)CG binds to DNMT1 in the presence of AdoMet and traps the enzyme into a stable DNMT1-AdoMet-DNA complex that does not proceed through catalysis (52–54). As a result, this causes inhibition. On the other hand, the DNA sequence and methylated pattern is identical to that of (CGG-CmCG)\(_{12}\), the substrate that produced the complex enzymatic patterns in Figs. 6 and 7. Thus, it was of interest to determine if [F/MeCG]\(_{12}\) acted as an inhibitor, an activator, or both. Eleven nM to 40.0 μM of modified CG steps from [F/MeCG]\(_{12}\) was added to 4.00 to 25.0 μM CG from supercoiled pRW3602 and 6.67 or 30.0 μM AdoMet. Fig. 10, A and B, shows the intercept and slope replots from double-reciprocal plots where pRW3602 was the variable substrate and [F/MeCG]\(_{12}\) the fixed inhibitor (like substrate and inhibitor). The Lineweaver-Burk plots were linear. [F/MeCG]\(_{12}\) caused a decrease in both intercepts and slopes at 6.67 μM AdoMet (filled circles) and had no effect at 30.0 μM AdoMet (open circles). Interestingly, the decreases in intercepts occurred at nanomolar concentrations of the added oligonucleotide, suggesting that [F/MeCG]\(_{12}\) was far more potent as an activator than as an inhibitor.

To verify this conclusion, control reactions were performed in
Michaelis-Menten patterns of methylation with hemimethylated DNA complexes have been proposed, based on non- and 56). Furthermore, allosteric transitions in DNMT1-meth molecules (Refs. 36 and 37 and references therein and Refs. 55 33018 an ordered mechanism. Therefore, the dissociation constant cannot be assigned to either the DNA or AdoMet. The data with (CGG-Cm5CG)12 that methylated DNA is accessible and/or, (F/MeCG)12 duplex DNA.

In summary, these product inhibition studies support the reaction scheme in Fig. 2 and velocity equations (Equations 1–3) developed according to steady-state assumptions that require the addition of both substrates before any product is released. However, the scheme is not sufficient to explain the curved responses obtained with (CGG-Cm5CG)12 and d(I-C-I-...
It was reported that DNMT1 is processive, based on the observations that methylation rates increase with the length of the DNA, that NaCl inhibits the reaction in a concentration-dependent manner (57, 60), and that DNA-protein associations are rather stable (61, 62). The linear velocity patterns obtained here with the various DNA templates, including the closely spaced CG steps in (CCG-CCG)$_n$, were accounted for by a reaction scheme whereby the enzyme dissociates from the DNA after each reaction cycle. Thus, additional terms associated with processivity were not necessary in the final velocity equation. The higher $V_{\text{max}}$ observed for longer, rather than shorter, polymers such as (CCG-CCG)$_{32}$ versus (CCG-CCG)$_{12}$ (45) does not require a processive mechanism. The one-dimensional limited diffusion (63–66) and/or intersegment transfer (67–70) processes, characteristic of DNA-binding enzymes, account for this result. The theory underlying such mechanisms states that macromolecular collision in solution is not elastic, so that when a protein collides with a DNA molecule, it stays along the contour of the DNA, that NaCl inhibits the reaction in a concentration-dependent manner. The higher $V_{\text{max}}$ observed for longer, rather than shorter, polymers such as (CCG-CCG)$_{32}$ versus (CCG-CCG)$_{12}$ (45) does not require a processive mechanism. The one-dimensional limited diffusion (63–66) and/or intersegment transfer (67–70) processes, characteristic of DNA-binding enzymes, account for this result. The theory underlying such mechanisms states that macromolecular collision in solution is not elastic, so that when a protein collides with a DNA molecule, it stays along the contour of the DNA, that NaCl inhibits the reaction in a concentration-dependent manner.