Base flipping was previously shown to bend its cognate DNA sequence by 52° and stabilize the target adenine in an extrahelical orientation. We describe the characterization of an EcoRI DNA methyltransferase mutant in which histidine 235 was selectively replaced with asparagine. Steady-state kinetic and thermodynamic parameters for the H235N mutant revealed only minor functional consequences: DNA binding affinity ($K_D^{DNA}$) was reduced 10-fold, and $k_{cat}$ was decreased 30%. However, in direct contrast to the wild type enzyme, DNA bending within the mutant enzyme-DNA complexes was not observed by scanning force microscopy. The bending-deficient mutant showed enhanced discrimination against the methylation at nontarget sequence DNA. This enhancement of enzyme discrimination was accompanied by a change in the rate-limiting catalytic step. No presteady-state burst of product formation was observed, indicating that the chemistry step (or prior event) had become rate-limiting for methylation. Direct observation of the base flipping transition showed that the lack of burst kinetics was entirely due to slower base flipping. The combined data show that DNA bending contributes to the correct assembly of the enzyme-DNA complex to accelerate base flipping and that slowing the rate of this precatalytic isomerization can enhance specificity.

The formation of protein-DNA complexes frequently requires distortion of the DNA from a B-conformation (1). Recently, the unstacking and subsequent extrahelical stabilization of a DNA base ("base flipping") has expanded the examples of enzyme-mediated DNA deformations. Extranshelical base stabilization enables access of the catalytic enzyme groups to inaccessible target DNA residues, providing an elegant mechanism whereby discrimination can be achieved. For S-adenosyl-L-methionine-independent DNA methyltransferases, base flipping also allows the modification of a large number of DNA sequences without the stereochemical constraint of cofactor-DNA interactions.

X-ray crystal structures reveal that the C6 cytosine-specific type II bacterial DNA methyltransferases HhaIII DNA cytosine methyltransferase and HhaI DNA cytosine methyltransferase stabilize an extrahelical base without significantly bending DNA (2, 3). Similarly, uracil DNA glycosylase stabilizes an extrahelical uracil without further distortion of the DNA configuration (4). In contrast, T4 endonuclease V recognizes thymine dimers, flips an adenine opposite to the lesion, and also bends the DNA by 60° (5). Functional studies of the N6-adenine EcoRI DNA methyltransferase (M.EcoRI) and the related EcoRV DNA adenine methyltransferase show that these adenine-specific base flipping enzymes also bend DNA (6, 7).

The enzyme-DNA cocrystal structures provide compelling evidence for the stabilization of distorted DNA conformations; yet, surprisingly little is known concerning the coordination between sequence-specific DNA base recognition and flipping. Determining the temporal coordination between the binding of the enzyme to the DNA and the distortion of the DNA conformation is clearly required to elucidate the functional consequences. A severe limitation in our understanding of these processes derives from the fact that both structural and steady-state kinetic studies overlook any conformational intermediates.

We report the identification and functional characterization of a bending-deficient M.EcoRI mutant (H235N M.EcoRI). Relatively minor functional consequences were revealed for H235N M.EcoRI at the level of the steady-state kinetic and thermodynamic parameters. In contrast, transient kinetic methods revealed a change in the rate-limiting step in the reaction that resulted in a profound enhancement in the discrimination against methylation at nontarget sequence DNA. Our data show that DNA bending may establish specificity by correctly assembling the enzyme-DNA complex to induce base flipping and thereby enhance catalysis.

MATERIALS AND METHODS

Site-directed Mutagenesis, Enzyme Expression, and Purification—WT M.EcoRI and H235N M.EcoRI were purified as described previously (8) from Esherichia coli strain MM294 harboring the overexpression constructs pXRI (9) and pKM(H235N) grown in LB medium supplemented with 100 μg/ml ampicillin. Enzyme expression was induced at early log phase (A600 = 0.4) by the addition of isopropyl thiogalactoside to a final concentration of 1.0 mm. pKM(H235N) was created by site-directed mutagenesis utilizing the method of Kunkle (10). Single-stranded uracil-containing DNA served as template for in vitro second strand synthesis with the following oligonucleotide: 5'-...
GTCTTATTAGGCTAAAGACTGCCC-3’. This oligonucleotide was used to convert codon 235 from histidine (CAU) to asparagine (AAU). Following plaque isolation and subcloning, the entire gene was sequenced on both strands to confirm that no undesired codon changes were present. Concentrations of the purified WT M.EcoRI (107 μM) and H235N M. EcoRI (5% w/w of protein) were detected spectrophotometrically utilizing the published extinction coefficient (11).

Confirmation of Amino Acid Replacement at Position 235—To confirm the presence of the histidine to asparagine substitution at the protein level, H235N M.EcoRI was digested with chymotrypsin followed by electrospray ionization mass spectrometric (ESI/MS) analysis (12, 13). Digestion was performed in 50 mM NH4HCO3 (pH 7.8) buffer for 4 h and terminated by heating the samples at 100 °C for 5 min. The digested protein samples (~100 pmol) were separated on a 1.0 x 150-mm 300-Å RP-C18 column (Microm BioResources, Inc.) utilizing a flow rate of 50 μl/min with a linear gradient (0–50% acetonitrile) in the presence of 0.1% trifluoroacetic acid. Eluted peptides were detected by ESI/MS in positive ionization mode over the mass range 300–19270, and the source temperature was maintained at 160 °C. The results of the ESI/MS analysis are summarized in Table I.

DNA Purification—Standard phosphoramidites and DNA synthesis were purchased from Glen Research and Amersham Pharmacia Biotech, respectively. Oligonucleotides were prepared on a Cyclone 8400 Plus DNA synthesizer (Milligen/Biosearch, Burlington, MA) using β-cyanophosphoramidites and purified by reverse phase high pressure liquid chromatography. DNA purity was assessed by 260 nm absorbance and visualized by autoradiography following 20% denaturing polyacrylamide gel electrophoresis. Concentrations were determined spectrophotometrically. The following oligonucleotides (14-mers) were used and contained a centrally located EcoRI site: top strand, oligonucleotide 1, 5’-GCGGAATTCCGGC-3’, and oligonucleotide 2, 5’-GCGGAATTCCGGC-3’; bottom strand, oligonucleotide 3, 5’-CCGGAATTCCGGC-3’, and oligonucleotide 4, 5’-CCGGAATTCCGGC-3’ (X represents 2-aminoarabinose, and A represents N9-methyladenosine). Oligonucleotides 1 and 3, 1 and 4, and 2 and 4 were annealed to form unmodified, hemethylated, and 2AP-substituted hemethylated 14-base pair DNA duplexes, respectively.

Thermodynamic Dissociation Constant Determinations (KdDNA and KAdoMet)—All thermodynamic binding, steady-state, and stopped-flow fluorescence experiments were performed with the cofactor analog sinecoccusin, and the catalytic cycle is limited to events leading to the chemistry step. Gel mobility shift assays were performed to determine KdDNA as described (14). Briefly, binding solutions contained 100 mM Tris, 10 mM EDTA, 1 mM DTT, 100 mM NaCl, 5% glycerol, 20 μM sinecoccusin, 50 μM 2AP-labeled oligonucleotide, and varied enzyme concentration. All preincubations were done at 20 + 2 °C for 30 min prior to loading the binding reactions onto a prerunning 12% polyacrylamide gel electrophoresis gel followed by electrophoresis at 4 °C for 1 h at 200 V. Ectrophoresis at 4 °C was shown to be very important for the stabilization of the H235N M.EcoRI-DNA complexes during electrophoresis to minimize complex dissociation (data not shown). The AdoMet-dependent quenching of protein tryptophan fluorescence was used to determine KAdoMet (15).

Scanning Force Microscopy Analysis—Enzyme-DNA complexes were observed by SFM with a Nanoscope III (Digital Instruments, Santa Barbara, CA) in tapping mode using Digital Instruments n silicon tapping mode probes at a resonance frequency of 300–400 kHz (6, 16). A 300-base pair DNA substrate containing a centrally located EcoRI site was generated by polymerase chain reaction amplification of plasmid pGEM-3. Complexes were prepared in a buffer containing 3 mM DNA, 20 mM Hepes (pH 7.6), 1 mM DTT, 10 μM sinecoccusin, and 50–100 nM mutant enzyme. Enzyme-DNA complexes were analyzed using the Nanoscope III software. Images were enlarged, two tangential lines were drawn along the center of the protruding DNA arms on both sides of the protein, and the bend angles were measured. The bend angle reported (0° to 28°) was calculated from the measurement of 130 complexes.

Steady-state Fluorescence Emission Spectra—Steady-state fluorescence emission spectra of hemimethylated DNA containing 2AP substituted at the target base were recorded and used to monitor the unstacking of the target base within the WT M.EcoRI and H235N M.EcoRI complexes as described (9). Briefly, excitation was at 310 nm, and fluorescence emission was scanned from 330 to 430 nm. The titration of the fluorescence emission intensity at λmax (a blue shift from ~370 to 360 nm is observed for both enzymes) was plotted versus enzyme concentration.

Stopped-Flow System and Photon Counting Fluorescence Detection—The stopped-flow unit and fluorescence detection system utilized for these stopped-flow studies have been described in detail elsewhere (14). Excitation was at 310 nm, and the fluorescence emission was collected through a 360-nm cut-off filter (Hoya Optics type L36). 2× solutions of enzyme (diluted into a degassed buffer containing 100 mM Tris, 10 mM EDTA, 100 mM NaCl, 1 mM DTT at pH 7.5), double-stranded duplex DNA, and buffer alone each were loaded into three separate syringes. Reactions were initiated by mixing equal volumes (100 μl) of enzyme and DNA solutions. Multiple runs were used to increase the signal to noise ratio. Background measurements were made by measuring the fluorescence emission of “preshot” controls that included buffer or duplex 2AP-containing DNA alone prior to mixing together the enzyme and DNA solutions in the stopped-flow.

In Vitro Methylation Specificity Analysis—The incorporation of [3H]-methyl groups into DNA under “standard” and EcoRI “star” reaction conditions were used to evaluate the discrimination against the methylation of the WT and H235N M.EcoRI Standard reaction conditions included 100 mM Tris (pH 8.0), 10 mM EDTA, 0.2 mg/ml bovine serum albumin, 1.0 mM DTT, 3.0 mM DNA, 3.2 mM enzyme, and 1.68 μM [3H]AdoMet. EcoRI star conditions were similar except that the solutions contained 152 mM enzyme and included 20% glycerol (19). Initial velocity studies assessed with the filter-binding assay of the two proteins at 5 μM [3H]AdoMet showed no significant differences from the results obtained at 1.68 μM [3H]AdoMet (data not shown). The DNA substrate, plasmid pBluescriptSK (+) II, was digested with HaeIII endonuclease to generate the fragments shown in Fig. 6. Following incubation of the enzyme and DNA, the restriction fragments were separated by electrophoresis and detected by fluorography as described (19).

RESULTS

Protein chemical modification data showed that incubation of M.EcoRI with the histidine-specific reagent diethylpyrocarbonate resulted in time-dependent enzyme inactivation. This inactivation occurred over 10-fold more slowly in the presence of DNA, strongly indicating that a histidine might be involved in DNA binding (2). Histidine 235 lies within a conserved motif common to the type II bacterial DNA methyltransferases (motif VIII) (20). An analysis of the adenine-specific TaqI DNA adenine methyltransferase-AdoMet covalent structure showed that the homologous peptide region forms a surface loop within the putative DNA binding cleft of the enzyme (21) (Fig. 1). Further structure-guided analysis of the HhaI DNA cytosine methyltransferase-DNA covalent complex revealed that motif VIII directly interacts with the extrahelical cytosine and the phosphate group 5’ to the target leading to the movement of the target base into and correct assembly of the enzyme active site (2). Given its plausible importance, histidine 235 was replaced with asparagine by site-directed mutagenesis removing the acid-base and hydrogen bonding properties of the histidine imidazole with the retention of some of the steric characteris-
tics. The H235N mutant enzyme was expressed and purified to apparent homogeneity, and the asparagine substitution was confirmed at the protein level by electrospray ionization mass spectrometry (Table I).

Steady-state kinetic parameters for WT and H235N M.EcoRI were determined, and the results are summarized in Table II. These data reveal that kcat for H235N M.EcoRI was reduced 30% and that the KdDNAa was increased approximately 10-fold. The specificity constant (kcat/KdDNA) for the modification of the target site was decreased approximately 14-fold for H235N M.EcoRI due predominantly to the changes in the KdDNA.
**TABLE I**

| CT peptide                  | Theoretical mass | Observed mass |
|-----------------------------|------------------|--------------|
| WT M.EcoRI                  | 1686.98          |              |
| H235N M.EcoRI               | 1682.96          |              |

**TABLE II**

| Parameter                     | WT M.EcoRI | H235N M.EcoRI |
|-------------------------------|------------|--------------|
| **K_cat (s⁻¹)**               | 0.11 (0.006) | 0.076 (0.009) |
| **K_D (nM)**                  | 28 (7)     | 274 (56)     |
| **K_adm (µM)**                | 0.27 (0.06) | 1.98 (0.27)  |
| **K_Dm (µM)**                 | 10 (2.0)   | 47 (12.0)    |
| **K_DN_specific** (nM)        | 1.08 (0.1)  | 10.56 (1.7)  |
| **K_DN/AAPDG** (nM)           | 0.51 (0.1)  | 1.6 (4.6)    |
| **K_DN/AAPDG** (nM)           | 0.29 (0.1)  | 3.13 (0.6)   |
| **K_DN/AAPDG** (nM)           | 12.8 (5.4)  | No shift     |

*From Ref. 17.*

*From Ref. 14.*

addition, H235N M.EcoRI showed a 5-fold increase in K_adm, indicating a slightly decreased affinity of the enzyme for its cofactor.

Two methods were used to evaluate equilibrium base flipping by the H235N mutant. Equilibrium dissociation constants (K_DN) were determined by gel mobility shift assays for WT and H235N M.EcoRI binding to a 14-base pair DNA duplex containing a centrally located hemimethylated EcoRI recognition site (Fig. 2). These binding data revealed that the bending-deficient mutant bound the DNA substrate 10-fold weaker than WT M.EcoRI (Table II). In addition to unmodified DNA, equilibrium dissociation constants were also determined for the binding of H235N M.EcoRI to DNA containing base analogs or an abasic site incorporated at the target base. As shown in Table II, tighter binding to DNA containing destabilized target base pairs was observed for H235N M.EcoRI. In contrast, no stable enzyme-DNA complexes could be detected by gel shift assay when the target base was substituted with 2,6-diaminopurine, an analog that increases the number of H bonds at the target base.

In addition, spectroscopic experiments were performed to assess the extrahelical stabilization of the target adenine. 2AP is a highly fluorescent adenine isomer that is an extremely sensitive spectroscopic probe for base flipping by M.EcoRI (9, 14). The fluorescence emission intensity of 2AP is highly quenched within double-stranded DNA relative to the nucleoside, due predominantly to intrastrand base stacking interactions (22). Following the titration of DNA substituted with 2AP at the target base with saturating H235N M.EcoRI, a large increase in the fluorescence intensity and a 10 nm blue shift in the emission λ_max were observed for both enzymes (Fig. 3). This significant increase in the 2AP fluorescence emission is consistent with an unstacking of the target base within the enzyme-DNA complexes. The quantum yield of the H235N M.EcoRI bound 2AP-substituted duplex was approximately ~30% greater than that observed for the WT enzyme-DNA complex.

DNA bending was visualized directly by SFM of enzyme-DNA complexes. This method can be used to calculate the statistical distribution of bend angles determined for a population of enzyme-DNA complexes (16). For WT M.EcoRI this SFM-based method reveals a bend angle (52°) identical to that of the more commonly used gel retardation analysis (6). Fig. 4 shows representative H235N M.EcoRI-DNA complexes (inset) and a histogram derived from direct measurement of the bend angle for 130 enzyme-DNA complexes. The calculated bend angle (0 ± 26°) was similar to that of unliganded double-stranded DNA determined under identical conditions by this method (6). The histogram in Fig. 4 clearly shows DNA bending was drastically altered for H235N M.EcoRI and that bent enzyme-DNA complexes were highly represented.

For WT M.EcoRI the methylation of DNA sites related by a single base pair substitution (e.g. 5’-AAATTC-3’) occurs up to...
20,000-fold more poorly than the target site (23). However, nontarget site methylation is greatly enhanced in the presence of solutes such as glycerol (19). Standard reaction conditions containing a relatively low enzyme concentration (3.2 nM) led solely to the detectable modification of the DNA restriction fragment containing the \textit{Eco}RI site by both enzymes (Fig. 5, \textit{30 min lanes}). However, at higher enzyme concentration (152 nM) in the presence of 20% glycerol, significant and time-dependent methylation of nontarget sites by WT \textit{M.\ Eco}RI was observed (Fig. 5, \textit{WT}, 10, 40, and 100 min lanes). Under identical reaction conditions \textit{H235N M.\ Eco}RI incorporated significantly fewer methyl groups at nontarget sequence DNA (Fig. 5, \textit{H235N}, 10, 40, and 100 min lanes). Under conditions of high enzyme concentration and excess DNA, WT \textit{M.\ Eco}RI shows a presteady-state “burst” of product formation corresponding to $k_{\text{methylation}}$ ($\sim 20$–$40$ s$^{-1}$) (18). This rapid burst phase is followed by a slower product release step that is rate-limiting for turnover (17). Manual presteady-state burst experiments were performed for both enzymes. As anticipated WT \textit{M.\ Eco}RI showed a burst of product formation that was followed by a slower steady-state rate of product accumulation (Fig. 6). In direct contrast, \textit{H235N M.\ Eco}RI showed no burst, indicating that the chemistry step or a prior event was rate-limiting for methylation by the mutant. These rapid quench experiments clearly indicate a change in the rate-limiting catalytic step for the bending-deficient mutant. Compar-
methyltransferase. Preincubation of 3.0 mM enzyme, 100 mM Tris, 10 mM EDTA, 1 mM DTT, 0.2 mg/ml bovine serum albumin, and 50 mM [\(^{3}H\)]S-adenosyl-L-methionine at 25 °C for 5 min. This was followed by a 50-fold dilution into an identical reaction mixture containing 200 nM of the DNA substrate. Reaction aliquots were quenched by spotting onto DE81 paper and processed as described (32).

For WT M.EcoRI base flipping is followed by the very fast methylation of the extrahelical base (14). Therefore under presteady-state conditions the precatalytic isomerization of the enzyme-DNA complex controls the rate of product formation. Hence, the lack of a burst for the mutant might not reflect a perturbation in methyl transfer to the extrahelical base per se but rather may reflect a slower transition of the target base to the enzyme active site. To directly determine whether the lack of burst kinetics was due to a perturbation of the chemistry step or a prior event, real time measurements of the base flipping transition were made. Stopped-flow fluorescence data obtained during the first 0.5 s of the experiment for both WT and H235N M.EcoRI (18) shows that the replacement of histidine 235 with asparagine decreased the apparent methylation rate constant by several orders of magnitude.

For WT M.EcoRI, the time course for product formation was determined by a filter-binding assay. Preincubation of 3.0 mM enzyme, 100 mM Tris, 10 mM EDTA, 1 mM DTT, 0.2 mg/ml bovine serum albumin, and 50 mM [\(^{3}H\)]S-adenosyl-L-methionine at 25 °C for 5 min. This was followed by a 50-fold dilution into an identical reaction mixture containing 200 nM of the DNA substrate. Reaction aliquots were quenched by spotting onto DE81 paper and processed as described (32).

**DISCUSSION**

Conformational changes in enzymes and substrates during catalysis are extremely difficult to characterize mechanistically. The interconversion and processing of the transient enzyme-substrate conformations can be extremely important for catalysis and form a critical part of many theories of how enzymes function (24, 25). Inevitably, a complete understanding of enzyme specificity must include, in addition to a description of the chemical and kinetic mechanisms, a dissection of the conformational mechanism. The study of a conformational mechanism is difficult because the transitions between intermediates often occur extremely rapidly, and no obligate changes in chemical structure are involved. An additional challenge is the identification of the specific amino acids that are critical for the initiation and subsequent stabilization of the precatalytic conformational changes.

**TABLE III**

| Protein | Amplitude | Rate | Amplitude | Rate |
|---------|-----------|------|-----------|------|
|         | \( \mu M \) | \( s^{-1} \) | % | \( s^{-1} \) |
| 0.3     | 46        | 0.56 | 54 | 0.091 |
| 0.6     | 34        | 0.19 | 66 | 0.035 |
| 0.9     | 25        | 0.22 | 76 | 0.045 |

DNA methyltransferases represent a true paradigm for large scale conformational changes induced upon formation of an enzyme-substrate complex: many of these enzymes require both significant DNA bending and base flipping prior to methyl group transfer. We initiated our studies based upon protein chemical modification data that showed a histidine residue to be important for DNA binding by M.EcoRI. Structure-guided sequence analyses of the type II DNA methyltransferases (20) suggested that motif VIII is involved in DNA binding. Histidine 235 lies within this conserved motif and was replaced by asparagine. Preliminary functional analysis of the H235N mutant revealed only small changes at the level of the steady-state kinetic parameters (Table II), implicating only minor perturbations in substrate binding and catalysis. However, in direct contrast to the WT enzyme, SFM analysis of DNA binding showed that H235N M.EcoRI enzyme-DNA complexes were indistinguishable from uncomplexed DNA, demonstrating a drastic difference in DNA bending (Fig. 5).

**DNA bending is frequently suggested to provide specificity (1, 16, 26–28). Interestingly, the bending-deficient mutant showed only a 10-fold weaker binding to its target sequence DNA (Table II). Based upon the frequently suggested importance of DNA bending to enzyme specificity, a much larger impact upon the \( K_{D}^{DNA} \) might have been anticipated. The overall equilibrium dissociation constant for proteins that must induce a change in DNA structure ultimately reflects the binding energy obtained in the distorted complex and the investment of binding free energy required to induce the conformational change. Hence, the relatively small difference in DNA binding free energy between WT and H235N M.EcoRI (\( \Delta \Delta G^{\circ} = +1.4 \text{ kcal mol}^{-1} \)) strongly implies that the energy “cost” to bend the DNA is essentially the same as the binding energy realized.
in the enzyme-bent DNA complex.

To determine whether the lack of DNA bending by the mutant affected the ability of the enzyme to stabilize an extrahelical base, functional analyses of base flipping were performed. $K_{D}^{DNA}$ measurements were determined for the binding of H235N M.EcoRI to DNA containing an abasic site or functionally modified purine bases. Tighter binding to oligonucleotides that destabilized the target base pair was detected (Table II). Similar binding trends have been observed for other DNA methyltransferases (7, 29) and for WT M.EcoRI (14), and our results are consistent with a base flipping mechanism for H235N M.EcoRI. Additional support for this interpretation was obtained from the large increase in the fluorescence emission intensity detected following the binding of H235N M.EcoRI to DNA containing 2AP substituted at the target base (Fig. 4) (9). The fluorescence of the mutant enzyme-DNA complex was ~30% greater than for the wild type, and no significant differences in the emission $\lambda_{\text{max}}$ were observed. This difference in steady-state emission might reflect a difference in the molecular environment surrounding the 2AP probe or may be of thermodynamic origin, and additional experiments are required to distinguish between these possibilities.

The ability of the bending-deficient mutant to bind the DNA, stabilize the extrahelical base, and methylate target sequence DNA without any serious functional impairment in thermodynamic and steady-state kinetic parameters left obscure the consequences of DNA bending. We sought to probe additional effects of the amino acid substitution at the level of substrate discrimination. Under standard conditions, the wild type enzyme is able to discriminate up to 20,000-fold against related DNA sequences (e.g. AAATTC) (23). The fluorography data in Fig. 6 clearly shows that the mutant is dramatically decreased in its ability to methylate such closely related DNA sequences. Although H235N M.EcoRI readily modified the restriction fragment harboring the target (5′-GAATTC-3′) site, little nontarget DNA was methylated even after extended incubation in the presence of higher enzyme concentrations of enzyme (Fig. 5). The significant reduction in nontarget sequence modification was accompanied by a minor decrease in the specificity constant ($k_{\text{cat}}/K_{M}$) for target site methylation (Table II). In sum, H235N M.EcoRI methylated target DNA less efficiently but showed a much greater qualitative discrimination against the methylation at nontarget DNA and therefore greater overall specificity.

The mechanism for this enhanced discrimination must derive from alterations in kinetic steps that are not revealed at the equilibrium binding or steady-state $k_{\text{cat}}$ level. For M.EcoRI, turnover is limited by a step subsequent to methyl group transfer (i.e. product release), and a large decrease upon the chemistry step could be completely obscured by steady-state kinetic data in isolation. To determine whether an impact upon the chemistry step had resulted from the asparagine substitution, the presteady-state rate of product accumulation was investigated by rapid quench methods (Fig. 6). Unlike the burst of product formation detected for WT M.EcoRI, no presteady-state burst kinetics were observed for H235N M.EcoRI. This lack of a burst showed that the chemistry step itself ($k_{\text{methylatio}}$) or a prior event, became rate-limiting for turnover. The single amino acid substitution that decreased the ability of H235N M.EcoRI to stabilize the bent DNA intermediate apparently also resulted in an enzyme-DNA interface that was not correctly or productively assembled for rapid catalysis. However, for WT M.EcoRI base flipping (or a prior step) is rate-limiting for the methylation reaction at target sequence DNA (14). Hence, the apparent decrease in $k_{\text{methylata}}$ may instead derive from a slower transition of the target base to the enzyme active site and not by a perturbation at the level of the chemistry step.

Having identified that the mutant enzyme showed a change in the rate-limiting step we sought to mechanistically distinguish between a direct impact upon the methyl transfer step from an impact upon a precatalytic conformational change. Stopped-flow fluorescence measurements enabled real time observation of the base flipping transition (14). These stopped-flow measurements clearly showed much slower 2AP total intensity time courses for H235N M.EcoRI compared with the wild type enzyme (see inset to Fig. 7) and conclusively demonstrate that the lack of DNA bending strongly decreases the rate of base flipping. Most interestingly, the observed rate constants for the slow 2AP fluorescence enhancement phase were nearly identical to $k_{\text{cat}}$ determined for H235N M.EcoRI under similar reaction conditions (Fig. 6) (Table III). Thus, although no direct comparison of $k_{\text{methylat}}$ for WT and H235N M.EcoRI is provided here, the lack of a presteady-state burst is understandable in the context of the slower flipping kinetics, which are the direct result of the inability of this mutant to bend the DNA.

That slowing the rate of an obligate precatalytic isomerization can enhance enzyme discrimination is consistent with classic theoretical predictions. As described by Ninio (30), either causing “incorrect” substrates to dissociate faster from the E-S complex or slowing down the conversion of the E-S complex to the E-P complex can enhance enzyme discrimination. For M.EcoRI the precatalytic base flipping transition is the dominant selection barrier because it leads to a highly committed methyl transfer step. Slowing the conversion of the E-S complex to the activated intermediate (E-S') effectively provides greater discrimination by allowing more frequent dissociation of the enzyme from the substrate. For the bending-deficient mutant the transition to the activated intermediate is rate-limiting at target sequence DNA, although the overall $k_{\text{cat}}$ is only slightly lower than that observed with WT M.EcoRI.

Further mechanistic insight into the recognition process can be gained by the elucidation of the temporal coupling between the distinct conformational intermediates. For example, does DNA bending facilitate base flipping or is DNA bending a mechanism whereby helical stability can be restored following the unstacking of a DNA base? Direct measurement of the temporal coupling between DNA binding and target base flipping by WT M.EcoRI shows that these processes occur in near synchrony with only a 4 ms delay between binding and flipping (33). Hence, DNA bending must also occur simultaneously with the formation of the enzyme-DNA complex. Although the lack of detectable DNA bending does not preclude H235N M.EcoRI from base flipping, the activation energy required to induce base unstacking apparently is significantly larger in the absence of concomitant DNA bending.

If H235N M.EcoRI is a more discriminating enzyme than the WT M.EcoRI, then why was this single amino acid substitution mutant not selected during the course of evolution? M.EcoRI is part of a bacterial type II restriction modification system, and the companion EcoRI endonuclease cleaves the identical (5′-GAATTC-3′) DNA sequence devoid of methylation. Endonuclease cleavage of nontarget DNA, although rare, is extremely harmful to the host cell. Hence, a more discriminating methyltransferase might leave nontarget sequence DNA exposed to potential cleavage. The selective pressure to protect all EcoRI sites against endonuclease cleavage in vivo requires an extremely efficient methyltransferase, whereas the need to modify some closely related sequence DNA at a decreased but significant level provides an additional level of selection. In sum, these selective pressures have probably both contributed to the evolution of this enzyme.
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