Non-additivity of sequence-specific enzyme-DNA interactions in the *EcoRI* DNA methyltransferase

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ABSTRACT

We describe a novel strategy to characterize protein-DNA interactions involving monomeric enzymes such as DNA methyltransferases (Mtases). This strategy is applied to our investigation of the *EcoRI* DNA Mtase, which binds its double stranded recognition site 5'-G-AATTC-3' and methylates the central adenosine of each strand using S-adenosyl-L-methionine as the methyl donor. We show that prior methylation of adenosine in either strand does not perturb catalysis. In contrast, substrates substituted with deoxyinosine at either guanosine position (T-BMI5 and TI5-BM) show the minor groove residing N2 amino group of both guanosines contribute to DNA recognition since specificity constants for the modified substrates are reduced 13 and 39 fold. Similar analysis of a substrate containing deoxyinosine at both positions (TI5-BMI5) clearly shows that some communication occurs between the sites. To determine the extent to which structural changes in the DNA alone contribute to this lack of additivity, we performed DNA melting analysis of the singly and doubly substituted substrates, and also found nonadditivity. Although our functional and structural analyses suggest that deoxyinosine incorporation causes long range conformational effects, the similarity of K_m^{AdoMet} for all substrates suggests that no large-scale structural changes occur in the Mtase-DNA-AdoMet complex. Our results support the following conclusions: 1) The non-additivity shown in this system contrasts with the widespread demonstration of additivity involving repressors [Lehming *et al.*, 1990; Takeda *et al.*, 1989; Ebright *et al.*, 1987], suggesting that sequence discrimination by enzymes may involve more complex mechanisms. Further, this non-additivity precludes quantitative assignment of individual interactions and we suggest that future analyses of this and related enzyme systems with base analogs include detailed information about the long range structural consequences of individual substitutions. 2) Although TI5-BM and T-BMI5 are shown to be radically different by thermodynamic analysis, the similar specificity constants with the Mtase suggest that the underlying structural differences (e.g., altered helical parameters of the DNA) are not critical for sequence-recognition. 3) The significance of minor groove Mtase-DNA interactions to specificity is confirmed.

INTRODUCTION

How do DNA binding and modifying proteins discriminate between DNA sequences with such incredible specificity? Our understanding of the underlying mechanisms to this question has progressed considerably, particularly with regards to prokaryotic repressors [Anderson *et al.*, 1987; Otwinowski *et al.*, 1988; Jordan and Pabo, 1988; Wolberger *et al.*, 1988; Rafferty *et al.*, 1989]. A common element of protein-mediated sequence-specific DNA binding is a complex network of hydrogen bonds and hydrophobic and ionic interactions [Seeman *et al.*, 1976; von Hippel & Berg, 1986], yet the diversity of observed recognition mechanisms suggests that no set of simple rules apply [Matthews, 1988]. Also, a quantitative understanding of how individual protein-DNA interactions contribute to specificity remains to be elucidated. This is particularly true for DNA-modifying enzymes whose sequence-specificity need not be determined solely by binding phenomena.

We are investigating the *EcoRI*¹ DNA Mtase to understand the molecular basis of enzymic DNA sequence discrimination. Detailed information about the protein-DNA interface for the *EcoRI* Mtase, any other DNA Mtase or any monomeric DNA-binding protein remain to be uncovered. The Mtase is part of the well studied *EcoRI* restriction-modification system. The Mtase is a 38,050 dalton protein which is active as a monomer and requires S-adenosylmethionine (AdoMet) and its DNA substrate for activity [Rubin and Modrich, 1977]. Methylation occurs at the N6 position of the second adenine in the double stranded DNA sequence 5'GAATTC3'. We recently demonstrated that initial binding of the enzyme to AdoMet and noncanonical DNA is random, but that canonical site recognition and subsequent catalysis requires AdoMet to be bound [Reich and Mashhoon, 1991]. The Mtase is an extremely efficient enzyme with a specificity constant ($k_{\text{cat}}/K_m^{\text{DNA}}$) greater than $10^8 \text{ s}^{-1} \text{ M}^{-1}$, and catalysis is limited by product release step(s) with the methyl transfer step being at least ten times faster than k_{cat} .

Structural data for the *EcoRI* DNA Mtase substrate and product provide an excellent basis for investigating the details of sequence-specific recognition. The X-ray [Wing *et al.*, 1980] and solution structures [Nerdal *et al.*, 1989] of a DNA sequence containing the canonical site are available (see Figure 1). The X-ray structure of the identical sequence in which both central adenosines are methylated shows that methylation causes minimal structural changes and highlights the proximity of the two methyl groups in the major groove [Frederick *et al.*, 1988] (see Figure 2).

Our approach to understanding Mtase-DNA interactions is by functional analysis of DNA substrates lacking structural features proposed to be critical for DNA recognition. Previous

investigations have implicated the exocyclic amine of guanosine, located in the minor groove, as being critical to the *EcoRI* Mtase [Modrich and Rubin, 1977; Brennan *et al.*, 1986b]. (See Figures 1, 2 and 3). This is a provocative finding since sequence-recognition by proteins is thought to occur largely through interactions in the major groove [Dickerson *et al.*, 1987]. Moreover, since methylation occurs in the major groove, the Mtase would be required to interact with both grooves simultaneously (Figure 1). For these reasons, as well as the prospect of testing our strategy with a conservative substitution (deoxyinosine replacement of guanosine), we investigated the contribution toward specificity which derives from Mtase-DNA interactions at this position.

We designed synthetic oligonucleotide substrates that allow investigation of a single binding orientation of the Mtase. Incorporation of a modified base (e.g., deoxyinosine for guanosine) within the canonical site should allow assessment of individual Mtase-DNA interactions. Our functional analyses of these modified substrates include true steady state parameters for AdoMet and DNA. The structural consequences of the functional group deletion are probed by comparisons of K_m^{AdoMet} , DNA stability using DNA melting and 'second site' analysis [Carter *et al.*, 1984].

MATERIALS AND METHODS

Materials

Phosphoramidites and ancillary DNA synthesis reagents were obtained from Milligen/Bioscience and American Bionetics, beta-cyanoethyl-N⁶-methyladenine from Pharmacia. Gamma [³²P]-ATP (5,000 Ci/mmol) was purchased from Amersham, and [methyl ³H]-AdoMet (78.5 Ci/mmol) from New England Nuclear. T4 polynucleotide kinase was from New England Biolabs. AdoMet was purchased from Sigma and DE81 filter papers were from Whatman. Unlabeled AdoMet was purified using an HRLC MA7S (50×7.8 mm) cation exchange column according to a modified procedure of Zappia *et al.* [1980]. Ammonium acetate buffer (0.1 M, pH 4.0) was used to elute AdoMet at a flow rate of 1 ml/min. The peak corresponding to AdoMet was collected, concentrated on a BioRex 70 cation exchange column (BioRad) and stored at -20°C in 0.1 N HCl [Reich and Everett, 1990]. *EcoRI* Mtase was obtained from an overproducing strain of *E. coli* and purified in our laboratory [Greene, *et al.*, 1978].

Oligonucleotide synthesis and purification

Oligonucleotides were synthesized on a Bioscience 3810 DNA synthesizer using beta-cyanoethyl phosphoramidites. Oligonucleotides were purified on a 10 micron 4.6×250 mm Vydac C-18 column, eluted with a 15%:85% to 40%:60% acetonitrile:triethylammonium acetate pH 7.0 (TEAAc) 20 minute gradient. The 5' dimethoxytrityl blocking groups were removed with 80% acetic acid in water, the DNA precipitated with 2.5 M ammonium acetate and 75% ethanol and dissolved in 10 mM Tris pH 7.5 1 mM EDTA (TE).

The double stranded substrates listed in Tables I and II were made by annealing the two appropriate single strands using a two-fold molar excess of the bottom strand to enhance the formation of the desired double stranded substrates. Mixtures of single strands were heated to 95°C and allowed to cool to room temperature over a 3 to 3.5 hour period.

Figure 1: Graphical representation of the *EcoRI* recognition site containing 'Dickerson dodecamer' [Dickerson and Drew, 1981]. The N2 amino group of one guanosine is shaded black while the sugar phosphate backbone is shaded light grey. Bases are dark grey. The two N6 amino groups of the central adenines are white. The methyl group of one thymine is also shaded black to identify the major groove.

Oligonucleotide characterization

Oligonucleotide purity was confirmed by denaturing polyacrylamide gel electrophoresis (PAGE) of [³²P] labeled oligonucleotides. Oligonucleotides were labeled by reaction with a 4 fold molar excess of gamma [³²P]-ATP and 0.1 units of T4 polynucleotide kinase per picomole of oligonucleotide using published reaction conditions [Maniatis *et al.*, 1982]. The labeled oligonucleotides were electrophoresed through a 25% denaturing polyacrylamide sequencing gel and an autoradiogram of the gel was used to estimate the homogeneity of each sample.

Double stranded substrate formation was confirmed by annealing a radiolabeled oligonucleotide to the complementary unlabeled oligonucleotide using the method described earlier. Samples were electrophoresed through a 14% nondenaturing polyacrylamide gel and analyzed by autoradiography.

Oligonucleotide melting analysis

The temperature dependent denaturation of double stranded oligonucleotides to single strands was monitored at 260 nm with a Shimadzu UV-265 UV/Vis spectrophotometer equipped with a water jacketed cuvette holder. The temperature of the oligonucleotide solution was controlled with a Haake A80 circulating bath connected to the cuvette holder. The output of this bath was regulated by a homemade 'temperature ramp generator' which was previously calibrated, and produced a 0.25°C/minute ramp from 15 to 88°C. Oligonucleotide solutions consisted of 10 mM phosphate, 0.1 mM EDTA, 20 mM NaCl pH 7.0 and double stranded oligonucleotide at various concentrations (from 6 to 9 different concentrations). Solutions were stirred throughout the analysis. The resulting melting temperatures (T_m) were determined using the sloping baseline-sloping plateau method [Breslauer, 1986]. The enthalpy and entropy terms were determined from the slopes and Y-intercepts respectively of $R \ln(4/C)$ vs. $1/T_m$ plots (R is 1.987 cal/mole/°K), such as those depicted in Figure 5, where C is the total concentration of DNA expressed as single strands and temperature is in degrees Kelvin [Aboul-ela *et al.*, 1985]. Our

Figure 2: Graphical representation of the structure of the methylated 'Dickerson dodecamer' [Frederick *et al.*, 1988]. The two methyl groups normally transferred by the *EcoRI* Mtase are highlighted black. The sugar phosphate backbone is shaded light grey. Bases are dark grey.

regression analysis afforded correlation values which were used to calculate the errors.

Kinetic analysis

EcoRI. Mtase activity was determined by an assay which monitors the transfer of the tritiated methyl group from [methyl³H]AdoMet to DNA. Mtase reactions contained 200 µg/ml BSA, 100 mM Tris pH 8.0, 10.0 mM EDTA, 10.0 mM DTT, 20 mM NaCl, 0.2 nM Mtase with five different concentrations of [methyl³H]AdoMet (specific activity ranging from 10,000 to 50,000 cpm/pmole) in a volume of 45 µL. The enzyme reactions were initiated by adding 5 µL of the DNA substrate (at five different concentrations). After five to seven minutes at 37°C these reactions were stopped by transferring 40 µL of the reaction mixtures onto Whatman DE81 filter papers. Filter papers were washed 3 times with 50 mM KH₂PO₄ and once each with 80% ethanol, 95% ethanol and anhydrous ethyl ether for 20 minutes each (150 mL for each wash). Labeled DNA bound to the filter papers was quantitated by determining the tritium content with a Beckman LS-1701 liquid scintillation counter and correcting for signal loss caused by the filter papers.

Data analysis

The raw cpm data was converted to velocity (V) in moles of methyl groups transferred per minute and fit to the Michaelis Menten equation using a Basic version of Clelands' kinetics program [Cleland, 1979]. Direct curve fitting was done with Clelands' HYPER equation to produce 1/V vs. 1/[Substrate] (1/[S]) plots which were used to determine V_{MAX} values. The true k_{cat} and K_m values for the reaction were determined from 1/V_{MAX} vs. 1/[S] replots. The results of replots for multiple independent assays were then averaged to produce the kinetic data in Tables I and II. All data are the mean values +/- one standard deviation (n = 3 to 6).

EXPERIMENTAL DESIGN

EcoRI Mtase methylates its palindromic hexanucleotide recognition site on both strands in separate catalytic events [Rubin and Modrich, 1977]. This observation and the fact that the Mtase

Figure 3: Graphical representation of the recognition site G-C base pair extracted from the structure of the 'Dickerson dodecamer' [Wing *et al.*, 1980]. The minor groove residing N2 amino group of the G is black. Bases are dark grey.

is active as a monomer led us to speculate that each strand is methylated from a unique binding orientation. This is schematically depicted in Figure 4 in which the Mtase binds the native substrate (A) in two orientations (B and C). If true, then functional parameters such as k_{cat}, K_m and k_{cat}/K_m will result from both orientations, making it impossible to comment on unique interactions. We addressed this problem by using hemi-methylated DNA substrates. Although nonproductive binding is expected to occur based on the inhibition constant for a doubly methylated site [Reich and Mashhoon, 1991], contributions to k_{cat} and K_m will cancel so that k_{cat}/K_m will not be influenced [Fersht, 1985]. The Mtase interacting with hemi-methylated substrates is shown in Figures 4D and 4E.

The initial substrate is shown in Figure 4A, and like all substrates used in this study, is non-selfcomplementary in sequence and fourteen basepairs in length. The use of hemi-methylated substrates described above requires non-selfcomplementary DNA strands which was achieved with the basepairs flanking the canonical hexanucleotide. Since our kinetic analyses are performed at 37°C and 20 mM NaCl, the substrates had to be long enough to ensure thermal stability. Moreover, Mtase-DNA interactions beyond the canonical hexanucleotide should be satisfied; preliminary DNA-footprinting results from our laboratory have shown a minimum of eight, and possibly ten basepairs are protected by the Mtase. Finally, to facilitate our structural analysis of modified substrates, the DNA sequence flanking the canonical site (Figure 4A) was selected based on the 'Dickerson dodecamer' which has been structurally characterized by both X-ray (Figure 2) [Wing *et al.*, 1980] and NMR [Nerdal *et al.*, 1989] techniques.

Our strategy should lead to assignment of DNA functionalities critical for recognition and catalysis by the *EcoRI* Mtase. More optimistically, assessment of the quantitative contributions toward specificity may be obtained [Fersht *et al.*, 1986]. The most compelling criticism of such quantitative interpretation is lack of structural information concerning the modified DNA and Mtase-DNA complex. We therefore studied the solution structures of the modified DNA substrates with thermal stability studies; comparisons were at the level of enthalpy and entropy changes. K_m^{AdoMet} was also determined to assess structural perturbations in the ternary complex (Mtase-DNA-AdoMet). Finally, we used 'second site' substitutions in the canonical site to determine any structural communication between sites [Carter *et al.*, 1984].

RESULTS

We submitted several synthetic DNA substrates of the *EcoRI* DNA Mtase to a structural and functional analysis. The substrates are all fourteen basepairs in length and the sequences shown in Tables I and II. The set in Table I was designed to determine

Figure 4: Schematic of possible modes of the *EcoRI* DNA Mtase binding to its non-methylated or hemi-methylated DNA substrate. **A**, shows the native DNA substrate. **B** and **C** show two possible productive orientations with the native DNA substrate. **D** and **E** show how hemi-methylation is used to selectively force one productive binding orientation. The black filled circles (**D** and **E**) represent the N6 associated methyl groups.

the effect of hemi-methylation on Mtase function. We define function as the catalytic turnover constant (k_{cat}), Michaelis constants (K_m^{DNA} and K_m^{AdoMet}) and specificity constant (k_{cat}/K_m^{DNA}) [Fersht, 1985]. The set in Table II was designed to assess the importance of the guanosine minor groove amine, which we approached by substituting deoxyinosine for guanosine and thereby replacing the amine with a hydrogen atom.

Preliminary characterization of DNA substrates and assay conditions

DNA substrates were shown to be greater than 98% pure by denaturing polyacrylamide gel electrophoresis of ^{32}P labeled oligonucleotides. Extent of annealings were shown to be greater than 95% by non-denaturing PAGE of ^{32}P labeled substrates annealed with a 2 fold molar excess of the bottom strand. *EcoRI* Mtase was shown to have maximal activity at pH 7.8 and 10–20 mM NaCl. For all conditions of DNA and AdoMet used in this report, the formation of methylated DNA was shown to be constant with respect to time. Enzyme activity was first order with respect to enzyme concentration. Under our assay conditions, no methylation was detected with single-stranded oligonucleotides. Enzyme activity was monitored as a function of annealing ratio (1:1, 1:2, 1:3 and 1:4; top:bottom strand), and followed a saturation curve saturating at a 1:2 ratio. (Data not shown).

Kinetic analysis

Figure 6 shows a representative double reciprocal analysis for the control substrate, CBM. This plot, the related double reciprocal analysis of velocity versus AdoMet concentration and the plots for the remaining DNA substrates are all consistent with the proposed kinetic mechanism [Reich and Mashhoon, 1991]. The resultant steady-state parameters are shown in Tables I and II. Our previous characterization of substrate C showed a significantly lower K_m^{DNA} [Reich and Mashhoon, 1991]. This previous analysis required a larger set of data and used Cleland's methods [1979] rather than the slope replot approach used in the present study. Application of Cleland's methods to the data collected for C in this study generates parameters comparable to our previously published values. Because we are obtaining specificity data for numerous substrates we used the replot approach which requires fewer data points. In any event, all

Figure 5: Concentration dependence of T_m for 14 bp DNA substrates. TI5-BM (circles) and T-BMI5 (triangles) are shown in the main figure. Inset shows the dependence for the control substrate CBM. R, gas constant; C, total oligonucleotide concentration in single strands; T_m , °K.

substrates in the present study have been submitted to identical data analysis methods and our specificity analysis is thus valid.

To assess the effect that hemi-methylation has on specificity, we determined the kinetic parameters for a native substrate (C) and the corresponding hemi-methylated substrates (CBM and C-TM), as shown in Table I. The data indicate that C, CBM and CTM have essentially identical k_{cat} values while K_m^{DNA} for both hemi-methylated substrates are approximately two times greater than that of the non-methylated substrate. The difference in available sites may be responsible for the observed K_m^{DNA} differences. The similarity of the corresponding specificity constants indicates that the Mtase is relatively insensitive to the N6 methyl group on the strand opposite to the site of methylation. Further support for the lack of significant structural changes comes from the minimal changes in K_m^{AdoMet} values. Thus, although canonical site recognition requires prior AdoMet binding, K_m^{AdoMet} is 250 fold smaller than K_D^{AdoMet} [Reich and Mashhoon, 1991], suggesting that AdoMet binding determinants in the Mtase-DNA-AdoMet complex are altered relative to the Mtase-AdoMet complex.

The minimal effects on the kinetic parameters with the hemi-methylated substrates support our use of one of these as a 'control' substrate. We selected CBM for comparison in our work with the deoxyinosine containing substrates and obtained the kinetic data shown in Table II. Replacement with deoxyinosine of either guanosine residue within the recognition site has little effect on k_{cat} . In contrast, the Michaelis constants for TI5-BM and T-BMI5 are significantly higher than for CBM. These results indicate that the specificity of the Mtase is decreased 13 or 39 fold when either guanosine is replaced with deoxyinosine.

The minor changes in K_m^{AdoMet} seen with T-BMI5 and TI5-BM suggests minimal changes in the conformation of the ternary complexes (Mtase-DNA-AdoMet) result from incorporation of deoxyinosine. In contrast, analysis of the doubly substituted substrate (TI5-BMI5) shows that the effects on k_{cat}/K_m^{DNA} are not additive when compared with the results from the individually substituted substrates. This clearly shows

Table I. Steady state kinetic parameters determined for the non- and hemi-methylated DNA substrates. Parameters were determined from double reciprocal data as shown in Figure 6; standard errors shown in parentheses. (* denotes methylated adenine).

Abbreviation	Sequence	DNA (nM)	K_M AdoMet (μ M)	k_{cat} sec^{-1}	k_{cat}/K_M^{DNA} $sec^{-1} M^{-1}$
C	5' GGCGGAATTTCGCGG 3' CCGCCTTAAGCGCC	24.4 (10.3)	0.280 (0.198)	0.14 (0.02)	4.51×10^6 (2.56×10^6)
CTM	5' GGCGGAATTTCGCGG 3' CCGCCTTAAGCGCC*	42.9 (12.9)	0.248 (0.099)	0.105 (0.006)	2.45×10^6 (0.89×10^6)
CBM	5' GGCGGAATTTCGCGG 3' CCGCCTTAAGCGCC*	43.0 (3.03)	0.322 (0.107)	0.13 (0.006)	3.05×10^6 (0.35×10^6)

Table II. Steady state kinetic parameters determined for the hemi-methylated control and the deoxyinosine containing substrates. Parameters were determined from double reciprocal data as shown in Figure 6; standard errors shown in parentheses. (* denotes methylated adenine).

Abbreviation	Sequence	DNA (nM)	K_M AdoMet (μ M)	k_{cat} sec^{-1}	k_{cat}/K_M^{DNA} $sec^{-1} M^{-1}$
CBM	5' GGCGGAATTTCGCGG 3' CCGCCTTAAGCGCC*	43.0 (3.03)	0.322 (0.107)	0.13 (0.006)	3.05×10^6 (0.35×10^6)
TI5-BM	5' GGCGIAATTTCGCGG 3' CCGCCTTAAGCGCC*	554 (6.4)	0.256 (0.062)	0.13 (0.01)	0.235×10^6 (0.021×10^7)
T-BMI5	5' GGCGGAATTTCGCGG 3' CCGCCTTAAGCGCC*	1428 (13.5)	0.322 (0.018)	0.11 (0.03)	0.077×10^6 (0.0025×10^6)
TI5-BMI5	5' GGCGIAATTTCGCGG 3' CCGCCTTAAGCGCC*	664 (18.9)	0.267 (0.045)	0.14 (0.021)	0.211×10^6 (0.025×10^6)

that communication occurs between each of the two individual sites. To quantitate this nonadditivity at the DNA level, we characterized the thermal stability of each substrate.

Substrate melting analysis

All thermodynamic constants were derived from the temperature-dependent double to single-stranded transition of the oligonucleotides assuming a true two state system. In all cases the absorbance versus temperature profiles were consistent with this assumption, in contrast to the complex behaviour observed with completely self-complementary oligonucleotides [Breslauer *et al.*, 1987]. Our analysis was initially used to confirm that greater than 80% of each substrate remains in the double stranded form under our assay conditions. As shown in Table III, the deoxyinosine substitutions change the Gibbs free energy for the double- to single-stranded transition by less than one kcal/mole (at 37°C). As with the k_{cat}/K_M^{DNA} analysis (Table II), comparison of free energy values shows the interdependence of each deoxyinosine substitution since TI5-BMI5 is more stable than T-BMI5. Hence, removal of one amino group perturbs the DNA structure enough to affect the inosine-cytosine basepair five bases away. To further probe the structural origins of this effect we determined the enthalpic and entropic components of the free energy term by characterizing the dependency of melting temperature on the DNA concentration [Breslauer, 1986; Petruska *et al.*, 1988]. As shown in Figure 5 and Table III the melting transitions of TI5-BM and T-BMI5 have decreased enthalpy and entropy terms relative to CBM. This type of 'compensation' is well known [Breslauer *et al.*, 1987; Petruska *et al.*, 1988], although an explanation at the molecular level is not available. Surprisingly, the changes for T-BMI5 relative to CBM are extremely large.

Figure 6: $1/V$ vs. $1/[DNA]$ plot for the control substrate CBM at 5 different AdoMet concentrations (3.0 μ M $\circ$, 1.0 μ M $\bullet$, 0.6 μ M $\triangle$, 0.45 μ M $\blacktriangle$, 0.3 μ M $\square$). The inset shows the $1/V_{max}$ vs. $1/[AdoMet]$ μ M $^{-1}$ replot used to determine K_M^{AdoMet} .

Table III. Thermal stability data determined for the hemi-methylated control and the deoxyinosine containing oligonucleotides obtained at pH 7.0 and 20 mM NaCl. Units for ΔH° and $\Delta \Delta H^\circ$ are Kcal/mole, ΔS° and $\Delta \Delta S^\circ$ are cal/(°K mole) $^{-1}$, and ΔG° and $\Delta \Delta G^\circ$ are Kcal/mole. All ΔG° values were calculated from ΔH° and ΔS° using $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$ at 310°K. Derivation of standard errors (parentheses) is described in Materials and Methods.

	ΔH° (Kcal)	ΔS° (cal)	ΔG° (Kcal) [37°]	$\Delta \Delta H^\circ$	$\Delta \Delta S^\circ$	$\Delta \Delta G^\circ$
CBM	80.74 (1.8)	227.8 (5.5)	10.09	---	---	---
TI5-BM	77.42 (2.3)	217.8 (7.2)	9.87	3.32	10.0	0.22
T-BMI5	36.69 (1.1)	88.8 (3.4)	9.04	44.05	139.0	1.05
TI5-BMI5	85.55 (3.2)	245.5 (1.0)	9.41	-4.81	-17.7	0.68

DISCUSSION

Investigations of enzymic specificity frequently exploit substrate and enzyme modifications. Modifications in either type of molecule have numerous attendant problems, although investigations with 'small' substrates have led to mechanistic insights [Fersht *et al.*, 1986; Kati and Wolfenden, 1989]. It is clear from studies with small substrate systems that a mechanistic understanding of specificity requires detailed quantitative data. The use of modified bases to assess contributions of individual protein-DNA interactions toward specificity has been widespread, particularly in the context of bacterial Type II restriction-modification enzymes [Brennan *et al.*, 1986a, b; Diekmann and McLaughlin, 1988; Modrich and Rubin, 1977; Fliess *et al.*, 1986; 1988; McLaughlin *et al.*, 1987; Seela and Kehne, 1987; Bodnar *et al.*, 1983; Berkner and Folk, 1979; Wolfes *et al.*, 1986; Newman *et al.*, 1990a, b; Cosstick *et al.*, 1990]. However, for a variety of reasons these past studies have been limited to a qualitative analysis. A major concern is the extent to which the desired structural modification causes long range conformational

changes, thereby complicating assignment of individual interactions. Also, in the absence of knowledge about the kinetic mechanism and rate limiting step(s), interpretation of specificity data is hampered. Two additional concerns are specific to studies of DNA Mtases: 1) These enzymes all use AdoMet as a cofactor and thus an accurate specificity analysis requires comparison of true kinetic constants, in contrast to the reported apparent values [Brennan *et al.*, 1986b; Modrich and Rubin, 1977; Newman *et al.*, 1990a, b]. 2) Most DNA Mtases are functional monomers which can bind to their canonical sites, and more importantly, modified sites, in two orientations. This has precluded investigations of unique Mtase-DNA complexes and an understanding of individual Mtase-DNA interactions.

Our strategy (see Results) addresses these concerns and was applied to an investigation of the contribution of the minor groove amino functionality of guanosine to substrate specificity in the *EcoRI* DNA Mtase system. The position of the amino group within the GC basepair is shown in Figure 3, and its location in the minor groove is shown in Figure 1. Figures 1 and 2 show structures derived from X-ray crystallographic analyses of the *EcoRI* Mtase DNA substrate [Wing *et al.*, 1980] and methylated product [Frederick *et al.*, 1988] respectively. Both dodecameric sequences contain the canonical site and three flanking basepairs identical to those found in our DNA substrates (see Figures 1, 2 and 4, and Tables I and II). Figure 1 shows the minor groove is quite narrow in this region, with the amino group near the bottom of the groove facing away from the site of methylation in the major groove (N6 position of centrally located adenines).

To determine the contribution of Mtase interactions with the guanosine amino group we substituted deoxyinosine for guanosine, effectively replacing the amine with a hydrogen. This is a conservative replacement, as suggested by the insensitivity of the *EcoRI* endonuclease to incorporation of deoxyinosine into the canonical site [Modrich and Rubin, 1977; Brennan *et al.*, 1986a]. We selected this analog to test our system since previous studies with the *EcoRI* Mtase have also used deoxyinosine [Modrich and Rubin, 1977; Brennan *et al.*, 1986a]. Modrich and Rubin [1977] used a plasmid DNA substrate with a single recognition site and found that activity with the deoxyinosine containing substrate was reduced 25 fold. Brennan *et al.* [1986a] found that a deoxyinosine containing octameric substrate was completely inactive. These investigations involved substrates in which both guanosines were replaced with deoxyinosine, analogous to our substrate TI5-BMI5.

Effect of hemi-methylation on Mtase function

Since our strategy requires the use of hemi-methylated substrates, we analyzed the functional characteristics of both hemi-methylated substrates (CTM and CBM) and compared these with an unmethylated substrate (C). Our kinetic analyses, shown in Table I, demonstrate no effects on k_{cat} and only minor changes on K_m (AdoMet and DNA). However, due to the methylation rate constant ($k_{methylation}$) being at least ten times faster than k_{cat} [Reich and Mashhoon, 1991], changes in $k_{methylation}$ less than ten fold would not have altered k_{cat} .

The apparent insensitivity of the Mtase to prior adenine methylation of one strand is surprising given the proximity of the two N6 methyl groups, shown in Figure 2 for the doubly methylated dodecameric product. The functional similarity of the hemi-methylated and native substrates supports a mechanism in which methylation of each strand occurs from a unique binding orientation. The alternative mechanism (methylation of either

strand from one binding orientation) is more likely to be sensitive to the presence of a methyl group on one of the potential sites of methylation.

Effect of minor groove guanosine amino on Mtase function

The results with the hemi-methylated substrates (CBM and CTM) supported the use of one of these as a control substrate in our specificity analysis. We selected CBM, and Table II shows that k_{cat} for the two singly-modified deoxyinosine-containing substrates (T-BMI5 and TI5-BM) are not significantly perturbed in comparison to CBM. In contrast, the significant increases in K_m^{DNA} with these substrates suggests that both guanosine amino groups contribute to Mtase-DNA interactions. A comparison of the specificity constants confirms that TI5-BM and T-BMI5 are poorer substrates by a factor of 13 and 39 fold respectively, equivalent to a loss in binding energy of the enzyme for the transition state of 1.56 and 2.23 kcal/mole. Although these apparent binding energies might be over- or under-estimations of the true binding energy, they do reflect differences in specificity [Fersht, 1986]. Thus, differences in k_{cat}/K_m^{DNA} directly implicate the deleted minor groove functionality in Mtase-DNA interactions since k_{cat}/K_m^{DNA} relates the conversion of free enzyme and free DNA to the transition state [Fersht, 1986]. Most structural data [Anderson *et al.*, 1987; Otwinowski *et al.*, 1988; Jordan and Pabo, 1988; Wolberger *et al.*, 1988; Rafferty *et al.*, 1989] and predicted models for sequence recognition [Seeman *et al.*, 1977; von Hippel and Berg, 1986; Dickerson *et al.*, 1987] implicate the DNA major groove as the site of protein-DNA interaction. Nevertheless, results from a variety of systems suggest that minor groove interactions do occur. Phage 434 repressor and Cro proteins both have an arginine which fits in the minor groove of the DNA, although a mutant repressor with an alanine at this position has wild-type specificity [Anderson *et al.*, 1987; Wolberger *et al.*, 1988]. X-ray crystallographic analyses of DNase I [Suck *et al.*, 1988] and the histones in the nucleosome core particle [Richmond *et al.*, 1984] show minor groove interactions. Finally, the tetrapeptide (Ser-Pro-Lys-Lys), found in histone H1, forms a structural motif which binds to the minor groove [Churchill and Suzuki, 1989]. Our results with the *EcoRI* DNA Mtase also support minor groove interactions and further implicate such interactions as determinants of sequence specificity.

Our analysis depends on the modified DNA substrate and Mtase-DNA-AdoMet complex remaining relatively structurally unperturbed. As for the hemi-methylated substrates, our functional analysis included comparisons of K_m^{AdoMet} . In support of the protein conformation remaining unaffected (in the Mtase-DNA-AdoMet complex), the K_m^{AdoMet} values for the different DNA substrates are essentially identical (Table II). An additional approach to determine the structural integrity of the modified substrates and/or Mtase-DNA complexes relies on the extent to which multiple substitutions cause additive functional changes. Previous application of this approach, limited to DNA-binding proteins and substitutions of entire basepairs, demonstrated additivity in the majority of cases [Sarai and Takeda, 1989; Lehming *et al.*, 1990; Takeda *et al.*, 1989; Ebright *et al.*, 1987], although nonadditivity has also been observed [Mossing and Record, 1985]. A more subtle application by Carter *et al.* [1984] with modified proteins was used to elucidate relatively minor structural changes. Here we combined the single modifications introduced in TI5-BM and T-BMI5 to generate TI5-BMI5 (Table II). Clearly, effects of the two substitutions are not additive since

$k_{\text{cat}}/K_m^{\text{DNA}}$ is greater than for T-BMI5. This implies that modification at one position alters the structure of the DNA, the enzyme or both sufficiently to perturb enzyme-DNA interactions at the second site. This precludes quantitative assignment of interactions probed with the individually substituted substrates. Further, our demonstration of the lack of additivity, at least with these substrates implicates a DNA recognition mechanism which is more complex than that found for the previously characterized repressor systems [Sarai and Takeda, 1989; Lehming *et al.*, 1990; Takeda *et al.*, 1989; Ebright *et al.*, 1987]. To investigate the nonadditivity at the level of the DNA substrate we undertook a detailed comparison of the thermodynamic parameters associated with the double- to single-stranded transition.

Thermodynamic analysis of DNA substrates

Our initial interest with the DNA melting analyses was to confirm the thermal stability of the modified substrates. This was verified (Table III) and the deoxyinosine substitutions change the Gibbs free energy for the double- to single-stranded transition by less than one kcal/mole at 37°C. Additivity analysis at this level also shows the interdependence of the individual substitutions since TI5-BMI5 is more stable than T-BMI5. Therefore, although the effects are small, our thermodynamic analysis suggests that the relatively minor single substitutions perturb the structure of the DNA sufficiently to alter the conformation at the non-substituted position. To characterize these effects further we determined the enthalpic and entropic components of the free energy term (Figure 5 and Table III) [Breslauer, 1986; Petruska *et al.*, 1988]. In comparison to CBM, TI5-BM and TI5-BMI5 show minor compensating alterations in enthalpy and entropy. Although well known [Breslauer *et al.*, 1987; Petruska *et al.*, 1988], we have found no generally accepted explanation for this type of compensation. Because the effects on the enthalpy and entropy for these substrates (and T-BMI5, discussed below) are larger than the free energy changes, the non-additivity is even more obvious.

In contrast to TI5-BM and TI5-BMI5, T-BMI5 when compared to CBM shows a large decrease in enthalpy and increase in entropy. A plausible explanation of this unusual behaviour assumes that the denatured strands of both CBM and T-BMI5 have comparable enthalpy and entropy terms. This is reasonable since although the structures of the denatured form(s) are not known, the most significant difference between the deoxyinosine- and guanosine-containing single strands should be the ability to form a small number of additional hydrogen bonds. The differences in enthalpy and entropy therefore derive predominantly from differences in the double stranded forms; hence, the chemical bonds comprising the T-BMI5 structure (and associated waters) are overall weaker by approximately 44 kcal/mole than those in the CBM structure. The smaller entropy change implies that the T-BMI5 structure (and associated waters) is significantly more disordered than the CBM structure (Table III).

Although extrapolation of our thermodynamic data toward a structural understanding of the modified DNA is not straightforward, our results show that conclusions based solely on free energy of melting data can be misleading [McLaughlin *et al.*, 1987; Cosstick *et al.*, 1990; Seela and Kehne, 1987; Newman *et al.*, 1990a, b; Seela and Driller, 1986; Fliess *et al.*, 1986, 1988]. Similar results have been reported for DNA-ligand interactions [Breslauer *et al.*, 1987]. Potential structural explanations of our thermodynamic data are based on the

extensive structural characterization of the *EcoRI* Mtase DNA substrate (Figure 1) [Wing *et al.*, 1980; Nerdal *et al.*, 1989]. The DNA has an extremely narrow minor groove in the central tetranucleotide region (AATT) in which a 'spine' of water molecules has been identified [Drew and Dickerson, 1981]. These water molecules are extensively hydrogen bonded with DNA functionalities and the guanosine amino group in a GC basepair precludes a water molecule from such interactions. This constraint is removed when deoxyinosine is used in place of guanosine, making the minor grooves of inosine-cytosine and adenine-thymine basepairs similar. A likely outcome of this is the extension of the spine of hydration to include five (TI5-BM and T-BMI5) or six (TI5-BMI5) basepairs. Such constrained waters might contribute to an increased degree of order in the inosine substituted substrates; our observed decreases in entropy (smaller changes in entropy, see Table II) with TI-BM and T-BMI5 are at odds with this prediction, suggesting the importance of additional factors.

The *EcoRI* DNA Mtase substrate shown in Figure 1 is completely self-complementary and contains two symmetrically positioned 5'CGA3'/5'TCG3' sequences [Wing *et al.*, 1980; Nerdal *et al.*, 1989]. This trinucleotide sequence has unusual helical parameters as identified by crystallographic analysis [Wing *et al.*, 1980; Dickerson and Drew, 1981; Frederick *et al.*, 1988], DNase digestion [Lomonosoff *et al.*, 1981], and N.M.R. analysis [Nerdal *et al.*, 1989; Chary *et al.*, 1987]. The large variations in helical twist (between the AT and GC basepair, 39 degrees; between GC and CG, 33 degrees) result from movement of the cytosine away from the adjacent thymine to increase its overlap with the adjacent guanosine [Dickerson and Drew, 1981]. In contrast to the dodecamer (Figure 1), the substrates studied in this report are not self-complementary outside the recognition hexanucleotide (Figure 4 and Tables I and II). Thus, 5'CGA3'/5'TCG3' appears only once, while 5'GGA3'/5'TCC3' occurs at the opposite end. Structural analysis of the 5'GGG-3' sequence shows no unusual features in the helical twist parameter [Nilges *et al.*, 1987]. This presumably results from the cytosine being adjacent to another cytosine rather than a guanosine. In the case of TI5-BM, the deoxyinosine replacement creates a 5'GIA3'/5'TCG3' segment, while in T-BMI5, deoxyinosine replacement occurs within the structurally anomalous 5'CGA3'/5'TCG3' segment, generating 5'CIA3'/5'TCG3'. Since each substitution only removes a single basepairing hydrogen bond, the differential changes in thermal parameters imply that the DNA (T-BMI5) and/or associated waters are conformationally altered. We are investigating the solution structures of CBM, T-BMI5 and TI5-BM with N.M.R. methods.

Although deoxyinosine incorporation into the 5'CGA3'/5'TCG3' segment (T-BMI5) may cause significant structural changes in the DNA, the specificity constants for T-BMI5 and TI5-BM vary only three fold (Table II). How, or the extent to which the 5'CGA3'/5'TCG3' segment is altered is not known, although some structural features of the DNA (e.g., helical twist, propeller twist, groove width, flexibility) will likely be affected. These structural features of DNA have been proposed to contribute to the mechanisms whereby proteins discriminate DNA sequences [Dickerson and Drew, 1981a; Dickerson and Drew, 1981b; Calladine, 1982; Sarai *et al.*, 1989]. Unfortunately, this intriguing suggestion is experimentally difficult to investigate. The functional similarity of T-BMI5 and TI5-BM provides evidence that these features within the canonical site may not be primary determinants of sequence specificity for *EcoRI* Mtase.

Relationship to previous analysis of the *EcoRI* DNA Mtase

Previous studies with the *EcoRI* Mtase relied on recognition sites containing deoxyinosine at both guanosine positions, equivalent to a nonmethylated version of T15-BMI5 [Modrich and Rubin, 1977; Brennan *et al.*, 1986a]. The plasmid substrate used by Modrich and Rubin contained a single canonical site and was 25 fold less active when guanosine was replaced with deoxyinosine. These results are surprisingly similar to ours (14 fold, Table II), since the plasmid data was obtained at single concentrations of DNA and AdoMet. Based on the 26 fold poorer binding of the T15-BMI5 substrate (Table II), the difference is easily explained by the lack of saturation with the inosine-containing plasmid. In contrast, the work by Brennan *et al.* [1986a] showed that a deoxyinosine containing octameric oligonucleotide was completely inactive. Comparison with our work is compromised by several factors. Their assays were done at 10°C, and catalytic turnover with an unmodified substrate was over eighty times slower than substrate C (Table I). Based on our footprinting data, their octameric substrates may preclude some Mtase-DNA interactions with the flanking DNA. This in turn may have altered Mtase interactions with the minor groove of the canonical site.

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