Kinetic Mechanism of Cytosine DNA Methyltransferase MspI*

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A kinetic analysis of MspI DNA methyltransferase (M.MspI) is presented. The enzyme catalyzes methylation of λ-DNA, a 50-kilobase pair linear molecule with multiple M.MspI-specific sites, with a specificity constant of (k_cat/K_M) of 0.9 × 10^6 M^-1 s^-1. But the values of the specificity constants for the smaller DNA substrates (121 and 1459 base pairs (bp)) with single methylation target or with multiple targets (sonicated λ-DNA) were less by an order of magnitude. Product inhibition of the M.MspI-catalyzed methylation reaction by methylated DNA is competitive with respect to DNA and non-competitive with respect to S-adenosylmethionine (AdoMet). The S-adenosylhomocysteine inhibition of the methylation reaction is competitive with respect to AdoMet and non-competitive with respect to DNA. The presteady state kinetic analysis showed a burst of product formation when AdoMet was added to the enzyme preincubated with the substrate DNA. The burst is followed by a constant rate of product formation (0.06 mol per mol of enzyme s^-1) which is similar to catalytic constants (k_cat = ~0.056 s^-1) measured under steady state conditions. The isotope exchange in chasing the labeled methyltransferase-DNA complex with unlabeled DNA and AdoMet leads to a reduced burst as compared with the one involving chase with labeled DNA and AdoMet. The enzyme is capable of exchanging tritium at C-5 of target cytosine in the substrate DNA in the absence of cofactor AdoMet. The kinetic data are consistent with an ordered Bi Bi mechanism for the M.MspI-catalyzed DNA methylation where DNA binds first.

A class of DNA methyltransferases, referred to as m5C-DNA MTases, catalyzes transfer of a methyl group from the physiological methyl donor, S-adenosyl-L-methionine (AdoMet), to C-5 of cytosine in the substrate DNA in a sequence-specific manner. In case of procaryotes, it primarily forms part of the restriction-modification system. But in higher organisms, it has been implicated to have a role in a variety of cellular functions such as developmental process (1), transposition (2), recombination (3), X-chromosome inactivation (4), and genomic imprinting (5). All the m5C-DNA MTases, whether from virus, bacteria, or higher organisms, share a common architectural plan (6). They have six highly conserved and four not so well conserved motifs (7). Although both adenine and cytosine DNA MTases show a degree of similarity in their architectural plan, there are important differences (8). Conservation of sequence is more prominent among members of the m5C-DNA MTase family than among those belonging to the N^6-adenine DNA MTase family (8). Kinetics of methyl transfer by M.HhaI (m5C-MTase) and M.EcoRI (N^6-adenine MTase) has been elucidated (9, 10). Despite both being bilobal and having a degree of similarity in structure, there are marked differences in the kinetic mechanism of these two methyltransferases. Both are known to be consistent with an ordered Bi Bi steady state mechanism. However, whereas the M.HhaI binds DNA first, the M.EcoRI binds AdoMet first (9, 10). Although the catalytic mechanism for m5C-DNA MTases is well understood, significant differences do exist among them from a kinetic standpoint. Two of the m5C-MTases, M.Dcm and M.BspRI, transfer the methyl group to themselves when incubated with AdoMet in the absence of DNA (11, 12). This unique feature of suicidal self-methylation has not been reported for any other enzyme of the m5C-DNA MTase family. The M.MspI displays many important features, which are uncommon to other m5C-MTases. For instance, it has the largest N-terminal sequence (107 bases) among the methylases of bacterial restriction/modification systems (7), it induces a bend in the substrate DNA in a sequence-specific manner (13), and it has also been shown to possess a topoisomerase activity (14). In view of these unique features of the M.MspI and the fact that it recognizes DNA substrates differently than its isochizomer M.HpaII, we attempted to investigate its kinetic mechanism. In the present communication, we report the kinetic parameters for methylation and tritium exchange reactions of the M.MspI, and a kinetic analysis of product inhibition and presteady state kinetics.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strain and Plasmid**

Escherichia coli K-12 strain ER 1727 (λ(mcr BC') hsd Rms rrr*)::Tn 10, mcrA1272:: Tn 10, FlacproAB lacI (lacZ-M15) was used for overexpression of the target gene which was placed downstream of the T7 promoter regulated by the lac operator in the expression vector pMSP (15). The E. coli strain ER 1727 harboring recombinant plasmid pMSP was cultivated in Luria Broth containing 150 μg ml^-1 ampicillin. The cells were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside and harvested as reported previously (13).

**Purification of MspI Methylase**

The harvested cells (0.5 g) were suspended in 2 ml of buffer containing 10 mM potassium phosphate (pH 7.4), 1 mM EDTA, 14 mM β-mercaptoethanol, 0.3 mM NaCl, and 10% glycerol. The cell suspension was sonicated for 3 min with burst and gap periods of 15 s at 14-μm amplitude in a Sonirep 150 (New Brunswick, Edison, NJ). The temperature during sonication was maintained at 5 ± 2 °C. The sonicated cell suspension was centrifuged at 31,000 g for 30 min. The cell-free extract, so recovered, was treated as a crude preparation from the MspI methylase. The enzyme was purified to apparent homogeneity on the following ion-exchange columns: phosphocellulose, S-Sepharose, and...
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Q-Sepharose as described previously (16). Active fractions were pooled and dialyzed against the buffer containing 50 mM Tris-HCl, pH 8.0, 0.1 mM NaCl, 10 mM EDTA, 1 mM dithiothreitol, and 50% glycerol for storage and further use.

**Optimum Reaction Conditions and Assays for the MspI Activity**

To determine the temperature where the MspI displayed optimum activity, methylation reactions were carried out at different temperatures in the range of 10–40 °C in NS buffer (50 mM Tris-HCl, pH 7.5; 10 mM EDTA; 1 mM β-mercaptoethanol with 200 µg/ml bovine serum albumin). The reaction mixtures were preincubated for 20 min at the corresponding temperature in a heat block (WWR Scientific) prior to enzyme addition. Disintegration rates were estimated at the disintegration/min value corresponding to the optimum reaction pH.

Quantitative assay of the methyltransferase activity was performed by measuring the incorporation of [3H]methyl group into the substrate DNA according to the procedures described elsewhere (9, 16) with a few modifications. Briefly, the following procedures were adopted for quantitative methyltransferase assays.

For procedure a, the methyltransferase reaction involving 50 nm 32P-DNA (1459-bp BstXI fragment from dX174 DNA) and 200 nm [3H]AdoMet (15 Ci/mmol) was carried out in a buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1 mM dithiothreitol, and 10% glycerol at 37 °C. The reaction mixture was withdrawn at intervals of 1 min for a total period of 30 min and added to a 300-µl slurry of DE52 (50% w/v in H2O). Prior to addition of the reaction mixture, a 1 ml solution of AdoHcy was mixed with the slurry in a 1:1 ratio to quench the methylase activity. The corresponding reaction pH.

**Determination of Kinetic Parameters**

Substrates for Determination of Kinetic Parameters—SalI, EcoRI, and SalI–HindIII fragments (121 bp) containing a single MspI-spe- scific site (5′-CCGG-3′), prepared from plasmid pBend2 (18), were used as DNA substrates. A third DNA substrate was prepared using XhoI digestion of pBend2 to produce a linear 121-bp fragment containing the 5′-CCGG-3′ sequence 19 bp from the 5′ end. This third substrate was different than the first two with regard to the position of the 5′-CCGG-3′ sequence along the molecule. Although the first two substrates had identical length as well as position of the 5′-CCGG-3′ along the molecule (70 bp from the 5′ end), they differed from one another with respect to bases flanking the canonical sequence. A larger substrate of 1459 bp was derived from dX174 DNA using BanI digestion (the 5′-CCGG-3′ sequence was 84 bp from the 5′ end). This substrate was cyclized (13) and digested with BstXI to obtain the same length DNA fragment with the 5′-CCGG-3′ position being 702 bp from the 5′ end. The hemi-methylated DNA was generated as follows. A synthetic oligonucleotide, 5′-CCTAG-3′, was ligated to one strand of the double-stranded 1459-bp BanI fragment to create size difference between the strands. It was subsequently methylated using excess of AdoMet and M.MspI. The strands of the modified as well as the unmodified 1459-bp fragments were separated on 5% polyacrylamide gel electrophoresis containing 6 M urea. The complementary strands were mixed in equimolar ratio to obtain hemi-methylated DNA. All the DNA substrates were purified from native polyacrylamide gel electrophoresis by electroelution in a dialysis bag (19) and reprecipitated. Their concentrations were determined spectrophotometrically (18) using aliquots after redissolving the precipitated DNA. The 1:1 annealing of the hemi-methylated substrate was confirmed by studying thermal denaturation/renaturation kinetics by determining the absorbance at 260 nm spectrophotometrically. Features of the various DNA substrates, obtained as above, are summarized in Table I.

**Kinetics of DNA Methylation Catalyzed by MspI Methylase**—The data from kinetic studies were analyzed as described by Wu and Santi (5). The FORTRAN program of Cleland (20, 21) was used in the current analysis. Kinetic studies were done using all of the DNA substrates that were prepared from plasmid pBend2 and dX174. The determination of initial velocity was made by performing the methyltransferase assay under conditions described earlier. In a series of identical reactions containing 0.03 nm MspI (specific activity ~145 × 10^6 units mg^-1) and 200 nm [3H]AdoMet, the concentration of the substrate DNA was varied in the range of 1–25 nm. A double-reciprocal plot of the initial velocity versus K_DNA and V_max. Similarly, initial velocities were obtained by varying the concentration of [3H]AdoMet in the range of 5–50 nm while keeping the DNA concentration fixed at 50 nm and keeping other reaction conditions identical. The double-reciprocal plot of initial velocity versus K_DNA and V_max. The catalytic constant (k_cat) was calculated as the ratio of V_max (0.1 nm min^-1) to the enzyme concentration used (0.03 nm), taking 49 KDa as the molecular mass of the M.MspI. The kinetic constants were also determined for intact and sonicated λ-DNA. While determining initial velocities, the product formation was measured under such conditions that overall inhibition of the reaction by AdoHcy (generated) was less than 5%. Thus the highest AdoHcy/AdoMet ratio allowed in the present experiments was −0.03.

**Product Inhibition Kinetics**—The 1459-bp BstXI fragment was used as the DNA substrate for the experiments pertaining to product inhibition kinetics. The methylated DNA (32P-DNA) fragments used for substrate inhibition studies were obtained by incubating 800 nm DNA with

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**Table I**

| Origin/source | Size | Fragment | Position of CCGG from the 5′ end |
|--------------|------|----------|---------------------------------|
| base pairs   |      |          |                                 |
| pBend2       | 121  | XhoI     | 19                              |
| pBend2       | 121  | SalI–EcoRI | 70                             |
| pBend2       | 121  | SalI–HindIII | 70                           |
| dX174        | 1459 | BanI     | 84                              |
| dX174        | 1459 | BstXI    | 702                             |
| dX174        | 1459:1464 | hemi-methylated | 702                         |
1.2 nM [3H]AdoMet in a reaction volume of 30 µl for a period of 100 min using 0.03 nM enzyme. After being extracted twice with chloroform it was precipitated with ethanol, dried, and used.

The product inhibition studies were performed under identical conditions as described for the quantitative MTase assay. Inhibition by AdoHcy was studied using 200 nM [3H]DNA while keeping the AdoHcy concentrations fixed (0.0, 2.5, 5.0, and 7.7 nM) and varying the concentration of substrate DNA from 1 to 25 nM for each of the fixed concentrations of AdoHcy. Similarly, another series of identical reactions included 50 nM substrate DNA (fixed concentration); AdoHcy concentrations fixed at 0.0, 1.0, 2.0, 3.0 nM and [3H]AdoMet concentrations varied in the range of 5–50 nM for each of the fixed concentrations of AdoHcy. The double-reciprocal plots of the initial velocity versus DNA ([3H]AdoMet concentrations were obtained at each concentration of the AdoHcy. These plots were used to determine the values of $V_{max}$ for DNA and for [3H]AdoMet by following the procedure described elsewhere (22).

For inhibition by [3H]DNA, the AdoHcy concentration was kept constant at 200 nM and that of the DNA was varied in the range of 1–20 nM against each of the chosen concentrations of the [3H]DNA as follows: 0.0, 2.5, 5.0, and 7.7 nM. Similarly, another series of identical reactions was carried out at the fixed concentration of DNA (50 nM) and different concentrations of [3H]AdoMet ranging from 5 to 100 nM against each of the fixed concentrations of the [3H]DNA as follows: 0.0, 3.0, and 7.0 nM. The data from these experiments were used to generate double-reciprocal plots of initial velocity versus variable substrate to determine the values of $K_{mDNA}$ for DNA and for AdoMet.

**Results**

**Kinetics of DNA Methylation Catalyzed by the MspI DNA Methyltransferase**—The MspI preparations purified to apparent homogeneity, as judged by SDS-polyacrylamide gel electrophoresis, were used for the kinetic analysis. These preparations displayed optimal activity at a pH value of 7.5 and at the temperature of 36 ± 1 °C. An activation energy of 15.7 kJ mol$^{-1}$ was obtained for the enzyme from the Arrhenius plot ($ln/V_{max}$ versus $1/T$; data not shown). The kinetics of methyl transfer on the C-5 of the target base (outer cytosine in the 5′-CCGG-3′ sequence) by the MspI has been investigated using a variety of substrates, which differed from one another with respect to (i) length in base pairs, (ii) position of the target sequence from the left end in the linear molecule, (iii) bases flanking the canonical sequence, and (iv) the methylation state of the strands. These variations in substrates allowed an examination of the kinetic parameters as affected by them and would further be helpful in elucidating the kinetic mechanism for the MspI.

**Progress Curve for the Methylation Reaction and Its Analysis**—The progress curve of the MspI-catalyzed methylation of the 1459-bp fragment was determined in order to evaluate the period during which the rate of product formation remains linear. The progress curve was obtained by measuring the amount of product formed at different time intervals. A plot of the methylated DNA product versus time was obtained. The MspI-catalyzed methylation of the 1459-bp fragment by AdoMet progressively decreases as the reaction proceeds (Fig. 1A). The inhibition appears to be competitive with respect to AdoMet, and the nonlinear kinetics is consistent with competition by the AdoHcy generated in the reaction.

The progress curves of the MspI-catalyzed methylation reaction fits Equation 1,

$$P' = \frac{V_{max}}{1 - (K_{d}/K)} - \frac{K_{d}(S + K)}{K_{m} - K} \ln S_{0} - P$$

(Eq. 1)

which describes a reaction wherein the product, AdoHcy, shows competitive inhibition with respect to the substrate, AdoMet (23). $P$ is the amount of AdoHcy formed at time $t$, $S_{0}$ is the initial AdoMet concentration, and $K$ 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 $\ln S_{0}/(S_{0} - P)$ provide a series of lines with positive slopes (Fig. 1B) indicating that the $K_{d}$ of AdoMet is much larger than $K$.

**Determination of Kinetic Parameters**—The kinetic parameters ($K_{M} \text{DNA}$, $K_{M} \text{AdoMet}$, and $V_{max}$) were estimated from the double-reciprocal plots of initial velocity versus substrate concentration. The catalytic constant ($k_{cat}$) was calculated as the ratio of initial velocity to that of protein concentration. The specificity constant was obtained as $k_{cat}/K_{M}$ values. The catalytic constant and turnover number for the MspI are synonymous as it is envisaged to have only one catalytic site per protein molecule. Values for all the kinetic parameters for different DNA substrates and AdoMet have been listed in Table II.

**Reverse Methylation and Catalysis of Tritium Exchange by MspI—**

A tritiated DNA was prepared by employing the reverse reaction of MspI methylase in the presence of 400 nM AdoHcy for a period of 5 min. The reaction mixture at 70 °C for 5 min. The DNA was recov-
Dependence of Initial Velocity on DNA and AdoMet Concentrations—These experiments were performed to determine the effect of different concentrations of DNA and AdoMet on the initial velocity. The initial velocity data also allowed a determination of the substrate inhibition of AdoMet on DNA and substrate inhibition of DNA on AdoMet, respectively (Fig. 2, A and B). Initial velocity data were determined at various concentrations of [3H]AdoMet and DNA (1459-bp BstXI fragment). The double-reciprocal plot of 1/V versus 1/DNA concentration (1459-bp BstXI) gave a series of lines intersecting close to but not on 1/V axis (Fig. 2A) and vice versa (Fig. 2B). These data therefore establish that AdoMet exerts noncompetitive substrate inhibition on DNA and vice versa. These data, therefore, suggest that a Ping-Pong mechanism is unlikely. All data were fit to PING-PONG, EQUARD and SEQUEN programs. However, the data were best fit to SEQUEN program, consistent with ordered steady state or random rapid equilibrium mechanisms.

Product Inhibition Kinetics—Product inhibition analyses were used to distinguish between the ordered and the random mechanisms. The double-reciprocal plots of initial velocity versus concentrations of DNA with respect to different fixed concentrations of methylated DNA yielded a series of lines intersecting at the y axis (Fig. 3A). The methylated DNA inhibition is, thus, competitive with respect to varying DNA as substrate. The methylated DNA inhibition with respect to varying AdoMet yielded a series of lines that intersect to the left of the y axis, indicating inhibition to be noncompetitive (Fig. 3B). The AdoHcy inhibition with respect to varying AdoMet concentration yielded a series of lines intersecting at the y axis (Fig. 3C). The AdoHcy inhibition is therefore competitive with respect to AdoMet. The AdoHcy inhibition with respect to varying DNA concentration yielded parallel lines (Fig. 3D) and is therefore uncompetitive with respect to DNA. The inhibition constant was obtained by equating the intersection point to \((1 + [I]/K_i)\) where [I] is concentration of the inhibitor. In the case of the uncompetitive inhibition (Fig. 3D), each point intersecting the initial velocity axis was equated to the above relationship to obtain the inhibition constant. The product inhibition analysis has been summarized in Table III.

Presteady State Isotope Partitioning Studies—Presteady state kinetic analysis was used to elucidate which steps limit the catalytic turnover. Preincubation of MspI with 2 \(\mu\)M labeled DNA results in a “burst” of product formation upon reaction initiation with addition of AdoMet [methyl-\(^3\)H]AdoMet and labeled DNA (Fig. 4, line A). The burst is followed by a constant rate of product formation (0.06 mol of meDNA per mol of enzyme s\(^{-1}\)) similar to catalytic turnover constant under steady state conditions (Fig. 4, line A). A decreased burst size was observed when unlabeled DNA and AdoMet [methyl-\(^3\)H] AdoMet was substituted in the chase. This burst is, however,
much smaller in size than that observed in A (Fig. 4, line B). The data indicate that the M.MspI-DNA complex is kinetically competent and preclude any kinetic mechanism that requires AdoMet to be bound first. The methyl transfer, as evident from presteady state analysis, precedes rate-limiting step(s). The DNA methylation is followed by a slower step (or steps), and the slower step is detected as the catalytic rate constant.

Reverse Reaction and Catalysis of Tritium Exchange by the MspI Methylase—The reverse reaction analysis was made to determine if the M.MspI catalyzes transfer of a methyl group from the [methyl-3H]DNA to AdoHcy. Within the sensitivity limits of our detection, we could not detect any transfer of a methyl from DNA to AdoHcy over a period of 100 min. Therefore, as far as our present investigation is concerned, the M.MspI-catalyzed reaction is irreversible under the experimental conditions described here.

The MspI methylase-catalyzed exchange of tritium from C-5 of the target cytosine with the hydrogen of water has been examined in the absence of AdoMet. Tritium exchange from the [3H]DNA substrates by the M.MspI was recorded in these experiments. The kinetic parameters for the M.MspI-catalyzed tritium exchange were obtained in a manner identical to that for methylation and are listed in Table IV. Evidently, the rate of tritium exchange is 3.3 times faster compared with the rate of target base methylation. The \( k_{\text{cat}}/K_M \) value of 7.4 \( \times \) \( 10^7 \) M\(^{-1}\) s\(^{-1}\) represents a minimum value for the association of DNA (Fig. 5 and Table IV). This tritium exchange demonstrates that the MspI MTase is capable of interacting with DNA in the absence of AdoMet. However, AdoMet when present, inhibits the tritium exchange (Fig. 6A). Furthermore, the velocity of tritium exchange is affected by AdoHcy (Fig. 5 and Fig. 6B).

**DISCUSSION**

**Substrate Variability and the Kinetic Properties of the MspI Methylase**—The MspI prefers the long chain substrates over the short chain ones, as revealed by the relative affinities in...
terms of the values of kinetic parameters (Table II). The relative preferences for a long chain over short chain molecules of DNA as substrates have been observed with other type II MTases as well. For example, the EcoRI MTase has a $K_M$ of 0.346 nM for the linear pBR322 compared with 2.44 nM for a 14-mer oligonucleotide (10), and the BamHI MTase has $K_M$ values of 3.2 and 11.2 nM with NdeI-linearized and EcoRI-linearized molecules of pBR322, respectively (24). The position of the target base along the linear DNA molecule also appears to affect the affinity of the M. MspI for the substrate. It was more evident with the 1459-bp substrate where the affinity for the centrally located target (BstXI fragment; $K_M^{DNA} = 2.28 \pm 0.03$ nM) was nearly twice compared with that when it was located toward the end (BanI fragment; $K_M^{DNA} = 4.34 \pm 0.18$ nM). For the EcoRI methylase, an increase in the values of $k_{cat}/K_m$ with increase in the length of DNA or with increase in the distance from the site to the nearest end was observed earlier (10, 25). Whereas length of the substrate DNA and the position of the recognition sequence along the molecule had significant affect on the MTase affinity, the bases flanking the canonical sequence did not apparently contribute toward enzyme affinity for the substrate since similar values for the kinetic constants were recorded for the SstI-EcoRI and the Sall-HindIII fragments (121 bp) in the M. MspI-catalyzed reaction (Table II). Furthermore, the methylation status of the recognition sequence had a profound effect on the kinetic parameters as well since a hemi-methylated 1459-bp fragment proved a better substrate for the enzyme. The higher affinity (1.85 nM) for hemi-methylated substrates, reported here, is in agreement with the previous observations (26, 27). This increased affinity for the hemi-methylated DNA, however, does not lead to an enhanced rate of methyl transfer. The methylation reaction of the M. MspI with intact λ-DNA yielded a catalytic constant of 0.17 s$^{-1}$ which was higher compared with the value of 0.056 s$^{-1}$ recorded with sonicated λ-DNA (Table II). The kinetic parameters obtained with the sonicated λ-DNA is comparable to those obtained with 1459-bp fragment. The kinetic behavior of the M. MspI with the intact and the sonicated λ-DNA suggested a processive mode of action for this enzyme rather than a distributive mode. This is in agreement with a previous report that, based on similar experiments, concluded that cytosine methylase isolated from rat liver possessed a processive mechanism (28).

**Kinetic Mechanism for the MspI Methylase**—The large specificity constants (of the order of $10^5$) for the M. MspI-catalyzed methylation are inconsistent with any random mechanism (where specificity constants are of the order of $10^3$) and suggest an ordered steady state mechanism (22). The methylated DNA in the M. MspI-catalyzed reaction inhibited DNA competitively and AdoMet in a noncompetitive/mixed manner with inhibition constants of 3.13 ± 0.05 and 2.78 ± 0.58 nM, respectively. The AdoHcy acted as a competitive inhibitor of AdoMet in the methylation reaction with $K_i$ value of 1.55 ± 0.2 nM. The competitive inhibition of AdoMet by AdoHcy is in accordance with the previous observations in the cases of M. HhaI and M. BsuRI with inhibition constants of 2.1 ± 0.1 nM (9) and 0.13 μM (29), respectively. The AdoHcy inhibition of DNA in the M. MspI-catalyzed reaction was competitive with inhibition constant of 1.51 ± 0.09 nM. The product inhibition pattern, summarized in Table III, is consistent with a steady state ordered mechanism in which DNA has to bind first. This argument requires AdoHcy to form a tight complex with the enzyme-DNA complex that has been previously reported (26). The mixed plot is envisaged for a random rapid equilibrium approach or for a random steady state mechanism; the competitive plot is not consistent with either of these mechanisms. All the kinetic data for the M. MspI-catalyzed methylation are consistent and best fit by the SEQUEN program of Cleland (21). Applying the statistical criteria of Cleland, the standard deviations are least for the SEQUEN program. In the pre-steady state analysis, preincubation of M. MspI with DNA results in a burst of product upon addition of AdoMet. The burst is followed by a constant rate of product formation, which is similar to the catalytic constant measured under steady state conditions. Thus, starting from an MTase-substrate complex, the rate constant for product formation is significantly larger during the first few seconds than the catalytic rate constant. The simplest interpretation of the data is that a slower step or steps follow DNA methylation, which is detected as the catalytic rate constant.

**Triptium Exchange Catalysis by the M. MspI**—The tritium exchange reaction catalyzed by the M. MspI indicated that the enzyme was capable of performing catalysis at the C-5 of the target cytosine even in the absence of the co-substrate AdoMet. Interestingly, the M. HpaII, an isoschizomer of the M. MspI, does not perform this reaction (9). Addition of a nucleophile to C-6 of a pyrimidine generates a carbanion equivalent at C-5 that upon protonation by water gives the 5,6-dihydropyrimidine adduct (the Michael Adduct). Reversal of this reaction can lead to exchange of the 5-H of the pyrimidine for a water proton. The general mechanism of Michael adduct formation

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**TABLE IV**

| Parameter | Value |
|-----------|-------|
| $V_{max}$ | 0.33 ± 0.08 nM min$^{-1}$ |
| $k_{cat}$ | 0.185 s$^{-1}$ |
| $K_M$ | 2.5 ± 0.38 nM |
| $k_{cat}/K_m$ | 7.4 x 10$^{-7}$ M$^{-1}$ s$^{-1}$ |

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**FIG. 5.** Double-reciprocal plots of the rates of $5^3$H exchange versus concentration of the substrate DNA (1459-bp BstXI fragment) at different fixed concentration of AdoHcy. Reaction (30 μl) contained 1459-bp DNA, 0.03 nM MspI, and indicated amounts of AdoHcy. After incubation, samples were withdrawn and analyzed as described in "Experimental Procedures."
has been demonstrated for five enzymes (TS, dUMP hydroxymethylase, dCMP hydroxymethylase, m5C-DNA methyltransferase:M.HhaI, and tRNA-uracil methyltransferase) which catalyze electrophilic displacement reactions at C-5 of the pyrimidine. Hydrogen exchange has been observed for a number of enzymes that serve as models for enzymatic reactions involved in the formation of such intermediates (9, 30, 31). The stereoechemical consequences of 5-3H exchange reaction are important, since most enzymes are stereospecific. If C-5 of one face of the pyrimidine were protonated to form the adduct, the proton removed emanates from the original 5-H face of the pyrimidine, and the outgoing proton mimics that of the normal electrophile of the reaction, in which the stereochemical addition of the entering solvent and deprotonation may be nonstereospecific. Here, the observed rate of exchange will be slower than the true rate of formation/reversal of the dihydropyrimidine adduct. (ii) The observed rate of exchange could be maintained (30). The rate of tritium exchange for tRNA-uracil-5-methyltransferase is 100-fold slower than the methylation reaction (31). With M.MspI, the kcat of tritium exchange is 7-fold greater than that for methylation (9). In the case of M.MspI the kcat of tritium exchange is about 3.3 times that of methylation. In view of the faster tritium exchange compared with methylation, a detailed stereoechemical understanding of exchange is required for these m5C-MTases.

The investigation of the kinetic mechanism of M.MspI assumes significance since this enzyme displayed important differences when compared with other members of the m5C-MTase family and also in view of the fact that its isoschizomer M.HpaII does not catalyze the tritium exchange.

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