Conformational Transitions as Determinants of Specificity for the DNA Methyltransferase EcoRI

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Changes in DNA bending and base flipping in a previously characterized specificity-enhanced M.EcoRI DNA adenine methyltransferase mutant suggest a close relationship between precatalytic conformational transitions and specificity (Allan, B.W., Garcia, R., Maegley, K., Mort, J., Wong, D., Lindstrom, W., Beechem, J.M., and Reich, N.O. (1999) J.Biol.Chem. 27, 19269-19275). The direct measurement of the kinetic rate constants for DNA bending, intercalation, and base flipping with cognate and non-cognate substrates (GAATTT, GGATTC) of WT M.EcoRI using FRET and 2-aminopurine fluorescence studies reveals that DNA bending precedes both intercalation and base flipping, and base flipping precedes intercalation. Destabilization of these intermediates provides a molecular basis for understanding how conformational transitions contribute to specificity. The 3500 and 23,000-fold decreases in sequence specificity for noncognate sites GAATTT and GGATTC are accounted for largely by a ~2500 fold increase in the reverse rate constants for intercalation and base flipping, respectively. Thus, a predominant contribution to specificity is a partitioning of enzyme intermediates away from the Michaelis complex prior to catalysis. Our results provide a basis for understanding enzyme specificity, and in particular, sequence-specific DNA modification. Since many DNA methyltransferases and DNA repair enzymes induce similar DNA distortions, these results are likely to be broadly relevant.

DNA modifying enzymes have evolved a delicate balance between sequence specificity and efficient catalysis (1;2). Indeed, the lack of our understanding of the underlying causes for specificity may account in part for the general lack of success in re-engineering such enzymes, in spite of relative successes with other classes of enzymes (3-5). Many DNA modifying enzymes have specificity constants which approach the predicted values for diffusion controlled reactions (kcat/Km ~ 108-109M-1s-1) (6;7). DNA modifying enzymes have attained such efficiency in part because of facilitated diffusion mechanisms (8). Remarkably, such highly efficient enzymes also show significant discrimination between cognate and non-cognate sequences. These enzymes frequently undergo significant conformational changes upon binding their cognate sequence, a clear example of an induced fit mechanism. The resultant intermediates provide a theoretical basis for modulating specificity through the concept of kinetic proofreading, as proposed for several DNA polymerases (9). These checkpoints can directly impact specificity by modifying the partitioning of reaction intermediates prior to catalysis differentially between cognate and non-cognate substrates (9-11).

S-Adenosyl methionine-dependent bacterial DNA methyltransferases modify bases at cytosine (C5 and N4), and adenine (N6), and play important roles in many cellular pathways including mismatch repair, restriction modification, and gene regulation (12). A detailed structural and mechanistic characterization of DNA methyltransferases is also motivated by recent demonstrations that some bacterial and mammalian enzymes are viable candidates for antibiotic and cancer drug development respectively (13-16). The bacterial DNA adenine methyltransferase EcoRI forms part of a restriction/modification system and modifies the N6 position of the second adenine in the sequence GAATTC. The cartoon and kinetic mechanism of M.EcoRI presented in Scheme 1 represents the chemical and conformational transitions observed during the methylation of cognate DNA. M.EcoRI
Methylases use facilitated diffusion to locate and methylate its cognate DNA to protect the bacterial genome from the complement restriction endonuclease \((8;17)\). Upon location of the target sequence, M.EcoRI induces a 50° bend in the DNA sequence and flips the target adenine out 180° to an extrahelical position to facilitate catalysis. Solution-based FRET studies were used to demonstrate that along with bending the DNA, M.EcoRI also expands the duplex DNA structure presumably through an intercalative mechanism \((18)\), and intercalation and bending occur nearly simultaneously with DNA binding at 25°C \((18)\).

M.EcoRI’s specificity constant \((k_{cat}/K_M, \sim 10^7-10^9 \text{ M}^{-1}\text{s}^{-1})\) for cognate DNA is near the rate of diffusion \((8;17;19;20)\). Specificity constants for M.EcoRI toward several non-cognate sites are decreased 5-fold (A6: GAATCC), 3500-fold (A4: GAATTT) 23,000-fold (A3: GGATTC) which involve relatively minor changes in DNA affinity \((19)\). The molecular basis of this discrimination remains unclear, as is the sequence-discrimination for any DNA methyltransferase. We recently described an intriguing bending-impaired M.EcoRI mutant (H235N) whose ability to flip its target adenine is decreased 2000 fold compared to the WT enzyme, while the cognate modification was left largely intact \((21)\). Moreover, the mutant is at least 1000 fold more discriminating than the WT enzyme \((21)\). The molecular basis of how changes in precatalytic conformational transitions can result in such a profound increase in specificity is the focus of this study. We find that much of the discrimination of the WT enzyme against noncognate DNA occurs from the selective destabilization of precatalytic intermediates. Our results are presented in the context of kinetic proofreading concepts, which provide a quantitative framework for understanding sequence-specific DNA modification.

**MATERIALS AND METHODS**

**Enzyme Expression and Purification**

M.EcoRI was purified from MM294 E. coli cells with the plasmid pXRI as previously described \((18)\). 6 liters of culture were induced with 1mM IPTG at an OD\(_{600}\) nm 0.4 for 3 hours. Cells were sonicated in extraction buffer (200 mM NaCl, 6.5 mM K\(_2\)HPO\(_4\), 3.5 mM KH\(_2\)PO\(_4\), 1 mM EDTA and protease inhibitor mixture (Sigma)). The lysate was centrifuged (30 min at 4 °C), the supernatant was loaded onto a 20-ml phosphocellulose (Whatman) column and the protein was eluted with a salt gradient between 200 mM and 800 mM NaCl. Fractions containing M.EcoRI were loaded onto a 10-ml hydroxyapatite (Biorad) column equilibrated with extraction buffer. The protein was eluted with a gradient between 6.5 mM and 1M K\(_2\)HPO\(_4\), and dialyzed overnight in extraction buffer containing 10 % glycerol. The dialyzed protein was loaded onto a biorex (Biorad) column, and the protein was eluted with a gradient of 200 mM and 800 mM NaCl. Protein from this procedure was typically greater than 95% pure by densitometry. Enzymes were stored at –80 °C with 10 % (V/V) glycerol, 0.3-0.4 M NaCl, 10 mM potassium phosphate (pH 7.8), 1 mM EDTA, and 7.5 mM β-mercaptoethanol.

**DNA Synthesis and Fluorophore Coupling**

DNA oligonucleotides for fluorophore coupling were synthesized at Integrated DNA Technologies containing 5’ C-6 primary amino modifications. 2-aminopurine (2AP) containing oligonucleotides were purchased at Midlands DNA with the target adenine replaced by the 2AP nucleotide. 14-mer top strands cognate 5’-AGACGAATTCGAA, noncognates (A6) 5’-AGACGAATTCGAA, (A4) 5’-AGACGAATTCGAA, (A3) 5’-AGACGAATTCGAA. 14-mer bottom strands cognate 5’-TTCGGAATTCGTCT, noncognates (A6) 5’-TTCGGAATTCGTCT, (A4) 5’-TTCGGAATTCGTCT, (A3) 5’-TTCGGAATTCGTCT. All bottom strand target adenines were methylated to force the orientation of the enzyme to target the top strand. Cognate duplex DNA is defined as cognate bottom methylated (CBM). Oligonucleotides for FRET studies were coupled as previously described \((18)\). Briefly, oligonucleotides were resuspended in dH\(_2\)O, extracted with chloroform, ethanol precipitated, and resuspended to a final concentration of 25 μg/μl. 6-Alexa Fluor 488 succinimidyl ester (Molecular Probes) was coupled to the top strand, while 6-carboxytetramethylrhodamine succinimidyl ester (Molecular Probes) was coupled to the bottom strand. Coupling reactions were performed with 10 to 20-fold molar excess dye to oligonucleotide. Coupling was carried out overnight in 75 mM sodium tetraborate (pH 8.5). Overnight couplings were ethanol precipitated and resuspended in 0.1
M triethylammonium acetate. Resuspended coupling reactions were purified on an analytical Vydac C4 column with a Waters/Millipore HPLC system. Purified coupled oligonucleotide concentrations were determined by absorbance scans from 230-700 nm and determined to be 100% coupled.

Equilibrium Anisotropy- Anisotropy experiments were carried out on a Fluoromax-2 fluorimeter (ISA SPEX) equipped with an L-format autopolarizer. Equilibrium measurements were performed by monitoring the change in anisotropy of 20 nM singly labeled (Alexa-488) duplex DNA as increasing enzyme was titrated into the reaction mix. An excitation of 488 nm and emission of 515 nm with slit widths of 8 nm were used for data collection. Anisotropy was calculated using the equation \( R = \frac{I_{||} - L - I_{┴}}{2I_{||} - 2L} \) (22). Dissociation constants were determined by fitting data to a quadratic formula \( f = \frac{((a+x+b)^2-4*x*a))}{2} \); where \( b = K_D \), \( X = [\text{Enzyme}]*[\text{DNA}] \), and \( a = [\text{Enzyme-DNA}] \).

Equilibrium FRET- Equilibrium fluorescence experiments were performed as previously described (18). All measurements were collected with a Perkin Elmer LS50B luminescence spectrometer at 22 °C. Excitation at 485 nm was used to excite Alexa-488, and an emission spectrum from 500 nm to 650 nm was collected. Enzyme at a final concentration of 200 nM was mixed with 20 nM DNA and 1 μM sinefungin in reaction buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 100 mM NaCl, and 1 mM DTT). The AdoMet analogue sinefungin (Sigma Aldrich) was used for all equilibrium and transient experiments.

Stopped Flow FRET- Stopped flow measurements were collected on an Applied Photophysics SX.18MV stopped flow reaction analyzer outfitted with a single channel emission photomultiplier tube. An excitation wavelength of 485 nm and an emission cutoff filter of 515 nm were used for data collection. Coupled DNA was rapidly mixed with M.EcoRI preincubated with sinefungin in reaction buffer. The change of donor fluorophore emission was used to detect bending and intercalation due to the greater signal change for the donor fluorophore during FRET with the acceptor fluorophore (18). DNA concentrations were 20 nM for cognate DNA and 100 nM for noncognate DNA substrates. Enzyme and cofactor concentrations were varied from 100 nM to 2000 nM. Error measurements are from at least two experiments with the DNA and enzyme mixes freshly prepared each time.

Stopped Flow Flipping- Transient base flipping data for cognate and noncognate substrates were measured in a stopped flow apparatus by monitoring the increase in fluorescence of 2-aminopurine (2AP) as it transitions from base-stacked to extrahelical in the presence of enzyme. 2AP fluorescence is roughly 14-fold greater when extrahelical, and has previously been validated as a probe for the measurement of base flipping kinetics (23-26). An excitation wavelength of 310 nm and an emission cutoff filter of 320 nm were used for data collection. 100 nM 2AP containing DNA was rapidly mixed with M.EcoRI preincubated with sinefungin in reaction buffer, monitoring the emission above 320 nm. Enzyme and cofactor concentrations were varied between 500 nM and 2000 nM. Error measurements are from at least two experiments with the DNA and enzyme mixes freshly prepared each time.

Reverse Kinetics- Stopped flow measurements were collected with excitation wavelengths and cutoff filters as described for the FRET and 2AP studies. The rate of extrahelical base restacking was measured by monitoring the dissociation of the preformed 2AP containing DNA-enzyme complex. The loss of fluorescence is attributed to the restacking of the target base. A final concentration of 100 nM 2AP DNA, 100 nM enzyme, and 6 μM Sinefungin were mixed with 5 μM unlabeled competitor DNA. The unbending/unintercalation data was collected by monitoring the change in FRET as a 25 to 50-fold excess unlabeled cognate DNA was rapidly mixed into the system. All forward and reverse stop flow data was fit to either a single or double exponential equation using the program Sigma Plot.

Data Analysis and Simulation- Kinetic simulations to model bending and intercalation data were performed using Scientist (Micromath Inc., Salt Lake City, UT) and KinTekSim (KinTek Corporation Austin, TX). Global fitting analysis of 4 °C cognate bending and intercalation data were performed using a two intermediate reaction mechanism. Goodness-of-fit statistics were used to evaluate the fit of the data to the reaction
mechanism, yielding a R-squared value of 0.99. KinTekSim modeling was done for binding, bending and intercalation as shown in Scheme 1. Second and first order rate constants for the cognate DNA simulation were: \( k_{on} = 3.0 \times 10^7 \text{M}^{-1}\text{s}^{-1} \), \( k_{bend} = 100 \text{ s}^{-1} \), \( k_{intercalate} = 10 \text{ s}^{-1} \), \( k_{unintercalate} = 0.02 \text{ s}^{-1} \). Simulations for noncognate substrates were performed by increasing the unintercalation rate constant to 2000 and 4000 fold.

RESULTS

Transient DNA bending is observable with cognate and noncognate substrates—Fluorescence Resonance Energy Transfer (FRET) between Alexa-488 and TAMRA coupled to the 5’ ends of 14mer oligonucleotides was previously used to examine the bending transition of the target DNA by M.EcoRI (18). Since cognate DNA bound by M.EcoRI is bent ~ 50° as determined by both AFM and gel shifting methods (21;27), an increase in energy transfer was anticipated. The opposite changes were observed, consistent with a decreased energy transfer upon protein binding (18) (Figure 1a). Further analysis revealed the decreased FRET was not dye dependent, and the anticipated increase in FRET does occur using an enzyme that recognizes the same DNA substrate but only induces DNA bending (18;28). Due to the robustness of the decreased FRET M.EcoRI-DNA data, an intercalative mechanism was proposed (18). Recent protein/DNA cocrystal structures of T4DAM and E.coli DAM reveal several residues intercalating into the DNA (29;30), supporting the concept of intercalation for methyltransferases.

Using a FRET based assay (18) we examined equilibrium and pre-equilibrium kinetics of binding, bending, and intercalation for the cognate sequence with the target adenine in the complement strand methylated (CBM) (GAATT) and 3 noncognate sequences; A6 (GAATCC), A4 (GAATT), and A3 (GGATT). The equilibrium FRET for cognate DNA previously demonstrated a decrease in FRET between the donor and acceptor fluorophores on the DNA. Noncognate A6 also has a decrease in FRET upon M.EcoRI binding similar to the cognate signal, while noncognate substrates A3 and A4 have no observable change in FRET (Figure 1a, b, & c). To ensure that all analysis on DNA bending, base flipping and intercalation are of a saturated enzyme substrate complex, the enzyme’s affinity for each substrate was determined. Enzyme \( K_D^{DNA} \) values were determined by equilibrium anisotropy (data not shown), and are within error of the previously determined \( K_D^{DNA} \) values obtained by gel mobility shift assays (19). Enzyme concentrations used in each experiment were more than 3-fold higher than the DNA dissociation constant (\( K_D^{DNA} \)) to ensure that the enzyme was bound to the substrate.

The lack of detectable equilibrium FRET changes with both A4 and A3 may be due to compensatory signal decreases and increases resulting from changes in bending and intercalation respectively. This was further investigated by measuring the pre-equilibrium kinetics of the FRET based bending / intercalation experiments on a stopped flow apparatus. Cognate and noncognate (A6, A4, and A3) substrates with dyes on both 5’ end, at a concentration of 100 nM were rapidly mixed with 500 nM M.EcoRI-sinefungin while monitoring the donor signal. We previously demonstrated that M.EcoRI binding to cognate substrate at 25 °C involves a protein-concentration dependent increase in the donor signal consistent with a decrease in FRET (Figure 2a) (18). Surprisingly, substrates A4 and A3 show a very rapid decrease in donor signal (130 s⁻¹ and 240 s⁻¹ respectively), followed by a slower increase (37 s⁻¹ and 22 s⁻¹ respectively) (Figure 2c & d). Noncognate A6 has a very rapid decrease in donor signal (rate constants are unattainable) followed by an increase in donor signal with a \( k_{obs} \) rate constant of 27 s⁻¹ (Figure 2b).

Based on classical FRET theory (18;22) we suggest that the initial decrease in the donor signal (increased FRET) is caused by the bending transition (18;28), while the increase in donor signal (decreased FRET) (18) is caused by intercalation. The assignment of DNA bending to the decrease in the donor signal is further validated in the following section when the decrease in the donor signal is observed for the cognate DNA with the temperature lowered to 4°C (Figure 3a). Further support for the assignment of the bending transition to the initial decrease in donor fluorescence is provided by the observed concentration dependence of this phase in the fluorescence data (Figure 3a). Finally, the cocrystal structure of the endonuclease R.EcoRV bound to the cognate DNA sequence for M.EcoRI GAATT reveals a significant degree of DNA bending; R.EcoRV binding to this same DNA...
shows the anticipated decreased donor signal (increased FRET) (28). Taken together, these observations support our assignment that the initial increase in FRET for M.EcoRI is due to DNA bending.

The $k_{obs}$ rate constant for the increase in donor signal with the rapid mixing of 20 nM cognate DNA with 500 nM M.EcoRI is 10 s$^{-1}$ (18). Under similar conditions the exponential fits for the donor signal increases of A4 and A3 (37 s$^{-1}$ and 22 s$^{-1}$ respectively) are faster than the cognate increase in donor signal (Figure 2a, c, &d). The faster rate constants for A4 and A3 arise from a faster approach to equilibrium for these noncognates versus cognate.

DNA bending precedes intercalation with the cognate site - Our previous results showed that cognate DNA binding, bending, and intercalation are nearly simultaneous (18). Furthermore, based on the biphasic FRET signal observed with the non-cognate substrates, tentatively assigned to rapid bending followed by slower intercalation (Figure 2b, c, & d), we sought to deconvolute these events with cognate DNA and M.EcoRI at 4 °C. Upon rapidly mixing M.EcoRI with cognate DNA, an initial decrease in the donor signal followed by a slower increase was observed. The small, but reproducible, decrease in signal occurs in the initial 25 ms of data collection (Figure 3a). The increase in FRET is, as stated in the prior section, most reasonably assigned to the anticipated changes resulting from a bending transition (28,31,32). This is followed by a decrease in FRET which has been assigned to the intercalation of the DNA by the enzyme (18). Measurement of the rate constant for intercalation with varying M.EcoRI concentrations (100, 300, 500, 700, and 1400 nM) and constant cognate DNA (20 nM) reveals that at high concentrations the rate of intercalation (donor signal increase) does not increase linearly with enzyme concentration (Figure 3b). The mild concentration dependence of the intercalation data suggests that intercalation is only partially second order (Figure 3b) (33). Further, because the bending transition remains concentration dependent, this step continues to be simultaneous with DNA binding.

The intercalation signal for cognate DNA is the only observable signal at 22 °C (Figure 2a), and it was previously shown that the rate of intercalation increases linearly with increasing enzyme concentration at 22 °C (18). The concentration dependence for intercalation of cognate DNA at 22 °C suggests that both bending and intercalation are nearly simultaneous with binding (18). The new signal at 4 °C (Figure 3a), which we assign to bending for cognate DNA demonstrates that bending precedes intercalation. We therefore focused subsequent bending and intercalation studies of non-cognate substrates at 4 °C (see following section). The decrease in the signal amplitudes for the intercalation phase with the noncognates A4 and A3 in comparison to cognate DNA (Figure 2c & d vs. Figure 2a), suggests a significantly altered protein/DNA interaction.

The intercalation rate constant for noncognate DNA is first order - The transient rates of bending and intercalation for noncognate DNAs A4 and A3 were examined at 4 °C by the rapid mixing of 100 nM DNA with 500, 700, 900, 1100 nM M.EcoRI (Figures 4a & b, A4 and A3). There is very little change in the observed rates of bending and almost no change in the observed intercalation rates for both substrates with increasing enzyme concentration (Figure 4 inset), which suggests that both bending and intercalation are no longer second order steps with A4 and A3 substrates. The noncognate rates observed for each curve were calculated by plotting the two signal directions as separate single exponentials (33;34). It is important to note that the level of amplitude change in the two phases is different for noncognate compared to the cognate DNA (Figures 3a vs. 4a & b). That is, the intercalation phase rises much higher than the bending phase for the cognate DNA than the noncognate DNA. Several scenarios could account for this; 1) Most of the bending transition for cognate DNA occurs within the dead time of the stopped flow measurement, giving the appearance of a greater intercalation amplitude. 2) The number of residues intercalating the noncognate substrate is less than the cognate substrate, decreasing the expansion of the DNA during intercalation and ultimately decreasing the amplitude of the signal change during intercalation. 3) The reverse rate constant for the intercalation step increases for the noncognate substrate, reducing the observable intercalated intermediate population.

Base flipping precedes intercalation - The use of 2-aminopurine (2AP) as a base flipping
probe has been well documented for both methyltransferases and repair enzymes (23-26;35). The base flipping rate constants for cognate and noncognate substrates were measured under pre-equilibrium conditions. The equilibrium signal of 2AP-containing A4 and A3 DNA bound by M.EcoRI is less than 35% of the cognate 2AP signal (data not shown). Based on the observed 2AP equilibrium data, pre-equilibrium experiments were designed to reveal tractable changes during stopped-flow experiments to measure the base flipping and restacking rate constants. The observed rate of base flipping was measured at different concentrations and time frames. An initial increase in 2AP fluorescence is observed in the first 100 ms of the flipping experiment (Figure 5a). There is an observable concentration dependence of the first phase of flipping, followed by a concentration independent phase (Figure 5a). The biphasic signal for 2AP flipping was previously assigned to flipping, followed by an isomerization event for the target base (26;35).

In order to assign a temporal order for bending and base flipping, we examined the enzyme-induced cognate base flipping at 4 °C by monitoring 2AP base flipping, and compared this to the FRET signal for cognate bending (only observed at 4 °C). 2AP containing DNA was rapidly mixed with M.EcoRI preincubated with sinefungin (Figure 5b). A short ~4 ms lag is observed before the increase of 2AP fluorescence (Figure 5b), which is consistent with previous observations (26). This lag persists even with increasing concentrations of 500, 1000, 1500, 2000 nM M.EcoRI. Closer inspection of the cognate FRET data at 4 °C (Figure 3a) reveals that no lag is observed and the bending event is near completion within this time frame. Furthermore, under similar conditions to the FRET experiments, the transient flipping experiment takes more than 200 ms to reach equilibrium. This data clearly shows that the DNA bending event precedes base flipping, as previously suggested (21;36). Also, the observed rate of intercalation from the FRET signal plateaus with increasing enzyme concentration (Figure 3b). This plateau suggests that the intercalation step is a first order process. Furthermore, since the observed rate of base flipping does not plateau under similar conditions (Figure 5c) base flipping must precede intercalation. The concentration profile (Figure 5c) demonstrates that the event that we and others have assigned to base flipping (23;25;35) is concentration dependent with a $k_{on} = 3.6 \times 10^{7} \text{M}^{-1}\text{s}^{-1}$, which is similar to the previously reported $k_{on} = 2.1 \times 10^{7} \text{M}^{-1}\text{s}^{-1}$ as determined by anisotropy (18;36) and FRET(18). The intercept which approximates $k_{off} = 40 \text{s}^{-1}$ is very close to the restacking transition of >50 s⁻¹ previously calculated from global fitting (36). Since we observe concentration-dependent behavior for both bending and base flipping, we suggest that the $k_{obs}$ rate of base flipping would eventually plateau if high enough enzyme concentration were achievable.

**Unbending and restacking rate constants increase for noncognate DNA**
The reverse rate constants for unbending and restacking were examined by trapping experiments. A preformed complex of DNA-M.EcoRI-sinefungin was rapidly mixed with 25 to 50 fold excess unlabeled DNA at 22 °C. The change in fluorescence for either the doubly labeled DNA or the 2AP containing DNA was attributed to unbending and restacking respectively. The rate constants of unbending for noncognates A6, A4, and A3 were all extremely fast compared to cognate (Table 1, Figure 6a, b, c). The A6 noncognate causes an decrease in the donor signal (increased FRET), opposite the signal observed for the forward rate (Figure 2b), as would be predicted. The change in the donor signal for A4 and A3 noncognates increases, suggesting that A4 and A3 are only unbending (Figure 2c &d).

Restacking rate constants were obtained through similar trapping experiments used to monitor unintercalation and unbending, except that the DNA substrates used for the preformed complex contained 2AP in substitution for the target adenine, and did not have fluorophores coupled to the 5’ ends. The equilibrium 2AP signals upon binding by M.EcoRI with all three noncognate substrates were uniformly small, and A3 could not be reproducibly obtained; hence, restacking rate constants were limited to the cognate and noncognates A6 and A4 (Figure 7a, b, c).

**DISCUSSION**
The hallmarks of enzyme function, specificity, rate enhancements, and regulation,
demand a molecular understanding to provide a basis for their rational redesign (37-39). The redesign of enzyme specificity (40-44) reflects a deep understanding of enzyme function, leading to the potential practical application of such powerful catalysts. DNA methyltransferases provide an excellent platform for the study of mechanisms leading to enzyme specificity: they are challenged with the daunting task of modifying a single base within a particular recognition element, which itself is embedded in a large amount of competing substrate, they do so by exploiting large scale conformational changes both within the enzymes and the target DNA, and the bacterial and mammalian enzymes form part of critical epigenetic pathways essential to bacterial pathogenesis, human development, and tumorigenesis. The redesign of sequence-specific DNA methyltransferases to modify unique positions within the genome may enable in vivo site-specific DNA modification and gene regulation for basic research and biomedical applications (45;46).

M.EcoRI-induced changes in DNA conformation, including bending, flipping, and intercalation provide a compelling basis for regulating sequence discrimination (10;18;47). Early evidence for this was provided by a bending-impaired H235N M.EcoRI whose decreased apparent base flipping constant also resulted in a dramatic increase in the mutant’s substrate discrimination. (21). We previously hypothesized that these transitions may play important roles in determining the WT enzyme’s discrimination against noncognate methylation (21).

WT M.EcoRI modifies the noncognate DNA substrates with varying levels of efficiency ($k_{cat}/K_M$ (cognate/non-cognate)): A6 (GAATCC, = 5), A4 (GAATTT, = 3500), and A3 (GGATTC, = 23000) (19). Only minor contributions towards this discrimination derive from changes in affinity (19). In order to reconcile the differences in noncognate specificity and affinity we examined the known conformational changes induced by M.EcoRI binding to each of these substrates.

**Observable bending / decrease in Intercalation Amplitude** – Examination of reaction intermediates and conformational changes prior to methylation by multiple fluorescence techniques presented here have allowed us to better understand the role of conformational changes in the reaction mechanism of M.EcoRI. Furthermore, these studies address fundamental questions pertaining to DNA modifying enzymes, specificity, and transitions between intermediates on a reaction pathway. Our studies further probe the widely held view that structurally accessible intermediates define an enzyme’s specificity (2;34).

FRET based experiments with noncognate substrates were performed to examine the rates and amplitude changes of DNA bending and intercalation. In accordance with the limited change in M.EcoRI’s specificity towards noncognate A6 we see limited changes in the equilibrium (Figure 1b) and pre-equilibrium (Figure 2b) bending and intercalation, compared to the cognate site. The lack of any change in the A4 and A3 equilibrium FRET (Figure 1c) is further supported by the kinetic pre-equilibrium FRET data (Figure 2c & d), which shows the intercalation signal reaching an equilibrium equivalent to the start of the bending phase, suggesting that the population of intermediates between the bent and intercalated states of the enzyme with A4 and A3 differ from the populated intermediates of bent and intercalated A6 and cognate with enzyme.

**Temporal order is bending first, followed by flipping and intercalation** – The use of 2-aminopurine as a probe for base flipping kinetics of methyltransferases has been well documented (23;25;26;35). There are minimal changes in M.EcoRI substrate affinity when the target base is replaced with 2-aminopurine, further validating its use as a relevant probe for studying base flipping (26). Also, the appendage of FRET fluorophores to the substrate DNA has not affected any observable catalytic parameters of M.EcoRI (18). The lack of any gross perturbation to the enzyme/substrate complex by the fluorophores further allows us to compare data collected using the two fluorescence techniques.

In order to provide temporal order to the steps of bending, flipping and intercalation, we compared our FRET based experiments at 4 °C (Figure 3a) with our base flipping experiments at 4 °C (Figure 5b). The observation of a lag in the flipping signal in the initial 4 ms is consistent throughout the concentration profile (Figure 5b) and has previously been reported (36). The bending signal (Figure 3a) is near completion.
within 10 ms and no lag is observed, indicating that bending precedes base flipping. The curvature in the concentration profile of the intercalation phase at 4 °C observed by FRET (Figure 3b) and the lack of any curvature in the concentration profile for base flipping at 4 °C (Figure 5c) demonstrates that intercalation follows base flipping (33). However, base flipping rates are anticipated to eventually plateau with increasing enzyme concentration since base flipping also follows bending. The assignment of the temporal order for the intermediates by collecting rate constants for observable DNA bending, base flipping and intercalation at temperatures of 22 °C and 4 °C also reveals that a significant portion of the energy barriers for these enzyme induced conformational changes is entropic.

**Reverse rate constants dictate specificity** – The kinetic rate constants for the reversal of the bending, intercalation and flipping transitions were determined by trapping experiments. The total change in amplitude for the FRET signal observed for A4 and A3 (unbending / unintercalation) (Figure 6c & d) is the same as the amplitude for the FRET signal observed with the forward step of bending (Figure 2c & d). Further, the changes occur in the opposite direction (Figure 6c & d) of those observed in forward experiments (Figure 2c & d). The partitioning of the enzyme/DNA intermediates back towards the unbent intermediate and which involves no intercalation is favored with these noncognate substrates. Fitting the trapping data to single exponentials reveals that the reverse rate constants for A4 and A3 are 2500 and 1900 fold faster than cognate respectively (Table 2). We reconfirmed the reverse rate kinetics by performing the same trapping experiment, but using the 2AP containing substrates. Indeed, the A4 noncognate substrate produces a restacking rate constant 2500 fold greater than the cognate substrate (Figure 7 Table 2). We reconfirmed the reverse rate kinetics by performing the same trapping experiment, but using the 2AP containing substrates. Indeed, the A4 noncognate substrate produces a restacking rate constant 2500 fold greater than the cognate substrate (Figure 7 Table 2). Interestingly, both restacking (Table 2) and unbending (Table 1) transitions for A4 show similar noncognate/cognate enhancements. Also, the increase in the reverse rate constant for A4 vs. cognate is very close to the reported 3500 fold difference in specificity towards the cognate substrate, which suggests that enzyme specificity may arise from the destabilized enzyme/noncognate complex and thus a more rapid partition away from the catalytic complex. The inability to collect A3 base flipping and restacking data may be caused by additional instability of the base-flipped enzyme complex, thereby showing the greatest difference in specificity from the cognate substrate of 23,000-fold. Reverse kinetic steps have been shown to control enzyme specificity, (28;39;48), and the specificity can be dominated by a single step in the overall mechanism (48;49). In the case of cytosine methyltransferases, changes in the intermediate populations preceding chemistry were previously shown to impact the steady state rate of catalysis (50). Our data suggests that intermediate populations are different between cognate and noncognate substrates bound by M.EcoRI, particularly the intercalated intermediate. Due to the increased unintercalation rate constants for noncognate substrates we observe the reaction intermediates partition away from the chemistry step, thereby changing the intermediate populations so that chemistry does not occur as readily as it does with a cognate substrate (Figure 8c).

**Simulation and Conclusion** – In order to obtain rate constants for the transitions between the intermediates of bending and intercalation, the bending and intercalation data for the cognate substrate obtained at 4 °C (Figure 3) was fit globally to a two intermediate reaction mechanism using the program Scientist. The goodness-of-fit statistics obtained when solving for 4 different rate constants (Figure 8a) yields an R-squared value of 0.99, further supporting our assignment of the two FRET phases to bending followed by intercalation. The obtained rate constants were used to simulate the bending and intercalation phases with 20 nM DNA and 300, 500, and 700 nM enzyme (Figure 8a). The calculated rate constants yield simulated FRET data that mimic the observed FRET data in shape and amplitude (Figure 8a vs. Figure 3a).

Global fitting analysis was also applied to the base flipping data obtained at 4 °C (Figure 5b). Use of a two intermediate reaction mechanism allowed for the incorporation of an intermediate prior to the flipping step which produces a lag in both the raw flipping data (Figure 5b) and the simulated flipping data (Figure 8b). Unfortunately the lag in Figure 8b is poorly defined due to large errors for the rate constant of the reverse step for the first intermediate. The calculated rate constants
for flipping (Figure 8b; $k_3 = 550 \text{ s}^{-1}$) vs. the calculated rate constant for intercalation (Figure 8a; $k_3 = 97 \text{ s}^{-1}$) further supports our assignment of base flipping preceding intercalation.

Prior work, based on modeling experiments with the computer program KinTekSim, assigned a lower limit of $10 \text{ s}^{-1}$ for the rate of bending (18). Expansion of this KinTekSim modeling exercise in combination with using the obtained rates from the global fit as lower limits (lower limits since this data is collected at 4 °C), allows us to further probe the influence of reverse rates on the population of the intermediates of bent and intercalated DNA. Figure 8c shows the simulated FRET data for the cognate substrate (solid line). When the rate constants for unintercalation are increased 2000 fold (dashed line; as seen for noncognate substrates A3 & A4) and 4000 fold (dotted line; doubling of observed noncognate rate constants for perspective) the $k_{\text{obs}}$ for the approach to equilibrium increases, while the total amplitude change for the intercalation phase decreases (Figure 8c). We suggest that the noncognate substrates destabilize the intercalated intermediate, thereby populating the bent intermediate to a greater extent. The change in the population distribution for the noncognate vs. cognate substrates tracks very closely with the changes in M.EcoRI’s sequence discrimination. Thus, M.EcoRI’s specificity is predominantly dictated by the reverse rate constants of the intercalation step (Table 1 Figure 6 & 8d). However, since the forward bending and intercalation $k_{\text{obs}}$ rates with A4 and A3 don’t increase linearly with increasing enzyme concentration, the forward transitions may have also been impacted with the noncognate substrates, thereby contributing to the enzyme’s discrimination. The observed temporal order of bending, flipping, then intercalation, along with increase unintercalation for the noncognate substrates suggests that by intercalating residues the enzyme stabilizes a base-flipped complex. In support of this idea we observe increased restacking rate constants for noncognate substrates which closely match the unintercalating rate constants. The caveat to this argument is that flipping and restacking data are obtained with a base analogue which may skew the rate constants for flipping, yielding the mechanistic step order of flipping preceding intercalation.

Our proposal that DNA sequence-dependent modulations of the enzyme’s partitioning away from the intermediate prior to methylation (Figure 8d) is consistent with M.EcoRI using a facilitated diffusion mechanism to locate its specific site (8;17). Noncognate sites do not cause the enzyme to dissociate from the DNA, rather stabilizing enzyme/DNA intermediates which occur prior to the catalytically relevant complex, involving a bent, flipped and intercalated conformation. This form of kinetic proof reading allows for the efficient selection of the cognate site over the noncognate sites and involves conformational checkpoints which contribute to the enzyme’s specificity prior to catalysis.

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FIGURE LEGENDS

Scheme 1. Kinetic mechanism of M.EcoRI. E\*DNA* indicates a bent enzyme DNA complex. E\*DNA** indicates a base-flipped complex. E\*DNA*** indicates an intercalated complex. DNA-CH3 indicates that the N6 of the adenine is methylated. FRET was used to measure bending and intercalation. Flipping was measured by 2-aminopurine fluorescence. The chemistry step is measurable by single turn-over experiments. Brackets indicate that flipping and intercalation may be occurring simultaneously due to the biphasic signal for 2AP flipping. Cartoons are provided to facilitate conceptualization of the various intermediates in the reaction mechanism.

Figure 1. Equilibrium FRET of 14mer 5´-Alexa-488 / 5´-TAMRA DNA. Dark line = 20 nM DNA alone, light line = 20 nM DNA + 200 nM M.EcoRI + 1 µM Sinefungin. (A) Cognate (GAATTC), (B) A6 (GAATCC), (C) representative of A3 (GGATTC) and A4 (GAATTT), which show no observable change in FRET. Cognate and A6 DNA show the characteristic decrease in energy transfer previously demonstrated to be due to an intercalation event.

Figure 2. Stopped flow observation of bending and intercalation of cognate and noncognate DNA by M.EcoRI at 22 ºC monitoring donor fluorescence. 14mer 5´-Alexa-488 / 5´-TAMRA was mixed rapidly with M.EcoRI preincubated with sinefungin (A) 20 nM cognate (GAATTC) mixed with 500 nM M.EcoRI. (B) 30 nM A6 (GAATCC) mixed with 400 nM M.EcoRI. (C) 100 nM A4 (GAATTT) mixed with 500 nM M.EcoRI. (D) 100 nM A3 (GGATTC) mixed with 500 nM M.EcoRI. A4 and A3 clearly show a decrease in the observable fluorescence prior to the intercalation phase of the fluorescence, while A6 fluorescence is similar to cognate DNA. The light gray line in figure A is of cognate DNA alone. The noncognate DNA alone stopped flow observations have been omitted for clarity.

Figure 3. Stopped flow observation of bending and intercalation of cognate DNA by M.EcoRI at 4 ºC. (A) 20 nM cognate 14mer 5´-Alexa-488 / 5´-TAMRA was rapidly mixed with 100, 300, and 500 nM M.EcoRI preincubated with 6 µM sinefungin. (B) Concentration profile for the rate of intercalation as observed by FRET. The rate of intercalation does not increase linearly with increasing enzyme concentration.

Figure 4. Stopped flow observation of bending and intercalation of noncognate DNA by M.EcoRI at 4 ºC. (A) 100 nM A4 noncognate DNA rapidly mixed with 500 nM M.EcoRI-sinefungin. (B) 100 nM A3 noncognate DNA rapidly mixed with 500 nM M.EcoRI-sinefungin. A and B Insets shows the intercalation phase for A4 and A3 have no concentration dependence with 500, 700, 900, 1100 nM M.EcoRI Insets. Residual analysis for a single exponential equation was done for data between 60-500 ms.

Figure 5. Base flipping kinetics for cognate DNA by M.EcoRI. (A) 200 nM cognate 14mer 2-aminopurine (2AP) containing DNA was rapidly mixed with 800, 900, 1000, 1100 nM M.EcoRI (data bottom to top) preincubated with 6 µM sinefungin at 22 ºC. The First phase shows a concentration dependence, while the second phase is independent of concentration. (B) At 4 ºC 100 nM cognate 14mer 2-aminopurine (2AP) containing DNA was rapidly mixed with 0, 500, 1000, 1500, 2000 nM M.EcoRI preincubated with 6 µM sinefungin, bottom to top. After removal of the lag portion all data fit to a single exponential equation. (C) Concentration profile of the flipping signal. The rate of flipping is linear with increasing enzyme concentration yielding a k_{on} = 3.6*10^7 M^{-1}s^{-1}. (D) 100 nM DNA with 2000 nM M.EcoRI experiment at 4 ºC out to 20 seconds fits to a double exponential. The second phase in the flipping signal is not observable at 4 ºC until the collection time is extended past 5 seconds hence the 20 second experiment.

Figure 6. Stopped flow measurements of unbending / unintercalation for noncognate DNA (A) Cognate, (B) A6, (C) A4, and (D) A3. Cognate measurements were performed with 30 nM M.EcoRI-30 nM DNA-
1 μM Sinefungin rapidly mixed with 1.5 μM (50-fold excess) competitor DNA (unlabeled cognate DNA). Noncognate measurements were performed with 60 nM M.EcoRI-60 nM DNA-1 μM Sinefungin, rapidly mixed with 1.5 μM (25-fold excess) competitor DNA (unlabeled cognate DNA). The change in donor fluorescence was monitored. Rapidly mixing the enzyme/DNA/cofactor preformed complex with only buffer resulted in no change in fluorescence.

Figure 7. Restacking of the flipped out base for (A) cognate, (B) A6 noncognate, and (C) A4 noncognate. A preformed complex of 100 nM M.EcoRI-100 nM DNA-5 μM Sinefungin was rapidly mixed with 5 μM (50-fold excess) competitor DNA (unlabeled cognate DNA), and the loss of 2AP fluorescence was monitored. Rapidly mixing the enzyme/DNA/cofactor preformed complex with only buffer resulted in no change in fluorescence.

Figure 8. Data modeling of FRET experiments confirms that the kinetics for unintercalation can account for the differences in equilibrium FRET between the noncognate and cognate enzyme complexes. (A) Global fitting analysis of cognate DNA bending and intercalation by M.EcoRI at 4 °C. Rate constants (s⁻¹) obtained for a two step reaction mechanism are k₁ = 0.018 ± 0.0015, k₂ = 120 ± 17, k₃ = 97 ± 1.0, k₄ = 0.67 ± 0.061. Simulations using 20 nM DNA and three enzyme concentrations of 300 nM 500 nM and 700 nM are shown light gray to black respectively. Compare to Figure 3a. (B) Global fitting analysis of cognate DNA base flipping by M.EcoRI at 4 °C. Rate constants (s⁻¹) obtained for a two step reaction mechanism are k₁ = 0.079 ± 0.020, k₂ = 380 ± 170, k₃ = 550 ± 130, k₄ = 47 ± 17. Simulations using 100 nM DNA and four enzyme concentrations of 500 nM 1000 nM and 2000 nM are shown light gray to black respectively. Compare to Figure 5b. (C) Solid line = modeling with cognate rate constants, dashed line = modeling with a 2000-fold increase in the unintercalation rate constant (as observed for noncognate substrate, A3 & A4), dotted line = modeling with a 4000-fold increase in the unintercalation rate constant. The 4000 fold increase (dotted line) was added to show that an even greater increase in unintercalation will increase the approach to equilibrium and reduce the total amplitude change for the intercalation signal. Note that the concentration of DNA for this simulation has increased to 100 nM to normalize the amplitude changes between cognate and noncognate substrates. (D) An illustrative energy diagram of reaction intermediates for cognate and noncognate substrates. Solid line = cognate DNA, dashed line = noncognate DNA, TŞ chem = transition state to the methylation step. Energy wells for base flipped and intercalated DNA are raised for the noncognate DNA substrates relative to the cognate DNA substrate since the reverse rate constants change significantly with noncognate DNA (Figures 6 & 7), with minor changes in the forward rate constants (Figure 2). Only differences between the cognate and noncognate substrates at the base-flipped and intercalated intermediates are revealed in the energy diagram as these are the only intermediates definitively addressed by the data from this work. No other intermediates are changed as we have no evidence to suggest other wise.
Table 1: Reverse rate constants for the transition of bending and intercalation at 22 °C

|       | $k_{off}$ s$^{-1}$ | NonCog/Cog |
|-------|-------------------|-------------|
| CBM   | 0.016             | —           |
| A6    | 7.9 ± 1.2         | 490         |
| A4    | 40 ± 1.5          | 2500        |
| A3    | 30 ± 2.5          | 1900        |
Table 2: Reverse rate constants for the base flipping transition at 22 °C

|        | Relative Amplitude 1 | Rate constant 1 (s⁻¹) | NonCog/Cog | Relative Amplitude 2 | Rate constant 2 (s⁻¹) | NonCog/Cog |
|--------|----------------------|-----------------------|------------|----------------------|-----------------------|------------|
| CBM    | 38 %                 | 0.089                 | —          | 62 %                 | 0.022                 | —          |
| A6     | 56 %                 | 2.4                   | 27         | 44 %                 | 0.92                  | 42         |
| A4     | 29 %                 | 220                   | 2500       | 71 %                 | 17                    | 772        |
| A3     | nd                   | nd                    | —          | nd                   | nd                    | —          |

nd = not determined
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FIGURES

Scheme 1
Figure 1

A

Relative Intensity

GAATTC

Wavelength (nm)

B

Relative Intensity

GAATCC

Wavelength (nm)

C

Relative Intensity

GAATTT & GGATTC

Wavelength (nm)
Figure 2

A

Relative Intensity

GAATTC

Time (sec)

B

Relative Intensity

GAATCC

Time (sec)

C

Relative Intensity

GAATT

Time (sec)

D

Relative Intensity

GGATTC

Time (sec)
Figure 5

A

Relative Intensity

Time (sec)

0.0 0.5 1.0 1.5 2.0

B

Relative Intensity

Time (sec)

0.00 0.02 0.04 0.06 0.08 0.10

C

k.obs s⁻¹

Concentration M

0 1×10⁻⁶ 2×10⁻⁶

D

Relative Intensity

Time (sec)

0 5 10 15 20 25
Figure 7

A

Relative Intensity

GAATTC

Time (sec)

B

Relative Intensity

GAATCC

Time (sec)

C

Relative Intensity

GAATT

Time (sec)
Figure 8
Conformational transitions as determinants of specificity for the DNA methyltransferase EcoRI
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