Targeted Base Stacking Disruption by the *Eco*RI DNA Methyltransferase†

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ABSTRACT: We describe a novel fluorescence-based assay for detecting DNA conformational alterations within enzyme−DNA complexes. The target adenine for *Eco*RI DNA methyltransferase (GA^{A}TTC) was replaced with 2-aminopurine, which fluoresces upon excitation at 310 nm. Addition of the methyltransferase to the duplex binding site results in a 14-fold increase in fluorescence intensity with a 10 nm blue shift. The fluorescence is ∼50% of that observed with equimolar free nucleoside, consistent with extrahelical stabilization of the target base in the enzyme−DNA complex. The shift in λ_{max} further implies the base is placed into a low dielectric environment. For adenine-specific DNA methyltransferases, a hydrophobic pocket composed of highly conserved amino acids lies proximal to the cofactor binding site. Substitution of 2-aminopurine adjacent to the target base also results in detectable changes in fluorescence emission following complex formation with the methyltransferase. Thus, other classes of enzymes hypothesized to utilize base flipping can be investigated by this method.

Distortion of DNA conformation by proteins can enhance binding specificity and for some enzymes is essential for catalysis (Steitz, 1990). Recently, a novel protein−DNA interaction implicating extrahelical base stabilization as a catalytic strategy was elucidated. The extrahelical conformation was covalently trapped, using a mechanism-based inhibitor of the cytosine C^5-methyltransferases M.*Hha*I[1] (Klimasauskas et al., 1994) and M.*Hae*III (Reinisch et al., 1995), or stabilized as in the T4 endonuclease V cocrystal complex (Vassylyev et al., 1995). For DNA methyltransferases, the open, extrahelical conformation positions the target base adjacent to the cofactor. Base flipping has also been suggested for T4 β-glucosyltransferase (Vrielink et al., 1994), Ada-O^6-methylguanine−DNA methyltransferase (Moore et al., 1994), *Taq*I DNA adenine N^6-methyltransferase (Labahn et al., 1994), uracil DNA glycosylase (Savva et al., 1995), and DNA ligase (Subramanya et al., 1996).

The static cocrystal structures leave several intriguing mechanistic issues unresolved. Do enzymes catalyze the extrusion of the target base, or do they simply stabilize the extrahelical base subsequent to spontaneous base pair opening? What is the functional and temporal relationship between extrahelical base stabilization and additional DNA deformations, such as base pair rearrangements (Reinisch et al., 1995) and DNA bending (Garcia et al., 1996; Vassylyev et al., 1995)? Does base flipping enhance sequence specificity, and if so, what is the molecular basis for this? These questions clearly require a dynamic, solution-based assessment of base flipping.

DNA methyltransferases belong to a large class of AdoMet-dependent enzymes. They methylate DNA at adenine N^6, cytosine C^5, or cytosine N^4 (Jost & Saluz, 1993). All three types of DNA methyltransferases are found in bacteria, in which adenine methylation in particular has diverse biological roles, including transcriptional regulation, DNA mismatch repair, regulation of DNA replication, and restriction−modification. In contrast, only cytosine C^5 methylation has been detected in higher eukaryotes where it is involved in transcriptional regulation, genetic imprinting, and oncogenesis. The structure of the adenine-specific M.*Taq*I (target site TGC**A**)[1] shows the proposed DNA binding cleft and the bound cofactor (AdoMet) to be separated by ∼15 Å (Labahn et al., 1994). Thus, methyl transfer from AdoMet to the DNA requires significant conformational changes within the protein, DNA, or both. DNA methyltransferases are known to undergo conformational changes upon DNA binding (Reich et al., 1991; Klimasauskas et al., 1994) and following catalysis (Szczelkun et al., 1995). For M.*Eco*RI (target site GA**A**TTC), binding to its cognate site induces an ∼52° bend in the DNA (Garcia et al., 1996). This is slightly less than the ∼62° bend angle determined for the adenine-specific M.*Eco*RV (Cal et al., 1996).

Bacterial DNA methyltransferases are ideal enzymes for dissecting the molecular basis of sequence-specific DNA modification. The catalytic cycle involves large scale conformational changes within the DNA that contribute to catalysis and specificity. We present a solution-based assay which can provide dynamic information of one such conformational change, base flipping. Our results suggest that M.*Eco*RI, an adenine DNA methyltransferase, stabilizes the target base in an extrahelical position. A strategy to extend the assay to other enzyme systems is proposed and tested.

MATERIALS AND METHODS

Protein Expression and Purification. Wild type M.*Eco*RI, H235N, and *Eco*RI endonuclease were purified from *Escherichia coli* strain MM294 harboring the overexpression constructs pXRI, pKM(H235N), and pPG440, respectively

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[1] The target base within the canonical site is shown in bold. Abbreviations: LB media, Luria Bertani media; IPTG, isopropyl thiogalactoside; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; M.*Eco*RI, *Eco*RI DNA adenine methyltransferase; R.*Eco*RI, *Eco*RI endonuclease; M.*Taq*I, *Taq*I DNA adenine methyltransferase; M.*Eco*RV, *Eco*RV DNA adenine methyltransferase; M.*Hha*I, *Hha*I DNA cytosine methyltransferase; M.*Hae*III, *Hae*III DNA cytosine methyltransferase; 2Ap, 2-aminopurine.

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(Greene et al., 1978). Construction of pPG440 has been described previously (Greene et al., 1978), and pKM(H235N) will be described elsewhere.[2] The wild type M.*Eco*RI overexpression construct, pXRI, was created by cleavage of pPG440 with *Pac*I followed by treatment with mung bean nuclease to generate a blunt-ended linear DNA that was subsequently cleaved with *Hin*dIII. The resultant ~1.0 kb M.*Eco*RI-encoding fragment was purified by agarose gel excision and ligated into pSE380 (Invitrogen) that was prepared by *Nco*I cleavage, treatment with mung bean nuclease, *Hin*dIII digestion and dephosphorylated with calf intestinal phosphatase. This placed M.*Eco*RI expression under control of the IPTG-regulated trc promoter, nine base pairs downstream of the ribosome binding site. All enzymes were purchased from New England Biolabs with the exception of the DNA ligase, which was from Boehringer Mannheim. Bacteria were grown at 37 °C in LB media supplemented with 100 μg/mL ampicillin. Expression was induced at early log phase ($OD_{600} = 0.4$) by the addition of IPTG to 1.0 mM. Concentrations of the purified wild type M.*Eco*RI (100 μM), H235N mutant (9.5 μM), and *Eco*RI endonuclease (14 μM) were determined spectrophotometrically utilizing the published extinction coefficients (Rubin & Modrich, 1977).

DNA Synthesis and Purification. Standard phosphoramidites and ancillary DNA synthesis reagents were obtained from Millipore/Biosearch. 2-Aminopurine phosphoramidite was purchased from Glenn Research and N^6-methyladenosine was from Pharmacia. Oligonucleotides were prepared on a Biosearch 3810 DNA synthesizer using β-cyanoethyl phosphoramidites and purified by reverse phase HPLC. DNA purity was assessed by ^{32}P radiolabeling and visualized utilizing 20% denaturing PAGE and autoradiography. Concentrations were determined spectrophotometrically. The sequences of the oligonucleotides (14mers) used are as follows: top strand, 5′-GGCGG**Ap**ATTCGCGG-3′, 5′-GGCGGA**Ap**TTCGCGG-3′; bottom strand, 5′-CCGCGAATTCCGCC-3′, 5′-CCGCGA**A**TTCCGCC-3′, 5′-CCGCG**Ap**ATTCCGCC-3′ (**Ap** = 2-aminopurine, **A** = N^6-methyladenosine). Hemimethylated complexes were used for methyltransferase titrations, whereas unmethylated duplexes were used in endonuclease experiments. The 2-aminopurine riboside (2-amino-9-β-D-ribofuranosylpurine) was purchased from Sigma and quantitated spectrophotometrically using the manufacturer's $E^{1mM}{}_{244nm}$ of 5.77 and $E^{1mM}{}_{306nm}$ of 6.72 in 0.1 M phosphate buffer (pH 7.0). Concentration determinations utilizing both extinction coefficients differed by less than 1%.

Steady-State Fluorescence Spectra. Fluorescence measurements were performed utilizing a Perkin-Elmer LS50 B spectrophotometer. Titrations were done at 22 °C in ~3.0 mL with stirring. Excitation was at λ_{310nm}, and fluorescence emission was scanned from 330 to 430 nm. Slit widths of 5.0 nm (excitation) and 5.0 nm (emission) were used for all experiments. Reactions were carried out in 100 mM Tris, 10 mM EDTA, 1 mM DTT, and 10 μM sinefungin at pH 8.0. Annealings were performed by titration of the 2Ap-containing strand (400 nM) with excess non-2Ap-containing strand until maximal fluorescence quenching was observed. M.*Eco*RI does not bind single-stranded DNA with any detectable affinity (Reich & Danzitz, 1992). Protein aliquots were subsequently added until no further perturbation of the protein-induced fluorescence was detected. Saturation required an approximately 2-fold molar excess of protein.

Thermodynamic Dissociation Constant Determinations. K_d's were determined using the gel mobility shift assay described previously (Surby & Reich, 1996). Briefly, DNA substrates were radiolabeled with ^{32}P using T4 polynucleotide kinase and [γ-^{32}P]ATP. Following excess ATP removal and annealing with the complementary strand, binding cocktails containing 50 pM DNA, 100 mM Tris, 10 mM EDTA, 200 μg/mL BSA, 10 μM sinefungin, and 10 mM DTT at pH 8.0 and varying concentrations of methyltransferase were prepared. Reaction mixtures (10 μL) were incubated at 25 °C for 10 min followed by 12% nondenaturing PAGE and autoradiography. Exposed films were scanned with a LBK Ultroscan XL laser densitometer and the relative amounts of ternary complex formed at each enzyme concentration fitted to a standard hyperbolic binding expression.

RESULTS AND DISCUSSION

We have developed an assay suitable for the detection of base-specific, DNA conformational alterations within enzyme−DNA complexes. 2Ap replacement of adenine is an ideal conformational probe as it base pairs to thymine (Nordlund et al., 1989; Guest et al., 1991) and causes minimal structural changes within the DNA duplex (Nordlund et al., 1989), and its fluorescence is extremely responsive to the localized environment (Ward et al., 1969). Unlike the four standard bases, irradiation of duplex DNA containing 2Ap at 310 nm produces a strong fluorescence emission spectrum with a λ_{max} at ~370 nm. Single-stranded DNA containing 2Ap has ~2-fold greater fluorescence intensity relative to 2Ap base paired within duplex DNA, while the fluorescence of equimolar free nucleoside is 20−125-fold greater (Nordlund et al., 1989; Guest et al., 1991).

An oligonucleotide with 2Ap in place of the adenine normally methylated by M.*Eco*RI (GA**Ap**TTC) was prepared and its steady-state fluorescence measured (Figure 1). Annealing of this oligonucleotide with the complementary strand results in an ~3-fold decrease in fluorescence intensity at 369 nm (Figure 1). M.*Eco*RI binds this hemimethylated, 2Ap-substituted duplex with a K_D of 150 pM, approximately 3 times more tightly than the hemimethylated sequence lacking 2Ap (Surby & Reich, 1996). The 2Ap·T base pair undergoes spontaneous base opening 7-fold more rapidly than the A·T base pair (Nordlund et al., 1989). Thus, there appears to be an inverse correlation between the enzyme's affinity for a particular sequence and base pair stability. A similar observation has been made for M.*Hha*I (Klimasauskas et al., 1995). Stoichiometric addition of M.*Eco*RI to the double-stranded DNA, in the presence of the cofactor analog sinefungin (Reich & Mashhoon, 1990), results in a 14-fold increase in fluorescence intensity and a 10 nm shift to a shorter wavelength (Figure 1). No further fluorescence enhancement is observed upon addition of excess M.*Eco*RI (data not shown). The free nucleoside at the same concentration has an ~2-fold greater fluorescence intensity compared to the M.*Eco*RI−2Ap-substituted DNA complex (Figure 1). This difference may be derived from M.*Eco*RI binding in two orientations relative to 2Ap, since hemimethylated and unmethylated substrates are bound with equal

[2] Allan et al., manuscript in preparation.

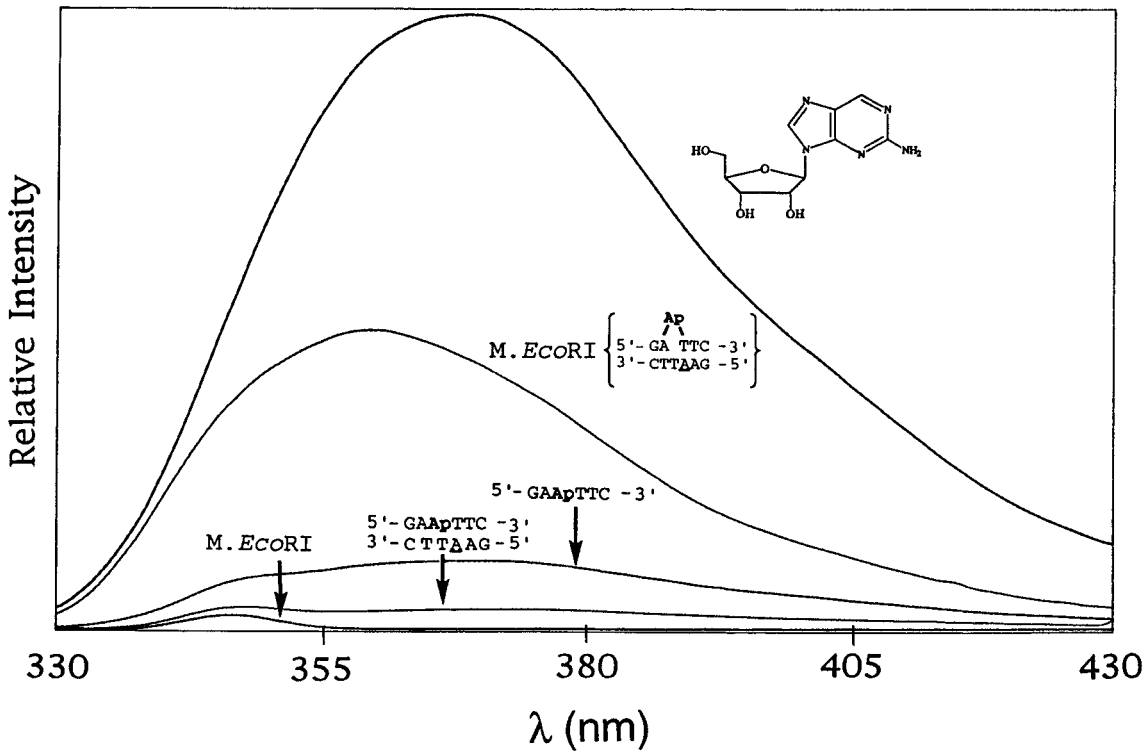


FIGURE 1: Steady-state fluorescence emission spectra using 2-aminopurine-substituted DNA and M.*Eco*RI (wild type). Annealing of the 2Ap-containing DNA (5′-GGCGGA**Ap**TTCGCGG-3′, 400 nM) to its complementary strand (5′-CCGCGA**A**TTCCGCC-3′) results in an ~3-fold decrease in fluorescence intensity at λ_{369nm} (**Ap** = 2-aminopurine, **A** = N^6-methyladenosine). The sequences shown in the figure only display the central hexanucleotide recognition site. Addition of enzyme to the hemimethylated duplex results in a 13.8-fold increase in emission intensity and a 10 nm blue shift in emission λ_{max}. The relative fluorescence intensity of the free nucleoside at the same concentration is ~2-fold greater. Reactions were done in 100 mM Tris, 10 mM EDTA, 1 mM DTT, and 10 μM sinefungin (3.0 mL) at pH 8.0 and 22 °C using $\lambda_{310\ nm}$ (excitation). The λ_{max} values of the free nucleoside and single- and double-stranded DNA are ~369 nm. The λ_{max} values of the M.*Eco*RI complex and Raman peak are 359 and 347 nm, respectively.

affinity (Reich et al., 1992). The large M.*Eco*RI-induced fluorescence enhancement is consistent with an unstacking of the 2Ap, suggesting that the enzyme induces base pair disruption and subsequent stabilization of 2Ap in an extrahelical conformation.

The emission λ_{max} values of the free nucleoside and 2Ap-containing DNA both occur at ~369 nm (Figure 1). Thus, the shift in emission λ_{max} upon formation of the enzyme−DNA complex implies not only that the 2Ap is extrahelical but also that the 2Ap is positioned into an environment with a significantly lower dielectric constant (Ward et al., 1969). M.*Taq*I is proposed to stabilize the extrahelical adenine through hydrophobic interactions with Tyr108 and Phe196, which are immediately adjacent to the bound cofactor (Figure 2) (Labahn et al., 1994). Tyr108 and Phe196 are found in highly conserved peptide motifs among adenine N^6-methyltransferases (Malone et al., 1995), and by homology, M.*Eco*RI has a Phe142 and a Phe232 at these positions, respectively. The fluorescence data are consistent with the stabilization of the target base in the hydrophobic cavity created by these amino acids. This low dielectric environment may help activate the adenine exocyclic amine for nucleophilic attack on the AdoMet methylsulfonium. Also consistent with the distal positioning of the cofactor and DNA is the insensitivity of K_m^{AdoMet} to changes in the DNA sequence (Reich et al., 1992). Further, unmethylated and hemimethylated substrates have identical rate constants of methylation (Reich & Mashhoon, 1993), which is difficult to reconcile with the 3.4 Å separation between the N^6-adenine functionalities within duplex DNA. Extrahelical placement of the target adenine circumvents this stereochemical constraint.

The *Eco*RI endonuclease−DNA cocrystal structure shows significant localized DNA kinking and perturbation of B-DNA parameters (Kim et al., 1990). Structural perturbations of this magnitude might be expected to cause a significant increase in the fluorescence intensity resulting from localized base unstacking. *Eco*RI endonuclease cleaves unmethylated GA**Ap**TTC duplexes (Brennan et al., 1986), and complex formation between the endonuclease and DNA under non-catalytic conditions causes only a minor fluorescence increase (Figure 3). The large M.*Eco*RI-induced increase in fluorescence emission intensity is therefore unlikely to be due to localized structural perturbations of a similar magnitude as that caused by the endonuclease under noncatalytic conditions. Figure 3 also shows that a mutant M.*Eco*RI (H235N), which unlike the wild type M.*Eco*RI shows no detectable DNA bending (Garcia et al., 1996; footnote 2), causes an even greater fluorescence enhancement. Thus, enzyme-induced DNA bending by the wild type enzyme is unlikely to cause the significant increase in fluorescence.

We anticipated that positioning 2Ap adjacent to a proposed extrahelical base might let it serve as an additional conformational probe and expand the versatility of the assay. The extrahelical conformation of the adjacent adenine (GAp**A**-TTC) should leave one face of the 2Ap unstacked, thereby possibly enhancing the fluorescence. Single 2Ap substitutions are possible adjacent to the target adenine or the N^6-methyladenine product. Substitution of 2Ap adjacent to the

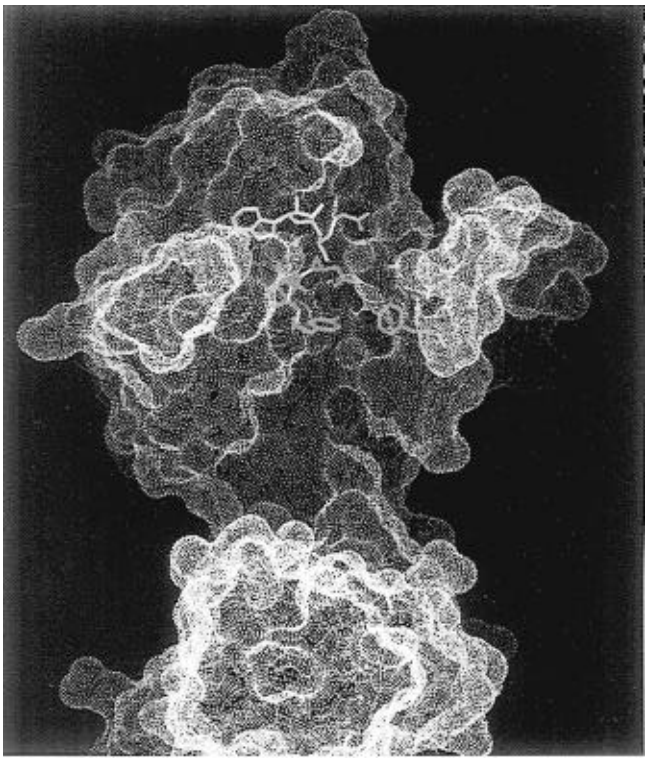


FIGURE 2: Computer graphics model of the M.*Taq*I−AdoMet structure (Labahn et al., 1994). The putative DNA binding cleft separates the larger N-terminal AdoMet-binding, catalytic domain (top) and the smaller target recognition domain (bottom). The cofactor, AdoMet (yellow), is modeled in the rearranged conformation thought to be required for catalysis (Labahn et al., 1994). The conserved NPPY motif (amino acids 105−108 in M.*Taq*I and by homology residues 139−142 in M.*Eco*RI) and a highly conserved phenylalanine are shown in green. The solvent-accessible surface (white) was created using a 1.4 Å radius for water, in the absence of cofactor and highlighted residues, using InsightII.

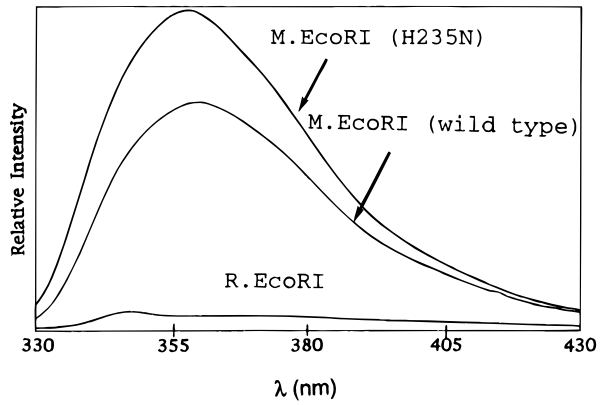


FIGURE 3: Steady-state fluorescence emission spectra of 2-aminopurine-substituted DNA with M.*Eco*RI (wild type), M.*Eco*RI (H235N), and R.*Eco*RI. Reaction conditions were identical to those in Figure 1, with the exception that unmethylated complementary strand was used for R.*Eco*RI. The bending-deficient mutant causes a greater fluorescence enhancement than the wild type, while the R.*Eco*RI-induced effect is negligible.

N^6-methyladenine, which cannot undergo methylation, results in a detectable M.*Eco*RI-mediated fluorescence increase (Figure 4).[3] Transient unstacking or extrahelical stabilization

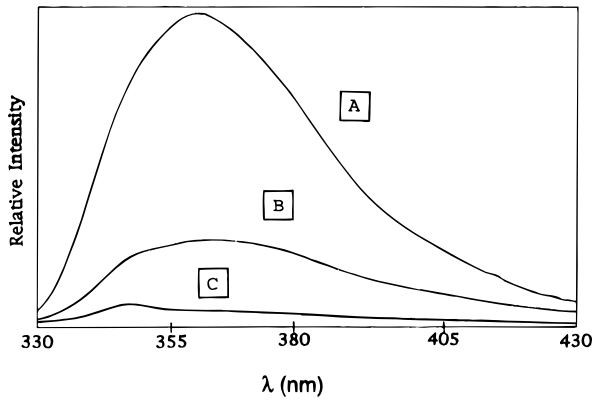


FIGURE 4: Steady-state fluorescence of M.*Eco*RI interaction with DNA substituted at various positions with 2-aminopurine: (A) 2Ap substitution at target base, (B) 2Ap substitution adjacent to target base, and (C) 2Ap substitution (on opposite strand) adjacent to a methylated target base.

of the N^6-methyladenine could cause sufficient exposure of the adjacent 2Ap base surface to account for the increased fluorescence emission. Figure 4 also shows that the fluorescence of the duplex substrate is in fact significantly decreased upon M.*Eco*RI binding when 2Ap is placed adjacent to an unmethylated target adenine. The lack of a M.*Eco*RI−DNA cocrystal structure precludes definitive interpretation of the observed fluorescence quenching. However, base pair rearrangement as seen in the M.*Hae*III cocrystal structure (Reinisch et al., 1995), interaction with a proximal protein side chain, or a combination of both is certainly possible.

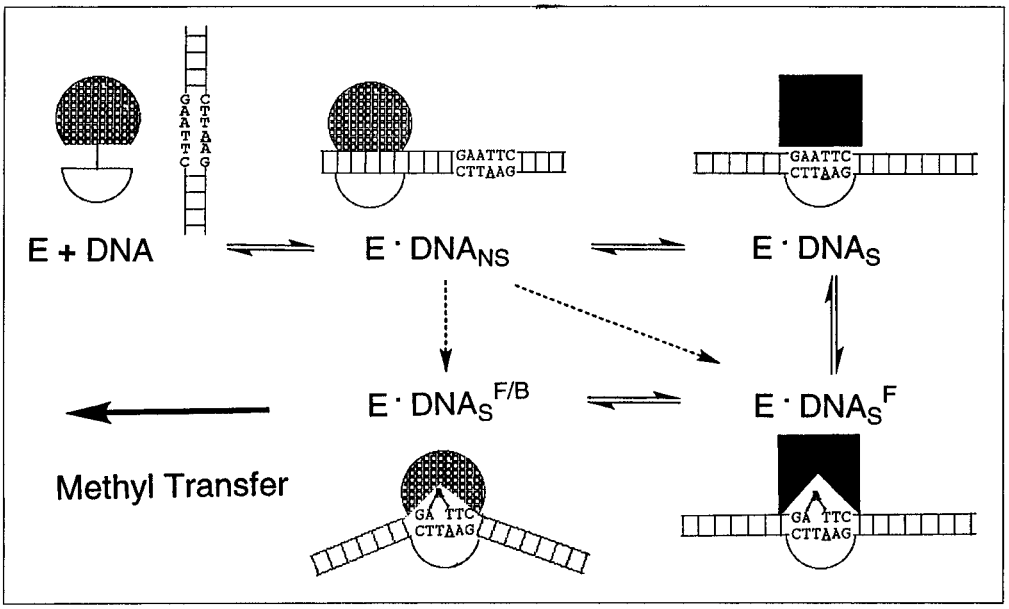
The different fluorescence behavior resulting from 2Ap substitution adjacent to a methylated (Figure 4, line B) or an unmethylated (Figure 4, line C) adenine target implicates unique protein−DNA interactions in the enzyme−substrate and enzyme−product complexes. Our strategy of positioning 2Ap adjacent to a base proposed to undergo extrahelical stabilization may be useful for the investigation of other enzymes thought to stabilize guanines, cytosines, or thymines.

Scheme 1 provides a general framework for understanding the relationship between sequence recognition, base flipping, DNA bending, and enzyme specificity. M.*Eco*RI binds nonspecific DNA and utilizes a facilitated diffusion mechanism to locate its cognate site (Surby & Reich, 1996). A significant protein conformational change occurs upon site-specific binding (Reich et al., 1991). Sequence recognition involving an extrahelical base is unlikely, as this requires k_{cat}/K_m^{DNA} to be greater than 10^{13} M^{-1} s^{-1}.[4] Thus, a sequence-specific enzyme−DNA intermediate is shown prior to base flipping. A mechanistically indistinguishable process of simultaneous sequence recognition and base flipping is also shown (diagonal dashed line, $E{\cdot}DNA_S^F$). We suggest that DNA bending follows base flipping since the H235N

[3] M.*Eco*RI binds these 2Ap-containing substrates 2- and 6-fold more weakly than unsubstituted substrates, dependent on whether 2Ap is placed next to an unmethylated or methylated target base, respectively (see Figure 4).

[4] The closed form of an A·T base pair is favored by a factor of 10^5 over the open form (Gueron et al., 1987). k_{cat}/K_m^{DNA} is greater than 10^8 M^{-1} s^{-1} for M.*Eco*RI and is equivalent to k_{on}, representing the diffusion-controlled collision between free enzyme and the specific site (Reich & Mashhoon, 1991; Surby & Reich, 1996). k_{cat}/K_m^{DNA} was determined considering every target site a potential substrate (Surby & Reich, 1996). Assuming that the enzyme only recognizes sites with a flipped-out adenine, the actual k_{cat}/K_m^{DNA} can be calculated by multiplying the observed k_{cat}/K_m^{DNA} by 10^5 since the latter describes the ratio between the closed and opened forms of the A·T base pair.

Scheme 1: Proposed Catalytic Cycle Showing Initial DNA Binding, Canonical Site Recognition, Base Flipping, DNA Bending, and Methyl Transfer[a]



[a] M.*Eco*RI has a large N-terminal domain (pattern) and a smaller C-terminal domain (open half-circle). The free enzyme (E) binds nonspecific DNA (DNA_{NS}) to locate its cognate site ($E{\cdot}DNA_S$) with a facilitated diffusion mechanism. This is followed by base extrusion ($E{\cdot}DNA_S^F$) and DNA bending ($E{\cdot}DNA_S^{F/B}$). The underlined A represents N^6-methyladenine, which creates a hemimethylated site. The catalytically productive binding orientation is depicted. Protein conformational changes are indicated schematically by sole alteration of the N-terminal domain. The two dashed lines are described in the text and reflect concerted alternative pathways.

M.*Eco*RI mutant, which does not bend DNA (Garcia et al., 1996; footnote 2), apparently stabilizes the extrahelical base (Figure 3). The concerted process involving sequence recognition, base flipping, and DNA bending (vertical dashed line) seems improbable given the results for the H235N mutant. Base flipping is a reversible process, as shown by our demonstration that the enhanced fluorescence emission (Figure 1) is inhibited by subsequent addition of competitor DNA lacking 2Ap (data not shown). Formation of the catalytically competent $E{\cdot}DNA_S^{F/B}$ complex is followed by methyl transfer. The proposed transformation between $E{\cdot}DNA_S^F$ and $E{\cdot}DNA_S^{F/B}$ (Scheme 1) is predicted by molecular modeling and energy calculations for DNA alone to favor the $E{\cdot}DNA_S^{F/B}$ form (Ramstein & Lavery, 1988). This energetically favorable process is apparently precluded for the H235N mutant. Since H235N is unaffected in k_{cat}, whereas K_m^{DNA} is 40-fold weaker,[2] DNA bending is not essential for catalysis but rather may contribute to overall enzymatic specificity. Enhancement of enzyme specificity might be derived from the formation of additional, unique protein−DNA interactions within the $E{\cdot}DNA_S^{F/B}$ complex. Similarly, the $E{\cdot}DNA_S^F$ complex might also contribute to enhanced specificity, as protein contacts with the extrahelical adenine will likely involve hydrophobic interactions not normally used in protein−DNA recognition. The proposed intermediates in Scheme 1 represent unique enzyme−substrate interfaces during the catalytic cycle that can lead to enhanced discrimination between correct and incorrect DNA sequences (Hopfield, 1974).

The rate constants for the proposed transformations between $E{\cdot}DNA_S$, $E{\cdot}DNA_S^F$, and $E{\cdot}DNA_S^{F/B}$ (Scheme 1) have not been characterized. Rate constants for the spontaneous base pair opening range from 20 to 200 s^{-1}, and the closed form is normally favored by 10^5-fold (Gueron et al., 1987; Guest et al., 1991). Because k_{cat} for M.*Hha*I is 0.02 s^{-1}, others have suggested that base flipping does not require enzyme assistance (Reinisch et al., 1995; Winkler, 1994). However, the relevant rate constant is $k_{methylation}$, which can be several orders of magnitude greater than k_{cat} (Reich & Mashhoon, 1993). Interestingly, unlike the T4 endonuclease V, neither the cytosine-specific M.*Hha*I or M.*Hae*III significantly bends the DNA. Perhaps concomitant DNA bending is a widespread mode of helical stabilization following extrusion of the bulkier purine nucleotide. Also, base pair opening in the M.*Hha*I−DNA complex is thought to occur via the minor groove (Klimasauskus et al., 1994), whereas in the absence of protein, base pair opening is energetically favored via the major groove (Ramstein & Lavery, 1988). Thus, the actual mechanism of base pair opening within enzyme−DNA complexes remains obscure.

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