DNA methyltransferases are excellent prototypes for investigating DNA distortion and enzyme specificity because catalysis requires the extrahelical stabilization of the target base within the enzyme active site. The energetics and kinetics of base flipping by the EcoRI DNA methyltransferase were investigated by two methods. First, equilibrium dissociation constants (K_DNA) were determined for the binding of the methyltransferase to DNA containing abasic sites or base analogs incorporated at the target base. Consistent with a base flipping mechanism, tighter binding to oligonucleotides containing destabilized target base pairs was observed. Second, total intensity stopped flow fluorescence measurements of DNA containing 2-aminopurine allowed presteady-state real time observation of the base flipping transition. Following the rapid formation of an enzyme-DNA collision complex, a biphasic increase in total intensity was observed. The fast phase dominated the total intensity increase with a rate nearly identical to k_methylation determined by rapid chemical quench-flow techniques (Reich, N. O., and Mashoon, N. (1993) J. Biol. Chem. 268, 9191–9193). The restacking of the extrahelical base also revealed biphasic kinetics with the recovered amplitudes from these off-rate experiments matching very closely to those observed during the base unstacking process. These results provide the first direct and continuous observation of base flipping and show that at least two distinct conformational transitions occurred at the flipped base subsequent to complex formation. Furthermore, our results suggest that the commitment to catalysis during the methylation of the target site is not determined at the level of the chemistry step but rather is mediated by prior intramolecular isomerization within the enzyme-DNA complex.

Protein-DNA complexes reveal diverse mechanisms leading to sequence-specific interaction. Direct readout of DNA base functionalities within the major groove and the indirect readout of sequence-dependent phosphate backbone geometry are thought to contribute binding discrimination (1, 2). For DNA modification and repair enzymes the correct assembly of active site residues frequently demands the insertion of protein side chains into and rotating of a base completely out of the DNA helix (3, 4). The stabilization of an extrahelical base is often coupled to sequence-dependent DNA base pair rearrangement (5) and DNA bending (6). However, the energetic cost of the enzyme-mediated DNA deformations integrating site-specific recognition and catalysis are only now being elucidated.

The mechanism leading to the stabilization of an extrahelical base is thought to involve a multi-step binding process with discrete conformational intermediates (4, 7). Enzyme-mediated weakening or breakage of Watson-Crick hydrogen bonds at the target base pair and intercalation of amino acid side chains into the DNA helix are likely to be critical to the initiation of the base flipping process (8). The enhanced discrimination provided by the major groove readout of DNA base functional groups (9) appears for DNA-modifying enzymes to require the sterically encumbered process of extruding a base via the DNA minor groove (3, 5). In contrast, DNA repair enzymes use minor groove readout and extrude the base via the DNA major groove (4, 6). Tighter binding to DNA containing mismatches or otherwise modified target base pairs is observed for DNA-modifying enzymes (10–12). Similarly, enhanced specificity (k_cat/K_m) with substrates that reduce the investment of binding energy required to “flip out” a base has been detected for DNA repair enzymes (13, 14). These studies suggest that the energetic cost of base flipping substantially affects the equilibrium for enzyme-DNA complex formation. However, the elusive nature of structural transitions such as nucleotide flipping, base pair rearrangement, and DNA bending combined with the lack of suitable detection methodologies leaves obscure the underlying kinetics and catalytic consequences.

EcoRI DNA methyltransferase (M.EcoRI) catalyzes methyl transfer from S-adenosyl-L-methionine (AdoMet) to adenine N^6 within double-stranded DNA (15). The methyl-transfer step (k_methylation) of this essentially irreversible reaction is significantly faster than k_cat, showing that product release or prior conformational change limits turnover (16). The chemical mechanism proceeds by direct attack of adenine N^6 upon the methylsulfonyl moiety of AdoMet (17), inducing an inversion in configuration of a chiral labeled methyl group (18). The conserved active site residues between the N^6 adenine and N^4 cytosine DNA methyltransferases indicates that these enzymes are likely to share a common mechanism of exocyclic amino modification (19).

2-Aminopurine (2AP) is a strongly fluorescent adenine isomer that is highly quenched within duplex DNA due largely to intrastrand base stacking interactions (20). The sensitivity of the 2AP probe to localized DNA conformation and dynamics has been exploited to monitor insertion and excision kinetics by DNA polymerases (21, 22), RNA polymerases (23, 24), helicase activity (25, 26), and conformational changes within the hammerhead ribozyme (27). 2AP-substituted oligonucleotides re-

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1 The abbreviations used are: M.EcoRI, EcoRI DNA N^6 adenine methyltransferase; DTT, dithiothreitol; 2AP, 2-aminopurine; 2,6-DAP, 2,6-diaminopurine; AdoMet, S-adenosyl-L-methionine.
tained B-form helical parameters and are cleaved by the EcoRI endonuclease (28–30). Using a steady-state 2AP-based base flipping assay, we recently demonstrated that M.EcoRI stabilizes the targeted base extrahelically in a low dielectric environment (7). Herein we extend this assay to the presteady-state, providing assessment of enzyme-assisted base-flipping dynamics continuously in real time. Our data suggest that the rate determining step for methyl-transfer is a first-order isomerization within the enzyme-DNA complex.

EXPERIMENTAL PROCEDURES

Enzyme Expression and Purification—M.EcoRI was overexpressed from pXRI (7) and purified essentially as described (31) with the addition of another anion exchange column (Bio-Rex), yielding an enzyme of >99% homogeneity. The purified enzyme was dialyzed extensively in a buffer containing 100 mM NaCl, 10 mM potassium phosphate, pH 7.0, 1 mM EDTA, 7 mM β-mercaptoethanol, 1 mM sodium azide at 4 °C. The enzyme preparation was confirmed to be free of detectable contaminating AdoMet by restriction analysis of pBR322 DNA (contains an EcoRI site) following incubation of the DNA and the enzyme with and without added AdoMet. In the absence of added AdoMet, the plasmid DNA was linearized following challenge with the EcoRI endonuclease (data not shown). M.EcoRI concentration (108 μM) was determined spectrophotometrically at 280 nm utilizing the published extinction coefficient (15).

Oligonucleotide Synthesis and Purification—Oligonucleotides (14mers) were synthesized on an Biosearch 3810 DNA synthesizer using β-cyanoethyl phosphoramidites. Modified base analogs (N7-methyladenosine-1-β-D-2-deoxyriboside, 2-aminopurine-1-β-D-2-deoxyriboside, nebularine-purine-1-β-D-2-deoxyriboside), 2,6-diaminopurine-1-β-D-2-deoxyriboside, and abasic) were purchased from Glen Research (Sterling, VA), and standard phosphoramidites were from Pharmacia Biotech Inc. Oligonucleotides were purified on a Dynamax C18 reversed-phase PureDNA column (Rainin Instrument Co.). DNA purity was assessed by 260 radiolabeling and visualization by overexposure of 20% denaturing polyacrylamide gel electrophoresis and confirmed to be >99% pure by densitometric analysis utilizing a UVP (San Gabriel, CA) imaging system. Concentrations were determined spectrophotometrically (32). Complementary strands were annealed in 10 mM Tris, 1 mM EDTA, 100 mM NaCl utilizing an MJ Research (Watertown, MA) programmable thermocycler with a slight excess of the unmodified DNA strand. M.EcoRI does not bind single-stranded DNA with detectable affinity (33). The following 14-mer were used: top strands, d(GGCG-ATTCTGCCG) (X = 6-aminopurineadenine), 2-aminopurinadenine, 2,6-diaminopurine, nebularine (purine), or a stable abasic site (spacer); bottom strands, d(GCGCGAATTCGCCG), d(CCGCGGAATTCGCCG), and d(CCGCGZATTTCCCG) (A = N7-methyladenosine, Z = 2-aminopurine). Hemi-methylated substrates were used to facilitate the formation of unique binding orientations.

Equilibrium Dissociation Constants—KDNA values were determined in the presence of the cofactor analog sinefungin essentially as described (34) with minor variations; 100 mM NaCl was used in binding mixtures, all preincubations were done at 20 ± 2 °C for 30 min prior to sample loading onto a prerunning 12% polyacrylamide gel, and electrophoresis was at 4 °C for 1 h at 200 V. These variations minimize dissociation during electrophoresis (data not shown). Following electrophoresis, the relative amounts of free and bound DNA were determined by densitometric analysis of autoradiogram band intensities using a UVP (San Gabriel, CA) imaging system. The percentage of complex was plotted versus enzyme concentration, and the KD values were determined by fitting the data to a standard hyperbolic binding expression using KaleidaGraph 2.1.2 (Adelbeck Software).

Stopped Flow System and Fluorescence Detection—An SFM-3 stopped flow unit containing three stepper-motor driven syringes (Molecular Kinetics, Pullman, WA) with an FC.15 cuvette (50-μl volume) and a high shut hand was used for stopped flow reactions. Fluorescence detection for the stopped flow studies utilized a home-built single photon detector consisting of the following: a Hamamatsu R928 photomultiplier, a 5 × 300-MHz amplifier (Stanford Research SR445, Sunnyvale, CA), a discriminator (Stanford Research SR400) and a multichannel scaler (Tennelec, Model MCS-II, Oak Ridge, TN), interfaced to an 80486 microcomputer. The detection system was activated by an external synch-out pulse from a Molecular Kinetics stepper motor controlling unit. Data acquisition began at least 100 ms before sample mixing. Data were collected using 1–15-ms dwell times in 8000 total channels. A 250-W xenon arc lamp (SPEX Fluorolog model 1681) with fiber optic output directed into the 50-μl cell was used for excitation at 310 nm.

RESULTS AND DISCUSSION

M.EcoRI DNA Binding Affinity Is Strongly Influenced by the Stability of the Target Base Pair—DNA containing destabilized base pairs (abasic, mismatched, or modified bases) are bound tightly by DNA methyltransferases (10–12). This was first observed for the C6 cytosine-specific M.HpaI (10) following the determination of the enzyme-DNA cocrystal structure, which clearly shows that the cytosine to be methylated is stabilized in an extrahelical conformation (3). To further test the hypothesis that the N6 adenine-specific M.EcoRI utilizes a similar base flipping mechanism and confirm our previous conclusions (7), equilibrium dissociation constants (KtDNA) were determined for M.EcoRI binding to a series of oligonucleotides that are modified at the target base pair. A binding isotherm derived from the gel mobility shift data for the 2AP-substituted DNA duplex (inset) is shown in Fig. 1. The dissociation constants (KD), free energy differences relative to the unmodified site (∆Gt), and structures of the modified base pairs are summarized in Fig. 2.

As shown in Fig. 2, substitution of the target base with 2AP or nebularine (purine) resulted in approximately 2-fold tighter binding relative to the unmodified 6-aminopurine (adenine) base. The purine substitution decreases the number of Watson-Crick hydrogen bonds, thereby reducing the energetic cost of base pair disruption. The 2AP/thymidine base pair maintains two hydrogen-bonds (28), but undergoes spontaneous base pair opening 6-fold more rapidly than an adenine/thymidine base pair (35). In contrast, replacement of the target adenine with 2,6-diaminopurine (2,6-DAP) decreased complex stability approximately 12-fold (Fig. 2). The 2,6-DAP/thymidine base pair (36, 37), increases to three the number of hydrogen bonds to be broken during base pair disruption. These analogs (purine derivatives) form stable base pairs with the partner thymidine; therefore, the nearest neighbor stacking interactions are expected to remain relatively unperturbed (38). However, the
thymine : 2,6-diaminopurine

\[
K_D^{DNA} = 12.80 \text{ nM (5.43)} \\
\Delta G^0 = +1.46 \text{ kcal/mol}
\]

thymine : adenine

\[
K_D^{DNA} = 1.08 \text{ nM (0.10)} \\
\Delta G^0 = 0 \text{ kcal/mol}
\]

thymine : purine

\[
K_D^{DNA} = 0.61 \text{ nM (0.11)} \\
\Delta G^0 = -0.31 \text{ kcal/mol}
\]

thymine : H (abasic)\(^{pB}\)

\[
K_D^{DNA} = 0.26 \text{ nM (0.17)} \\
\Delta G^0 = -0.82 \text{ kcal/mol}
\]

\[
K_D^{DNA} = 0.29 \text{ nM (0.11)} \\
\Delta G^0 = -0.78 \text{ kcal/mol}
\]

**FIG. 2.** \(K_D^{DNA}\) and \(\Delta G^0\) determinations for enzyme-DNA interactions and structures of the modified target base pairs. \(K_D^{DNA}\) and standard error (shown in parentheses) were determined as described under “Experimental Procedures.” The binding free energy differences, relative to the unmethylated DNA duplex, for the ternary (enzyme-DNA-sinefungin) complexes were calculated from \(\Delta G^0 = -RT \ln(K_D^{DNA,mod} / K_D^{DNA,unmod})\), where \(R\) is the universal gas constant, \(\cdot\) hemi-methylated abasic duplex; \(\cdot\) unmethylated abasic duplex.

The overall inverse correlation between \(K_D^{DNA}\) and the strength of the base pair (Fig. 2) supports a base flipping mechanism for M.EcoRI (10–12), allowing limits to be set regarding the energetic cost of DNA distortion. Moreover, these results show that the disruption of the target base pair is a dominant energetic barrier to the tightly bound complex. The lack of steric exclusion of the modified target bases from the enzyme active site suggests that once the base is positioned extrahelically, the primary determinant for methyl-transfer is the presence of the \(N^6\) amino nucleophilic center. The minimal substrate thus appears to be a flippable purine base with an amino group at the \(N^6\) position. However, equilibrium binding determinations reveal only the sum of numerous thermodynamic effects and leave unresolved the rate of the base flipping step and the relevance of extrahelical base stabilization to sequence specificity and the catalytic cycle.

**Real Time Analysis of Base Flipping by Stopped Flow Fluorescence of 2AP**—Steady-state fluorescence of 2AP-substituted DNA showed a 14-fold increase in fluorescence emission intensity following titration of double-stranded DNA containing 2AP at the target base with saturating M.EcoRI (7). Although enzyme-assisted stabilization of an extrahelical base can only be confirmed by an X-ray diffraction structure of an enzyme complexed with double-stranded DNA, the large increase in 2AP fluorescence intensity clearly indicates an unstacking of the probe within the complex. Total intensity stopped flow fluorescence time courses of M.EcoRI interaction with 2AP-substituted duplex DNA are shown in Fig. 3. M.EcoRI was preincubated with the cofactor analog sinefungin (43) prior to mixing with the DNA. The biphase total intensity signal change (Fig. 3, upper panel) indicated that at least two kinetic processes were occurring. Approximately 75% of the observed signal increase was associated with a rate constant of \(21 \pm 2\) s\(^{-1}\), and the remaining 25% slowly increasing at 0.6 s\(^{-1}\). Stopped flow total intensity data obtained with 0.6, 1.2, and 4.8 \(\mu\text{M}\) M.EcoRI each yielded nearly identical fractional amplitude changes (75 \(\pm \) 2%) and rate constants for both phases. The fast phase of the 2AP fluorescence enhancement showed a weak dependence on enzyme concentration. In contrast, the slow phase was demonstrated to be concentration-independent. To directly determine the kinetic coupling between DNA binding and base flipping, spectroscopic binding anisotropy experiments were performed using oligonucleotides extrinsically labeled with the fluorescent probe Rhodamine-X. These stopped flow anisotropy experiments revealed that the fast 2AP fluorescence increase occurred subsequent to a more rapid DNA binding event. These results show that for the adenine-specific M.EcoRI, the base flipping process does not involve the capture of an extrahelical base but rather occurs subsequent to the assembly of the enzyme onto the target DNA site.

Because the intranstrand base stacking of 2AP within the DNA contributes significantly to the quenching of this probe (20), the dominant (75%) fast phase was probably associated with...
with a transition that directly disrupted base stacking interactions (i.e. the base flipping phase). This phase occurred at a rate nearly identical to the measured $k_{\text{methylation}}$ (24 ± 5 s⁻¹) determined for hemimethylated DNA by rapid chemical quench-flow methods (16). The slow (0.6 s⁻¹) intramolecular transition may reflect a rearrangement of the extrahelical base within the enzyme active site. The fact that the relative amplitudes of both phases are concentration-independent is consistent with a sequential base flipping mechanism. Thus, the biphasic nature of the total intensity increase (Fig. 3, upper panel) may be the result of a sequential process involving rapid targeted base stacking disruption, transition of the unstacked base to the active site pocket, and finally reorientation of the extrahelical base within the enzyme active site.

**Base Restacking and Enzyme-DNA Complex Dissociation**—Further support for the existence of two intramolecular kinetic phases associated with the base flipping process was obtained by examination of the off-rate kinetics. Rapid mixing of a preformed M.EcoRI-2AP-containing DNA complex with a 100-fold molar excess of the tightly bound abasic DNA duplex (Fig. 2) resulted in a biphasic decrease in the 2AP total intensity (Fig. 3, lower panel). Double exponential solutions (30% exp.(-0.011t) + 70% exp.(-0.0017t)) were statistically superior to monophasic fits at a confidence level >99.99% using an F-statistic test criterion (44). This fluorescence quenching clearly reflects the restacking of the extrahelical base within the DNA double helix. Interestingly the recovered off-rate amplitudes from this “flip off” experiment match very closely with the observed amplitudes (75% fast, 25% slow) associated with the 2AP “flip on” experiment (Fig. 3, upper panel). Although the coupling between base restacking and complex dissociation is not known, base restacking or a prior conformational change is probably the rate-limiting step for turnover. Comparison of the $K_{\text{D, DNA}}$ determined for the 2AP-substituted DNA duplex (0.51 nM, Fig. 2) with the two predicted dissociation constants (0.10 and 0.68 nM) obtained from the ratio of the measured kinetic dissociation constants (Fig. 3, lower panel) and association rate constant” shows that these results are internally consistent. The two off-rate phases suggest that two DNA-bound enzyme forms are in equilibrium on the DNA and that enzyme dissociation requires the interconversion from a tightly bound form to one that dissociates rapidly.

**Discrimination at the Binding Orientation Level—Single-turnover experiments initiated from the enzyme-AdoMet complex indicate that hemimethylated DNA is methylated to only 50% of the level of unmethylated DNA (16). These data suggest that under presteady-state conditions the monomeric M.EcoRI binds the asymmetric hemimethylated target site with equal probability in both orientations, leading to catalysis or the assembly of an unproductive dead end product complex.** To confirm and extend these observations at the level of the base flipping step, stopped flow total intensity data were obtained with DNA that has the 2AP probe incorporated in place of the adenine adjacent to a methylated target base was utilized in the stopped flow.
is followed by isomerization of the complex into a conformation activation step because this step is likely to be greater than the rate of spontaneous 2AP:T base pair opening. Methylation of the extrahelical base occurs rapidly subsequent to the stabilization of the target adenine within the enzyme active site. The chemistry step (k_{methylation}) is followed by a slow dissociation of the enzyme from the methylated DNA product (DNA\textsuperscript{M}).

**Scheme I. Proposed catalytic cycle showing DNA binding, isomerization, methyl-transfer, and product release for M.EcoRI.** Site-specific binding of the target site (E-DNA\textsubscript{a}) by M.EcoRI is followed by isomerization of the complex into a conformation activation step (E-DNA\textsubscript{s}). Methylation of the extrahelical base occurs rapidly subsequent to the stabilization of the target adenine within the enzyme active site. The chemistry step (k_{methylation}) is followed by a slow dissociation of the enzyme from the methylated DNA product (DNA\textsuperscript{M}).

**Implications of this Work—** Our model relating site-specific DNA binding, complex isomerization, methyl-transfer, and product release by M.EcoRI is depicted in Scheme I. DNA binding is shown in a multi-step mechanism involving the rapid formation of a "loosely" bound site-specific encounter complex, followed by one or more conformational changes within the enzyme-DNA complex. Because the majority of the base flipping transition occurred at a rate similar to k_{methylation} (16), all of the conformational changes required for catalysis must also be complete within this time frame. In addition to stabilizing an extrahelical base, M.EcoRI bends the DNA by 52° (39). DNA bending may occur simultaneously (46) with complex formation and possibly strain the DNA to destabilize the target base pair and induce base stacking disruption. Alternatively, DNA bending may occur in a sequential mechanism following site-specific complex formation or subsequent to base extrusion. Because the extrahelical conformation of the target base is clearly the preferred orientation at equilibrium (7), the rapid methyl-transfer step during the modification of the target site (5′-GAATTC-3′) indicates that the chemistry step has a high degree of commitment. Initial binding of the canonical site by M.EcoRI leads to an active configuration of the enzyme-DNA complex where the key catalytic residues converge into an alignment compatible with transition state stabilization. A value of >200 s\textsuperscript{-1} is tentatively assigned to the chemistry step because this step is likely to be greater than 10-fold faster than the prior first-order isomerization (21 s\textsuperscript{-1}). Because the rate of base flipping is approximately 50-fold slower than the rate of spontaneous 2AP:T base pair opening (1000 s\textsuperscript{-1}) (35), enzyme-assisted and spontaneous base pair opening most likely occur by different pathways. Although the base flipping trajectory for M.EcoRI is not known, the C\textsuperscript{5}-cytosine-specific DNA methyltransferases extrude the target base via the DNA minor groove (3, 5). This trajectory is distinct from the major groove route predicted to mediate spontaneous base pair opening (47). Although base flipping clearly occurs subsequent to complex formation, the ensuing events remain obscure.

The precataylatic isomerization of the enzyme-DNA complex is likely to serve as a determinant of enzyme discrimination between competing DNA sites. Because the investment of binding free energy required to contort the DNA into a catalytically activated conformation is probably sequence-dependent, discrimination may be modulated by the energetic cost of DNA distortion. A different or additional level of discrimination may be revealed at the transition state if the assembly of the active residues is also sensitive to sequence-specific enzyme-DNA interaction. For M.EcoRI the chemistry step (k_{methylation}) is significantly faster than turnover (16). A burst of product formation, followed by a steady-state increase in product accumulation is observed in a typical presteady-state experiment where the concentration of the substrate is 5-fold greater than that of the enzyme. However, no burst of product formation is detected with certain closely related sites (e.g. 5′-GAATTC-3′), showing that a step prior to product release is rate-limiting. Furthermore, the k_{cat}/K\textsubscript{m} observed for the methylation of non-target sites is decreased up to 20,000-fold (48). Thus, the overall discrimination against non-target site methylation is likely to be mediated at the level of the precataylatic isomerization and the chemistry step. The formation and interconversion among the conformationally distinct structural intermediates would serve to enhance sequence specificity relative to an enzyme that does not partition among multiple enzyme-DNA intermediates. Ultimately, a quantitative understanding of enzyme discrimination demands knowledge of the relative magnitude of both of these energetic barriers toward the modification of target and non-target sites.

The stabilization of an extrahelical base circumvents the problem of catalyzing a stereo-specific reaction at sterically occluded DNA bases or target lesions. For AdoMet-dependent enzymes, base flipping minimizes DNA-cofactor interaction by removing the bound AdoMet from the enzyme-DNA interface. This permits site-specific methylation at many different DNA sequences, which might otherwise not be possible if sequence-specific recognition elements involved the bound AdoMet molecule. This expansion of the recognition sequence diversity is not without energetic consequence. DNA methyltransferases typically bind DNA significantly less tightly than the related restriction endonucleases or repressor type proteins. The free energy differences separating the closed and open forms of the base pair and the large activation enthalpy (5–25 kcal mol\textsuperscript{-1}) associated with spontaneous base pair opening (49) provide a rationale for the differences in equilibrium binding affinities observed for these distinct classes of proteins. In conclusion, direct real time observation and rigorous solution-based characterization of the nucleotide flipping process allows mechanistic investigation into the origin of sequence specificity and enzyme discrimination derived from this dramatic protein-nucleic acid interaction.

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