Contribution of Facilitated Diffusion and Processive Catalysis to Enzyme Efficiency: Implications for the *Eco*RI Restriction−Modification System†

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ABSTRACT: The contribution of nonspecific DNA to enzyme efficiency (k_{cat}/K_m) is described for a sequence-specific DNA-modifying enzyme. Our investigation focuses on the *Eco*RI DNA methyltransferase which transfers a methyl group from the cofactor *S*-adenosylmethionine to the second adenine in the double-stranded DNA sequence GAATTC. k_{cat}/K_m increases 4-fold as DNA length increases from 14 to 429 base pairs and increases 2-fold as the distance from the site to the nearest end is increased from 29 to 378 base pairs. No changes in k_{cat}/K_m result from further increases in either case. A facilitated diffusion mechanism is proposed in which the methyltransferase scans an average of <400 base pairs prior to dissociation from a DNA molecule. The methyltransferase was found to methylate two sites on a single DNA molecule in a distributive rather than a processive manner, suggesting that the enzyme dissociates from the DNA prior to release of the reaction product *S*-adenosylhomocysteine. A direct competition experiment with the *Eco*RI endonuclease shows the methyltransferase to be slightly more efficient at specific site location and catalysis. A rationale for the role of facilitated diffusion in this type II restriction−modification system is proposed.

The *Eco*RI DNA methyltransferase (MTase)[1] is an extremely efficient DNA-modifying enzyme, with an apparent second-order association rate constant (k_{cat}/K_m) of 4.1×10^8 s^{-1} M^{-1} when a linearized 4361 base pair (bp) plasmid (pBR322) containing a single, centrally located *Eco*RI site is used as a substrate (Reich & Mashhoon, 1991). This rate constant is 8-fold higher than that observed with a 14 bp oligonucleotide. Placement of the specific site within a large excess of nonspecific DNA should inhibit a three-dimensional search by the MTase to some extent due to the overall structural similarity between the nonspecific DNA and the target sequence. These results suggest that the mechanism of specific target site binding is not limited to a standard three-dimensional search.

A plausible mechanism invokes a reduction in the dimension of the search process from three to one (von Hippel & Berg, 1989); nonspecific binding to any point on the DNA is followed by movement along the DNA until the target sequence is located or the protein dissociates. The effective target size of the DNA is increased to that which is typically "scanned" during a single binding event, enhancing the association rate constant for specific site binding. One-dimensional facilitated diffusion of this type is utilized by several proteins that interact with DNA, including *lac* repressor (Riggs et al., 1970), *Eco*RI endonuclease (ENase) (Jack et al., 1982), RNA polymerase (Ricchetti et al., 1988), *Bam*HI ENase (Nardone et al., 1986), T4 ENase V (Dowd & Lloyd, 1990), and the T4 late enhancer complex (Herendeen et al., 1992). The MTase is essentially diffusion limited in terms of its catalytic efficiency, with a k_{cat}/K_m of 5.1×10^7 s^{-1} M^{-1} for a 14 bp DNA substrate (Reich & Mashhoon, 1991) and a predicted rate constant of 5×10^7 s^{-1} M^{-1} for the diffusion-limited collision of the MTase and its target sequence on DNA.[2] Therefore, k_{cat}/K_m represents the second-order association rate constant for specific target location by the MTase (Fersht, 1985). Observation of an increase in the association rate constant with an increase in DNA length is typically used to implicate a facilitated diffusion mechanism [Berg et al. (1981) and Winter et al. (1981)], making the MTase an ideal candidate for such a mechanism. MTase binding to nonspecific DNA can lead to a catalytically competent ternary complex at the specific site (Reich & Mashhoon, 1991), further supporting the potential importance of interactions between the MTase and nonspecific DNA.

The MTase is part of a type II restriction modification system and catalyzes the transfer of a methyl group from *S*-adenosylmethionine (AdoMet) to the N^6 of the second adenine in the double-stranded DNA sequence 5′-GAATTC-3′, making the site resistant to cleavage by the *Eco*RI ENase

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[1] Abbreviations: MTase, methyltransferase; bp, base pair(s); AdoMet, *S*-adenosylmethionine; ENase, endonuclease.

[2] The association rate constant was calculated using the following equation (von Hippel & Berg, 1989):

$$k = 4\pi\kappa af(D_{MTase} + D_{DNA})N_0/1000 \quad (1)$$

where κ is a unitless steric interaction factor, a is the interaction radius, f is a unitless electrostatic factor, D_{MTase} and D_{DNA} are diffusion constants for the two molecules, and N_0 is Avagadro's number. We used the cited estimates of κ, a, f, and D_{DNA}, while D_{MTase} was calculated using the following equation (Freifelder, 1982):

$$D_{MTase} = \frac{RTs}{M(1 - \bar{v}\rho)} \quad (2)$$

in which R is the gas constant, T is the absolute temperature, s is the sedimentation coefficient, M is the molecular mass of the MTase, $\bar{v}$ is the partial specific volume of the MTase, and ρ is the solution density. The following values from Rubin and Modrich (1977) were used: $s_{20,w} = 3.0$, $\bar{v} = 0.74$, and $M = 38\,000$ Da.

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(Rubin and Modrich, 1977). The ENase has been extensively characterized with regard to its use of facilitated diffusion (Jack et al., 1982; Terry et al., 1983, 1985). By examining the differences between the ENase and the MTase in terms of facilitated diffusion, some insight into how they function as a system might be attainable.

Analysis of k_{cat}/K_m for DNA of intermediate lengths between the 14 bp substrate and the full-length plasmid (4361 bp) enabled the characterization of the number of base pairs scanned during a typical binding event. Additionally, this information was used to quantitatively address the contribution of facilitated diffusion to enzyme efficiency. We also tested whether the MTase, once bound to DNA, will methylate multiple recognition sites processively and the ability of the MTase to compete with the ENase for the same DNA substrates at various ionic strengths. This work is supplemented by a further investigation of facilitated diffusion of the MTase under noncatalytic conditions as a more direct comparison with previous investigations of other protein-DNA systems (M. A. Surby and N. O. Reich, accompanying manuscript).

MATERIALS AND METHODS

Materials. *Eco*RI ENase and MTase were purified from *Escherichia coli* strain MM294 harboring plasmid pPG440 (Greene et al., 1978). Phosphoramidites and ancillary DNA synthesis reagents were obtained from Milligen/Biosearch. [γ-^{32}P]ATP (6000 Ci/mmol) was purchased from Amersham and [*methyl*-^{3}H]AdoMet (73.0 Ci/mmol) was from New England Nuclear. *Eco*RI, *Bam*HI, *Hin*dIII, *Pvu*II, and *Eco*RV Enases were purchased from Promega, and *Aat*II, *Ssp*I, and *Xmn*I ENases were from New England Biolabs.

DNA Substrates. Synthetic oligonucleotides were prepared on a Biosearch 3810 DNA synthesizer using β-cyanoethyl phosphoramidites and purified by C_{18} reverse-phase HPLC (Becker et al., 1985). The concentrations of single strands were determined spectrophotometrically. Confirmation of the double-stranded form was through autoradiography with nondenaturing polyacrylamide gel electrophoresis. The melting temperature (T_m) of the 14 bp DNA substrate that contained the flanking sequence of the *Eco*RI site from plasmid pBR322 was determined as described previously (Reich & Danzitz, 1991). Plasmids pBR322, pBR322$(RI)_2$ (Terry et al., 1985), and pBR322(ΔRI), were isolated from *E. coli* strain MM294 by alkaline lysis and were purified using QIAGEN maxi-prep columns [*E. coli* transfected with pBR322$(RI)_2$ was a gift from P. Modrich, Duke University]. pBR322(ΔRI) is a modified version of pBR322 from which the *Eco*RI site was removed. The pBR322(ΔRI) vector was prepared by linearizing with *Eco*RI ENase and removing the overhanging 5′-ends with mung bean nuclease; the linear plasmid was then circularized by blunt-end ligation and used to transform bacteria for large-scale plasmid isolation. The removal of the *Eco*RI site was confirmed by restriction analysis. Plasmid DNA concentrations were determined spectrophotometrically. Restriction digests were performed using the suppliers' buffers supplemented with bovine serum albumin (BSA). Double digests were performed either sequentially or in a buffer which optimized the activity of both enzymes. ENases were heat inactivated, and no further purification was performed.

K_m, k_{cat}, and K_i Determinations. The MTase catalyzed incorporation of tritiated methyl groups into DNA was monitored by using an ion-exchange filter binding assay (Rubin & Modrich, 1977). Reactions contained 100 mM Tris, pH 8.0, 10 mM EDTA, 200 μg of BSA/mL, and 10 mM dithiothreitol. DNA concentrations typically ranged from 0.5 to 10 times K_m, and MTase and AdoMet concentrations were 0.05 nM and 1.5 μM, respectively. The MTase was diluted in a buffer containing 20 mM potassium phosphate, pH 7.4, 200 mM NaCl, 0.2 mM EDTA, 200 μg of BSA/mL, 2 mM dithiothreitol, and 10% (v/v) glycerol. The diluted enzyme was added as 1/10 the volume of the final reaction mixture. The ionic strength of the assay mixture was determined to be 0.05 by comparison of the conductivity with a standard curve using solutions of known NaCl concentration. The total time of each reaction was 4 min. [*methyl*-^{3}H]AdoMet was 35 000−70 000 cpm/pmol. All K_m and k_{cat} data were determined on a Macintosh IIci using the KinetAsyst (IntelliKinetics, State College, PA) software package. The K_i for nonspecific DNA was determined by examining the amount of methyl group incorporation for a constant concentration of 14 bp DNA (3 nM) in the presence of various concentrations of plasmid pBR322-(ΔRI) (0.03 to 6 nM). The K_i of the plasmid was then determined with a Dixon plot of 1/velocity versus [I] (Segel, 1975).

Processive Catalysis Assay. This is a modified version of the assay used to demonstrate processive DNA cleavage by the *Eco*RI ENase (Terry et al., 1985). The DNA substrate for this assay was pBR322$(RI)_2$, which is a pBR322 derivative in which a second *Eco*RI site with 20 bp of identical flanking sequence on either side, has been inserted so that it is 51 bp from the original *Eco*RI site (Terry et al., 1985). The plasmid was cut with *Xmn*I and *Bam*HI ENases to produce a 975 bp fragment containing the two *Eco*RI sites. This fragment (21 nM) was incubated with 0.1 nM MTase and 3 μM AdoMet for varying lengths of time (0−10 min). The reaction was stopped by the addition of 10 nM *Eco*RI ENase and 10 mM Mg^{2+} to cleave any unprotected sites, and the resulting fragments were run on a 5% polyacrylamide gel. Controls showed complete cleavage of unmethylated DNA within 30 seconds and no cleavage of methylated DNA. The gel was stained with ethidium bromide and photographed with type 665 Polaroid film. The resulting negative was then scanned with an LKB laser densitometer to determine the relative DNA quantities in each band. DNA standards of known concentration were used to demonstrate a linear densitometric response over the expected concentration range. The assay was also performed using the *Eco*RI ENase with the modification that the reaction was stopped by the addition of the following components to these final concentrations: 8.5% glycerol, 0.2% SDS, and 8.5 mM EDTA.

ENase:MTase Competition Assay. Reaction vials containing 6 mM Tris, pH 7.5, 6 mM $MgCl_2$, 100 mM NaCl, 6 mM β-mercaptoethanol, 200 μg of BSA/mL, 40 μM AdoMet, 10 nM ENase, and 0−10 nM MTase were prepared. The ENase and MTase were diluted in a buffer containing 20 mM potassium phosphate, pH 7.4, 200 mM NaCl, 0.2 mM EDTA, 200 μg of BSA/mL, 2 mM dithiothreitol, and 10% (v/v) glycerol. The diluted enzymes were each added as 1/10 the volume of the final reaction mixture. To start the reaction, a ^{32}P-labeled 775 bp *Bam*HI/*Xmn*I restriction fragment from pBR322 containing a single *Eco*RI site was added to this mixture to a final concentration of 0.1 nM. The ionic strength of the assay mixture was determined to

Table 1: Kinetic Parameters Obtained with DNA Substrates with Different Flanking Sequences

substrate	k_{cat} (s^{-1})[a]	K_m (nM)[a]	$10^{-8} \times k_{cat}/K_m$ (s^{-1} M^{-1})
C,[b] 10 °C	0.12 (0.03)	1.4 (0.3)	0.86
CPBR,[b] 10 °C	0.08 (0.02)	3.0 (0.8)	0.27
pBR322, 10 °C	0.04 (0.01)	<0.2[c]	>2.0[c]
C, 37 °C	0.58 (0.07)	25 (4)	0.23
pBR322, 37 °C	0.23 (0.02)	1.3 (0.2)	1.8

[a] All values are apparent values determined under the following conditions: 100 mM Tris, pH 8.0, 10 mM EDTA, 200 μg of BSA/mL, and 10 mM dithiothreitol. DNA concentrations varied from 0.5 to 10 times K_m at a saturating AdoMet concentration of 1.5 μM and 0.05 nM MTase (Reich & Mashhoon, 1991). Values in parentheses are standard errors resulting from at least five determinations. [b] C and CPBR are 14-base pair synthetic oligonucleotides of the following sequences: C, 5′-GGCGGAATTCGCGG-3′, and CPBR, 5′-TCAAGAATTCTCAT-3′. [c] DNA concentrations required to determine the K_m were too low to determine an accurate value using this assay.

be 0.16 by comparison of the conductivity with a standard curve using solutions of known NaCl concentration. The solution was incubated at 37 °C for 1 min, and the reaction was stopped by the addition of the following components to these final concentrations: 8.5% glycerol, 0.2% SDS, and 8.5 mM EDTA. The solution was then incubated at 70 °C for 30 min to disrupt any MTase·DNA interactions at the higher MTase concentrations. Each sample was then loaded onto a 6% polyacrylamide gel that was run at 300 V for 2 h, dried, and exposed to X-ray film with an intensifying screen at −70 °C. The exposed film was scanned with an LKB Ultrascan XL laser densitometer to determine the relative quantities of uncut and cut DNA.

RESULTS

Enhancement in k_{cat}/K_m Observed for pBR322 Is Not Due to the Local Flanking Sequence around Its EcoRI Site. Although our initial observation that a 14 bp DNA substrate is modified 8-fold less efficiently than a 4361 bp plasmid containing a single *Eco*RI site (Reich & Mashhoon, 1991) suggests the use of facilitated diffusion, this change could plausibly be accounted for by differences in local flanking sequences. We therefore compared a 14 bp DNA substrate (CPBR) matching the sequence of the *Eco*RI site from pBR322 including the four bases to either side (5′-TCAAGAATTCTCAT-3′) with that of the 14 bp long DNA (C) used in the previous experiments (5′-GGCGGAATTCGCGG-3′) (Reich & Mashhoon, 1991) and with pBR322 linearized by *Pvu*II so that the *Eco*RI site was centrally located. We determined the T_m of CPBR to be 32 °C (data not shown); therefore, it was decided to perform the assay at 10 °C with this substrate. Assays using C and pBR322 were run at both 10 and 37 °C to characterize their differences under both conditions. The results in Table 1 show that CPBR does not have the enhanced specificity of pBR322, and, in fact, appears to be slightly less efficiently methylated than C. C and pBR322 display similar differences in both k_{cat} and K_m at both temperatures, suggesting that 10 °C is an appropriate temperature to compare C and CPBR.

K_i for a Single Nonspecific DNA Binding Site is 3 μM. The addition of nonspecific DNA might be expected to inhibit a search for a specific site due to the limited structural variability of DNA. To determine the magnitude of any possible inhibitory effect due to the presence of nonspecific

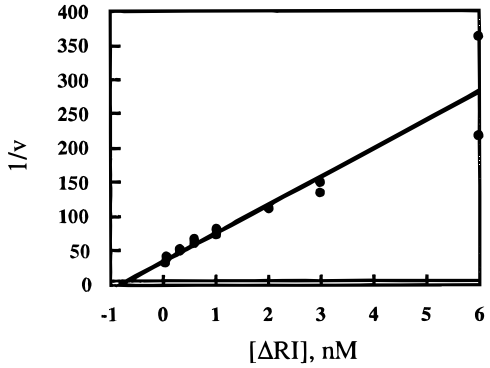

FIGURE 1: Dixon plot of the effect of pBR322(ΔRI) concentration on the observed velocity of 14 bp DNA methylation. All velocities were determined at 37 °C with 0.05 nM MTase, 3 nM 14mer, 1.5 μM AdoMet, varying concentrations of pBR322(ΔRI), and the following buffer conditions: 100 mM Tris, pH 8.0, 10 mM EDTA, 200 μg of BSA/mL, and 10 mM dithiothreitol. The horizontal line near the abscissa corresponds to $1/V_{max}$ of the 14mer under noninhibitory conditions. The intersection of the two lines yields a K_i for pBR322(ΔRI) of 0.7 nM.

DNA, methylation assays were performed with 3 nM C in the presence of various concentrations of a pBR322 derivative lacking the *Eco*RI site (ΔRI). The Dixon plot of this data is shown in Figure 1. The intercept of the plot of $1/v$ versus [ΔRI] and $1/V_{max}$ of C yields a K_i for ΔRI of 0.7 nM. This can be converted to a K_i for nonspecific DNA of 3 μM per nonspecific binding site, assuming that each bp of the plasmid can contribute to a new binding site. A similar K_i was determined when this experiment was repeated with ΔRI that had been digested into three fragments with *Pvu*I and *Xmn*I (data not shown), demonstrating that the inhibition was dependent upon the number of base pairs rather than the number of nonspecific DNA fragments, and validating our calculation of the K_i for a single nonspecific binding site.

Enhancement in k_{cat}/K_m Derives from Decreases in K_m. In order to determine the length dependency of the changes in k_{cat}/K_m, substrates of intermediate length were analyzed in addition to the previously investigated 14 and 4361 bp long DNA's. Several pBR322 derived restriction fragments were generated by first digesting pBR322 with *Hin*dIII so that the *Eco*RI site was always located 29 bp from one end while the distance to the other end was dependent upon the second restriction ENase used. The DNA substrates were not purified away from fragments lacking the *Eco*RI site created in the digestion. In this way, the number of nonspecific sites was the same in all experiments and any differences were due to changes in the chain length of the *Eco*RI site containing substrate. Any inhibition resulting from the nonspecific fragments would be manifested as contaminating competitive inhibition, which results in equivalent decreases in both k_{cat} and K_m and would therefore have no effect on k_{cat}/K_m (Segel, 1975).[3] The results of these experiments are seen in Table 2. There is a continuous increase in k_{cat}/K_m as the length is increased from 14 to 429 bp with no further enhancement as the substrate size changes to full-length *Hin*dIII linearized pBR322. The value of this enhancement is approximately 4-fold based upon comparison of the experimental means. The incremental changes in k_{cat}/K_m at intermediate DNA lengths support facilitated diffusion, while the leveling of the effect at 429 bp suggests a short average scan length (<400 bp) by the MTase.

Adding up to 375 bp to Both Sides of the Cognate Site Further Enhances k_{cat}/K_m. The above assay was modified

Table 2: Kinetic Parameters Obtained with DNA Substrates of Varying Lengths

substrate[a]	k_{cat} (s^{-1})[b]	K_m (nM)[b]	$10^{-8} \times k_{cat}/K_m$ (s^{-1} M^{-1})
14 bp	0.58 (0.07)	25 (4)	0.24 (0.05)
*Hin*dIII/*Aat*II, 106	0.25 (0.07)	5.9 (0.9)	0.42 (0.14)
*Hin*dIII/*Ssp*I, 222	0.16 (0.04)	2.1 (0.6)	0.76 (0.29)
*Hin*dIII/*Xmn*I, 429	0.25 (0.04)	2.4 (0.5)	1.0 (0.27)
*Hin*dIII/*Pvu*II, 2326	0.37 (0.02)	3.7 (0.9)	1.0 (0.25)
*Hin*dIII, 4361	0.30 (0.03)	3.2 (0.8)	0.94 (0.25)

[a] Oligonucleotides are designated by size while pBR322 derived restriction fragments are designated by the restriction ENases used to create them, followed by their size in bp. The 14-bp substrate is a double-stranded synthetic oligonucleotide with the sequence 5′-GGCG-GAATTCGCGG-3′. [b] All values are apparent values determined at 37 °C under the following conditions: 100 mM Tris, pH 8.0, 10 mM EDTA, 200 μg of BSA/mL, and 10 mM dithiothreitol. DNA concentrations varied from 0.5 to 10 times K_m at a saturating AdoMet concentration of 1.5 μM and 0.05 nM MTase (Reich & Mashhoon, 1991). Values in parentheses are standard errors resulting from at least five determinations.

Table 3: Kinetic Parameters Obtained with Linear pBR322 Substrates in which the *Eco*RI Site is Positioned at Various distances from the Nearest End

substrate[a]	k_{cat} (s^{-1})[b]	K_m (nM)[b]	$10^{-8} \times k_{cat}/K_m$ (s^{-1} M^{-1})
*Hin*dIII, 29	0.30 (0.03)	3.2 (0.8)	0.94 (0.25)
*Eco*RV, 185	0.18 (0.03)	1.6 (0.4)	1.1 (0.34)
*Bam*HI, 375	0.20 (0.02)	1.1 (0.3)	1.8 (0.53)
*Pvu*II, 2064	0.23 (0.02)	1.3 (0.2)	1.8 (0.31)

[a] All substrates are of equal length (4361 bp) and are listed by the restriction ENase used to linearize pBR322, followed by the distance from the *Eco*RI site to the nearest end in bp. [b] All values are apparent values determined at 37 °C under the following conditions: 100 mM Tris, pH 8.0, 10 mM EDTA, 200 μg of BSA/mL, and 10 mM dithiothreitol. DNA concentrations varied from 0.5 to 10 times K_m at a saturating AdoMet concentration of 1.5 μM and 0.05 nM MTase (Reich & Mashhoon, 1991). Values in parentheses are standard errors resulting from at least five determinations.

slightly in order to verify that the average scan length of the MTase is <400 bp. Substrates were prepared with stepwise increases in DNA length between the recognition site and the nearest end (Table 3). Once the distance from the specific site to the nearest end exceeds the average scanning distance, any enhancement should level out in a manner similar that observed with the substrates in Table 2. The maximal effect that could possibly be observed from this experiment is an enhancement of 2-fold due to a doubling of the DNA which results in a productive binding event. As can be seen in Table 3, there is a small overall enhancement in the k_{cat}/K_m observed between pBR322 linearized with *Hin*dIII and with *Pvu*II; no further enhancement is observed with substrates in which the *Eco*RI site is located farther than 375 bp from the nearest end. This clearly supports a short scan length of <400 bp for the MTase on DNA. In addition, when k_{cat}/K_m for the 14 bp substrate is compared with that for pBR322 linearized with *Pvu*II, there is an overall increase of 8-fold that is consistent with our earlier results (Reich and Mashhoon, 1991).

MTase Does Not Methylate Multiple Sites on the Same DNA Molecule in a Processive Fashion. An enzyme that can scan nonspecific DNA could potentially perform catalysis at multiple sites along the DNA prior to dissociation. In order to test whether the MTase could perform processive catalysis, experiments were carried out using a DNA fragment containing two centrally located *Eco*RI sites separated by 51 bp. Methylation at each site was determined by protection of that site from cleavage by the ENase (see Materials and Methods for details). Processive methylation by the MTase would result in a rapid accumulation of substrates methylated at both sites and little or no substrates methylated at single sites during early stages of the reaction (<30% conversion), while distributive methylation would be characterized by a greater number of substrates methylated at only a single site at early time points. As has been demonstrated previously (Langowski et al., 1983), these two outcomes can be simulated by treating catalysis at the two sites as two consecutive first-order reactions

$$\text{no sites methylated} \xrightarrow{k_1} \text{one site methylated} \xrightarrow{k_2} \text{two sites methylated} \quad (4)$$

controlled by the rate constants, k_1 and k_2. A distributive mechanism is characterized by $k_1 = k_2$ since methylation at the first site has no influence on the rate of methylation at the second site. Processivity is demonstrated by $k_2 > k_1$ with the extent of processivity increasing as the ratio of k_2 to k_1 increases. Computer generated curves representing the expected outcome of this experiment for either a distributive or processive methylation mechanism are shown in Figure 2a,b, respectively. A value of $k_2 = 10k_1$ was chosen to represent significant processivity since increases in the ratio of k_2 to k_1 beyond 10 showed very little change in the shape of the curve shown in Figure 2b. A negative image of an ethidium bromide stained polyacrylamide gel from a typical experiment can be seen in Figure 2c. The percent conversion of unmethylated fragment to both single- and double-methylated fragments, derived from densitometric analysis of the bands from the gel shown in Figure 2c, were plotted versus time of reaction (Figure 2d). This data was fitted to equations relating the generation of the two methylation products (with one or two sites methylated) to the rate constants k_1 and k_2 with the best fit corresponding to $k_2 = 1.01k_1$ ($R = 0.975$), indicating that the MTase clearly exhibits distributive methylation. No changes were observed in the distribution of single to double methylation when reaction conditions such as pH (7.0−9.5), NaCl concentration (30−100 mM), or AdoMet concentration (0.05−5 μM) were altered (data not shown). We were also able to reproduce the processivity observed using *Eco*RI ENase (Terry et al., 1985) with our assay, confirming its effectiveness in determining processive catalysis (data not shown).

Direct Competition Demonstrates that the MTase Is Slightly More Efficient than the ENase. The MTase is part of a restriction-modification system and must perform catalysis in the presence of the *Eco*RI ENase *in vivo*.

[3] The nonspecific DNA could be considered to act as a contaminating inhibitor which increases in concentration in proportion to any increases in substrate concentration. This type of inhibition will exhibit kinetics which conform to the following equation (Segel, 1975):

$$v/V^*_{max} = [S]/(K^*_m + [S]) \quad (3)$$

where $V^*_{max} = V_{max}/(1 + (xK_m/K_i)$ and $K^*_m = K_m/1 + (xK_m/K_i)$ and where x is equal to the ratio [I]/[S]. In this situation, both the V_{max} (and therefore the k_{cat}) and the K_m should be decreased by the same factor, which in the case of the 14mer versus pBR322 would be approximately 30-fold. However, we have observed that there is virtually no change in the k_{cat} while there is an 8-fold decrease in the K_m.

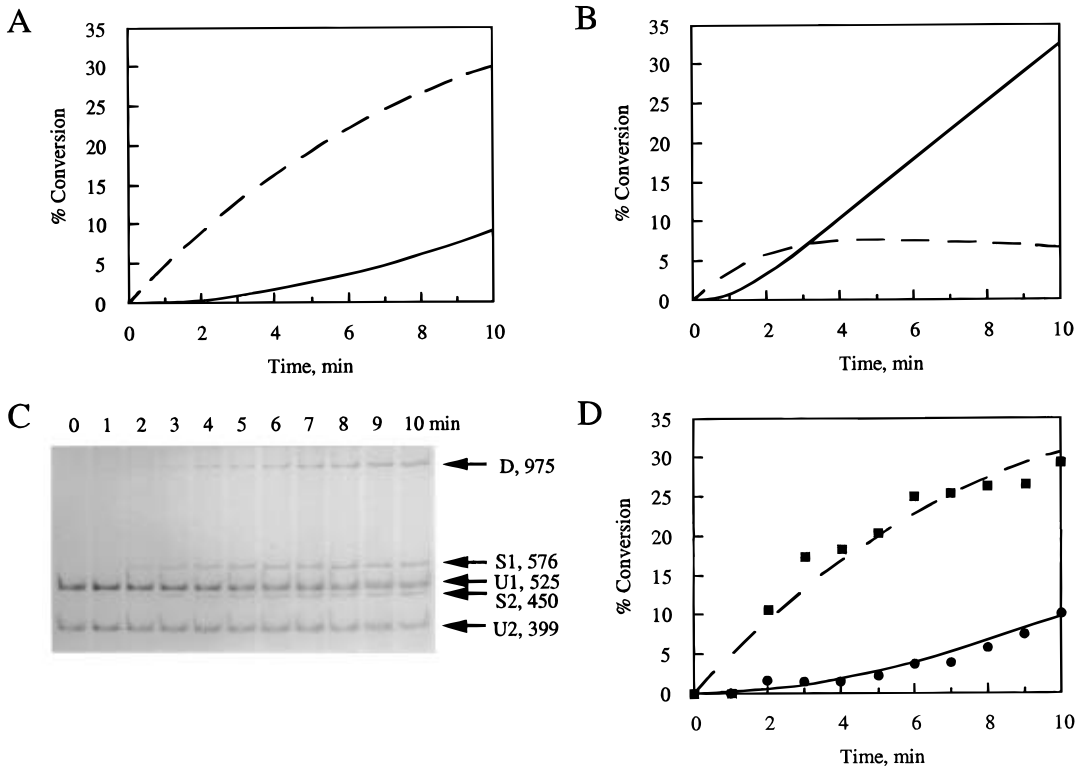

FIGURE 2: Comparison of results of processive catalysis assays with theoretical predictions. (A) Computer simulated plot of distributive methylation of a DNA molecule containing two *Eco*RI sites ($k_2 = k_1$; eq 4). (B) Computer-simulated plot of processive methylation of a DNA molecule containing two *Eco*RI sites ($k_2 = 10k_1$; eq 4). (C) Negative image of an ethidium bromide stained polyacrylamide gel from processive catalysis assay. The MTase was allowed to react for various times with a 975 bp fragment containing two *Eco*RI sites spaced 51 bp apart. The reaction was stopped by the addition of *Eco*RI ENase and Mg^{2+} which cut any unmethylated sites. The reaction mix was then run on a 5% polyacrylamide gel and stained with ethidium bromide. D, S1 and S2, and U1 and U2 correspond to the double, single, and unmethylated fragments resulting from ENase digestion, respectively. The numbers correspond to the length of each fragment in bp. (D) Plot of percent conversion of DNA fragments containing two *Eco*RI sites to single- and double-methylated products versus time. Relative intensities of the bands shown in C were determined using a laser densitometer. The intensity of D was halved to correct for its significantly longer length. The intensity of D and S1 + S2 were divided by the sum of D + S1 + U1 + S2 to determine the percent conversion of double (circles) and single (squares) methylated species, respectively, for a given time of reaction. The lines in all plots correspond to the percent conversion of unmethylated DNA to single (- - -) and double (—) methylated DNA versus time in minutes.

Whether an unmodified site is methylated or cut is essentially determined by which enzyme arrives at the site first since catalysis by both enzymes is diffusion controlled: a k_{cat}/K_m of 1.3×10^7 s^{-1} M^{-1} has been reported for the ENase with pBR322 as a substrate (Hager et al., 1990). Since both enzymes recognize the same DNA sequence and modification of this site by the MTase blocks cleavage by the ENase, a single-turnover assay was developed to assess the relative efficiencies of site location and catalysis by the two enzymes through direct competition. ENase and MTase concentrations were always greater than or equal to the concentration of DNA to ensure competition for a limited number of substrate molecules. Since the MTase was presumed to be more efficient due to a slightly higher k_{cat}/K_m, the ENase was held at 100-fold excess over DNA while the MTase was varied from 1- to 100-fold excess over DNA. This allowed for modulation of the concentration ratios of the two enzymes over a significant range while still keeping both enzymes in excess over the DNA. The two enzymes were preincubated with saturating concentrations of their necessary cofactors—Mg^{2+} for the ENase and AdoMet for the MTase—so that the determining factors in the competition between the two enzymes would be location and catalysis at the canonical site on the DNA rather than cofactor binding. Control experiments showed that AdoMet had no effect on ENase activity and that Mg^{2+} had no effect on MTase activity beyond those expected from ionic strength increases (data not shown). The percentage of DNA molecules protected by the MTase can be represented by the following equation:

$$\% \text{ protection} = \frac{[\text{MTase}](\text{efficiency})^{\text{MTase}}}{[\text{ENase}](\text{efficiency})^{\text{ENase}} + [\text{MTase}](\text{efficiency})^{\text{MTase}}} \times 100 \quad (5)$$

For example, if the two enzymes were at equivalent concentrations and had equal site location efficiencies, the equation predicts 50% protection. However, since the individual values of the apparent efficiency for the two enzymes could not be determined using this assay, eq 5 has been rearranged to express the percent protection of the DNA substrate as a function of the ratio of the efficiency of the MTase to that of the ENase:

$$\% \text{ protection} = \frac{([\text{MTase}]/[\text{ENase}])(\text{efficiency})^{\text{MTase/ENase}}}{1 + ([\text{MTase}]/[\text{ENase}])(\text{efficiency})^{\text{MTase/ENase}}} \times 100 \quad (6)$$

A plot of the percent protection versus the enzyme concentration ratio can be seen in Figure 3, for which the best fit of eq 6 corresponds to $(\text{efficiency})^{\text{MTase/ENase}} = 1.8$.

DISCUSSION

Enzymes exhibiting high catalytic efficiencies have specificity constants (k_{cat}/K_m) that approach limits set by the

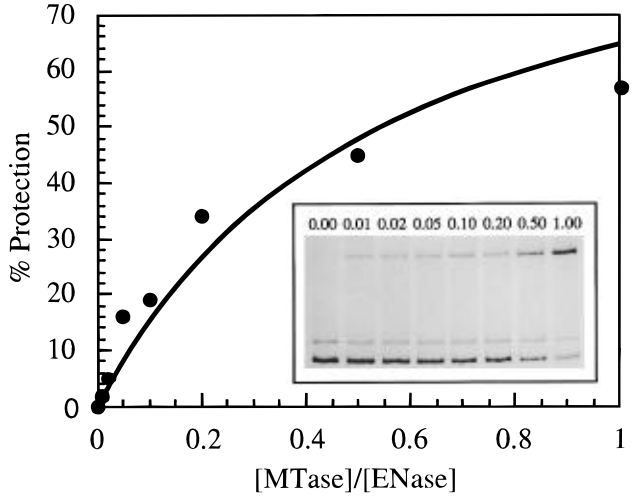

FIGURE 3: Plot of the percent protection of DNA by the MTase versus the ratio of [MTase] to [ENase] at 0.016 ionic strength. Each point was determined at 37 °C using the following conditions: 6 mM Tris, pH 7.5, 6 mM $MgCl_2$, 100 mM NaCl, 6 mM β-mercaptoethanol, 200 μg of BSA/mL, 40 μM AdoMet, 0.1 nM DNA, 10 nM ENase, and 0−10 nM MTase. The best fit to eq 6 was for $(k_{cat}/K_m)^{MTase/ENase} = 1.8$ with a correlation of $R = 0.94$. All points are the average of at least three determinations.

physical diffusion of enzymes and substrates to form enzyme−substrate complexes (Fersht, 1985). The bacterial *Eco*RI DNA MTase shows such behavior with plasmid DNA ($k_{cat}/K_m = 4 \times 10^8$ s^{-1} M^{-1}) (Reich & Mashhoon, 1991). Thus, the MTase must bind its specific site with a rate constant of this magnitude or greater (Fersht, 1985), in spite of the presence of an enormous excess of nonspecific DNA. Our previous demonstration that a short synthetic oligonucleotide is modified 8-fold less efficiently than a 4361 bp plasmid suggests that this large DNA molecule enhances the ability of the enzyme to locate its target site (Reich & Mashhoon, 1991). The predicted second-order association rate constant for the collision of the MTase and its target sequence embedded within a large DNA molecule[2] is 8-fold lower than the observed k_{cat}/K_m, lending credence to this argument. Here we sought to further investigate these observations by answering the following questions: Can sequences immediately flanking the canonical site account for these differences in efficiency? What is the magnitude of any possible inhibitor effect on k_{cat}/K_m by the addition of nonspecific DNA? What is the dependency of k_{cat}/K_m on DNA length and canonical site location? Does the MTase act processively when presented with DNA molecules containing more than one canonical site? How efficient is the MTase in the presence of the competing activity of the ENase?

Although our preliminary finding clearly shows a net enhancement in catalytic efficiency deriving from the addition of nonspecific DNA to the target molecule (Reich and Mashhoon, 1991), protein interactions with nonspecific DNA are reasonably expected to have a negative impact as well since such complexes would be a form of nonproductive binding. To address this, we determined the inhibition constant (K_i) for nonspecific DNA not physically attached to the specific site and obtained a value of 3 μM per nonspecific binding site (Figure 1). Assuming that DNA flanking the specific site functions only as a contaminating competitive inhibitor, we predict 30-fold decreases in both k_{cat} and K_m resulting from this inhibitory interaction.[3] Nonspecific DNA should therefore not alter k_{cat}/K_m, since any changes in the individual constants would be canceled out in their ratio. Our previous results (Reich & Mashhoon, 1991) and the results presented here clearly show increases in efficiency with increasing flanking DNA; therefore, this inhibitory effect must be overshadowed by positive consequences on k_{cat}/K_m. Interestingly, while K_m does decrease to some extent, k_{cat} remains largely unchanged [Reich and Mashhoon (1991) and see below]. Since nonproductive binding is predicted to decrease k_{cat} by lowering the concentration of the productive enzyme−substrate complex, we suggest that flanking DNA overcomes this effect and maintains the level of the productive complex.

Differences in the DNA sequences immediately flanking the *Eco*RI sites in the 14 bp and plasmid substrates (Reich & Mashhoon, 1991) could potentially account for the observed differences in their methylation efficiencies. However, Table 1 shows that this is not the case. In fact, the 14 bp substrate corresponding to the *Eco*RI site of pBR322 (CPBR) displayed a lower k_{cat}/K_m than the 14 bp DNA used in the previous experiments (C) (Reich & Mashhoon, 1991). This is possibly due to the larger number of A·T base pairs flanking the *Eco*RI site of CPBR, giving it low thermal stability when it is in a short DNA substrate. In pBR322, this sequence may be stabilized by the additional flanking DNA of the plasmid, and the MTase can then bind and modify this site as well as C. Although flanking sequences are known to be important for DNA recognition, this does not appear to be a component of the 8-fold difference in k_{cat}/K_m observed previously (Reich & Mashhoon, 1991) and investigated here.

Since k_{cat}/K_m is greater for plasmid DNA than a short oligonucleotide, it is likely that the presence of nonspecific DNA increases the association rate constant of the MTase and the specific site on the plasmid. Our analysis of the DNA length and site location dependence of the specificity constant further supports our earlier work (Reich & Mashhoon, 1991) and shows that DNA length affects the specificity constant at intermediate lengths as well. The enhancement is observed as the fragment size is increased to 429 bp with no additional effects as the length is increased further to 4361 bp (Table 2). This suggests that any facilitating mechanism involving nonspecific DNA is localized to within a few hundred base pairs of the target site. When the *Eco*RI site is located at a distance greater than approximately 300 bp from the nearest end of the DNA substrate there is an additional small enhancement of the k_{cat}/K_m (Table 3). This indicates that there needs to be sufficient nonspecific DNA on both sides of the specific site in order to attain the greatest site location efficiency. Thus, we have observed the following phenomena with regards to k_{cat}/K_m: (1) The previously observed enhancement is not due to changes in the local flanking sequence of the specific site; (2) there is approximately a 4-fold enhancement as the length of the DNA is increased to at least 429 bp; and (3) there is an additional approximately 2-fold enhancement as the site is moved at least 375 bp from the nearest end. The length dependence of the second-order association rate constant (k_{on}) is a standard method of demonstrating facilitated diffusion [Berg et al. (1981) and Winter et al. (1981)], and that for a diffusion limited enzyme such as the MTase, k_{cat}/K_m is a reasonable approximation of k_{on} (Fersht, 1985). Therefore, these observations support facilitated diffusion via sliding

along the DNA as a likely mechanism for DNA site location by the MTase. Additionally, the distances at which an enhancement of the specificity constant is observed for both substrate length and site location are reasonable estimates of the average number of DNA base pairs that are scanned by the MTase prior to dissociation. This yields an approximate upper limit of 400 bp for the scanning distance, although it is likely to be less than this since 400 bp is where the observed effects plateau. These distances are supported by our results determined under noncatalytic conditions (M. A. Surby and N. O. Reich, accompanying manuscript). An intriguing observation is that no negative effects are seen even when the DNA length is increased to more than ten times the estimated average scan length. Any potential negative effect may be offset by the fact that long DNA molecules can form "domains" wherein segments of DNA which are sequentially distant from each other may be spatially close (von Hippel & Berg, 1989). Through dissociation and reassociation with nearby DNA segments, the protein may quickly move from sequences far from the specific site to sequences that facilitate specific site location. The individual enhancements considered here are similar in magnitude to the 8-fold difference between pBR322 and the 14 bp oligonucleotide observed previously (Reich & Mashhoon, 1991) as well as those observed with other proteins that interact with DNA in which quantitative effects implicated to arise from the use of facilitated diffusion have been on the order of 2−9-fold (Ehbrecht et al., 1985; Jack et al., 1982; Nardone et al., 1986; Ricchetti et al., 1988).

An enzyme that uses facilitated diffusion to locate a specific site might reasonably be expected to locate another site on the same molecule by remaining bound and diffusing along the DNA chain. This would greatly enhance the efficiency of methylation at the second site by the MTase, since we have shown that k_{cat}/K_m is diffusion controlled. However, the MTase does not processively methylate two *Eco*RI sites located 51 bp apart during a single binding event (Figure 2). This result can be combined with previous data to infer additional information about the kinetic mechanism of DNA methylation by the MTase. When the MTase is bound to nonspecific DNA, it can subsequently bind AdoMet and form a catalytically competent ternary complex at the specific site (Reich & Mashhoon, 1991). Therefore, the MTase most likely dissociates from the DNA prior to release of *S*-adenosylhomocysteine (AdoHcy). If this were not the case, a percentage of the MTase bound to the DNA after release of AdoHcy would bind AdoMet and methylate the second site and some degree of processivity would be detectable. Furthermore, the MTase must leave the domain of the DNA molecule completely prior to binding to a new AdoMet molecule, since reassociation with the same DNA via this form of facilitated diffusion (von Hippel & Berg, 1989) would also be manifested as processivity.

Both the MTase and ENase must perform their catalytic functions on the same DNA sequence in the presence of the other enzyme *in vivo*. The competitive, single-turnover assay illustrated in Figure 3 shows the MTase to be slightly more efficient than the ENase at initial site location and catalysis: $(\text{efficiency})^{\text{MTase/ENase}} = 1.8$. Since the assay only measures whether a site is cut by the ENase or not, it is possible that some of the DNA molecules that were protected from cleavage by the ENase were not due to methylation by the MTase. One possibility that is easily dismissed is incomplete reaction time for the ENase since all of the DNA is cut in the lane containing no MTase (Figure 3; lane "0.00" in inset). However, it is possible that the MTase could arrive at a site first and protect the site not by performing catalysis but by simply denying the ENase access to the site. This seems unlikely given that the enzyme−DNA−AdoMet complex has a high commitment to catalysis (Reich & Mashhoon, 1991) and the methylation rate constant (41 s^{-1}) is fast relative to the time of the assay described in Figure 3 (Reich & Mashhoon, 1993). The small difference in efficiency shown in our direct competition assay is significantly different from the 30-fold larger k_{cat}/K_m measured for the MTase relative to the ENase (Reich & Mashhoon, 1991). However, the k_{cat}/K_m values for each enzyme were determined under different solution conditions, making the direct competition assay a better reflection of differences in enzyme efficiencies. Our competition results are presumably relevant to the *in vivo* condition since the MTase and ENase are found at a ratio of approximately two functional MTases for every ENase.

While the slightly greater efficiency (and potentially higher concentration) of the MTase provides a protective *in vivo* mechanism for the bacterial genome, it leaves the mechanism of foreign DNA restriction obscure. An analysis of the functional differences between the ENase and MTase, based in part on the results presented here, may help address this apparent paradox. The two enzymes display the following differences in terms of facilitated diffusion: (1) As stated above, the MTase is slightly more efficient than the ENase at physiological ionic strengths; (2) the average number of base pairs scanned by the MTase prior to dissociation ($<$400 bp) is much less than the 1300 bp observed for the ENase (Jack et al., 1982); and (3) the MTase does not show processive catalysis while the ENase does (Terry et al., 1985). Protection of the host genome requires methylation of all *Eco*RI sites; this is provided by the MTase because of its ability to act on hemimethylated DNA and effectively locate sites which are at great distances from one another due to its limited scanning distance. In contrast, the ENase cannot cleave hemimethylated DNA and remains bound to a particular stretch of DNA for longer periods of time. These differences work to the advantage of the ENase when restriction of viral DNA is considered. Although more efficient, the MTase is required to methylate all sites for protection while the ENase only needs to cleave once to be effective. Since the target sequence GAATTC appears with a frequency of approximately once very 4000 bp, it is to the advantage of the ENase to have a fairly long average scanning distance. Rapid dissociation works against the MTase here since it may dissociate from the viral DNA prior to methylation and become bound to another DNA molecule, allowing the ENase to cleave any unmethylated sites. Finally, the lack of catalytic processivity for the MTase means that other canonical sites on the viral genome will remain unprotected and can be recognized and cleaved by the ENase. Our kinetic analysis of the *Eco*RI MTase, coupled with past work on the ENase (Jack et al., 1982; Terry et al., 1985; Hager et al., 1990), provide a satisfying rationale for how this primordial "immune" system might function. Whether this model is generally applicable awaits the characterization of other restriction−modification systems. In support of our hypothesis, only bacterial MTases which are not part of a restriction−modification system, *Sss*I (Renbaum and Razin, 1992) and Dam (Bergerat et al., 1989),

act in a processive manner, while those that do have a corresponding endonuclease, *Hha*I, *Hpa*II (Renbaum & Razin, 1992), and *Eco*RI, are not processive.

Although the use of facilitated diffusion for promoter location has been directly observed for the *E. coli* RNA polymerase (Kabata et al., 1993), all other studies implicating this mechanism have relied upon correlating an increase in DNA length with an enhancement of either binding or catalytic parameters. These studies can be divided into three categories: (1) Thermodynamic and kinetic analysis of sequence-specific DNA-binding proteins (Berg et al., 1981; Herendeen et al., 1992; Riggs et al., 1970) or enzymes interacting with DNA under noncatalytic conditions (Jack et al., 1982; Ricchetti et al., 1988; Terry et al., 1983), (2) time course studies examining the DNA length dependence of reaction rates of sequence specific DNA modifying enzymes [Ehbrecht et al. (1985) and Nardone et al. (1986)]; and (3) examinations of the processivity of enzymes that react with DNA containing numerous sites on a single molecule (Brody et al., 1986; Dowd & Lloyd, 1990; Terry et al., 1985). Although most of these previous reports demonstrated facilitated diffusion *in vitro*, the existence of facilitated diffusion *in vivo* has also been shown (Dowd & Lloyd, 1990). Our work is distinct from these earlier reports in that it relates catalytic constants, k_{cat} and K_m, to DNA length and specific site location within the DNA molecule. Comparisons of k_{cat}/K_m provide the best assessment of relative catalytic efficiency (Fersht, 1985), and our results with the MTase clearly demonstrate the advantage gained by an enzyme that utilizes a limited one-dimensional search mechanism to locate its specific site on a large DNA molecule.

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