The coupling of tight DNA binding and base flipping: identification of a conserved structural motif in base flipping enzymes

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Summary

Valine$^{121}$ is positioned immediately above the extrahelical cytosine in HhaI DNA C$^5$-cytosine methyltransferase and replacement with alanine dramatically interferes with base flipping and catalysis. DNA binding and $k_{cat}$ are decreased $10^5$ fold for the Val$^{121}$Ala mutant which has a normal circular dichroism spectrum and AdoMet affinity. The magnitude of this loss of function is comparable to removal of the essential catalytic Cys$^{81}$. Surprisingly, DNA binding is completely recovered (increase of $10^5$ fold) with a DNA substrate lacking the target cytosine base (abasic). Thus, interfering with the base flipping transition results in a dramatic loss of binding energy. Our data supports an induced fit mechanism in which tight DNA binding is coupled to both base flipping and protein loop rearrangement. The importance of the proximal protein segment (His$^{127}$-Thr$^{132}$) in maintaining this critical interaction between Val$^{121}$ and the flipped cytosine was probed with single site alanine substitutions. None of these mutants are significantly altered in secondary structure, AdoMet or DNA affinity, $k_{methylation}$, $k_{inactivation}$, or $k_{cat}$. While Valine$^{121}$ plays a critical role in both extrahelical base stabilization and catalysis, its position and mobility are not influenced by individual residues in the adjacent peptide region. Structural comparisons with other DNA methyltransferases and DNA repair enzymes that stabilize extrahelical nucleotides reveals a motif which includes a positively charged or polar side chain and a hydrophobic residue positioned adjacent to the target DNA base and either the 5’ or 3’ phosphate.
Abbreviations: 

WT, wild type; **M.HhaI**, C^5_ cytosine methyltransferase type I from *Haemophilus haemolyticus*; **AdoMet**, S-adenosyl-L-methionine; **AdoHcy**, S-adenosyl-L-homocysteine; **MD**, molecular dynamics; **PCR**, Polymerase chain reaction

**Keywords:** M.HhaI, base flipping, correlated motions, alanine scan, mutagenesis, motif, induced fit
Introduction

The molecular basis of enzymatic catalysis is of broad interest, with implications for biocatalyst design and drug development. The abundance of detailed three-dimensional structures and investigational methods provide newly addressable aspects of enzymatic function. We are interested in the importance of protein motion, and particularly correlated motions, to catalysis. The underlying premise is that protein-solvent interactions are converted into peptide motions, resulting in the transient stabilization of active site elements with preferred reactivities(1;2).

Recent studies have provided highly suggestive evidence for this concept. Molecular dynamics investigations of dihydrofolate reductase (DHFR) demonstrate that strong coupled motions in the reactive complex disappear in the product complexes, indicating that these motions may be linked to catalysis(1). Mutants that alter the kinetics of particular catalytic steps are concentrated within segments of the protein structure shown to participate in highly correlated motions(1). Solid state NMR and solution NMR relaxation studies have measured substrate and protein dynamics that are matched to the turnover-time of the respective enzymes(3). Studies of hydrogen and electron tunneling during enzyme catalysis provide further evidence for the importance of protein dynamics to catalytic events at the active site(4;5).

Molecular dynamic simulations of catechol O-methyltransferase and M.HhaI DNA methyltransferase provided initial evidence for correlated motions within the active sites of these enzymes(6;7). We sought to test the importance to catalysis of motions made by specific distal residues (His\textsuperscript{127} - Thr\textsuperscript{132}) in facilitating active site chemistries by altering the position and orientation of critical residues such as Val\textsuperscript{121}. Alanine scan
point mutagenesis and kinetic characterization of individual steps in the catalytic cycle were used to probe the effects of such mutations and provide insights into the roles of correlated motions. M.HhaI provides an excellent structurally and functionally tractable enzyme to study various aspects of catalysis, including base flipping and the importance of motions to catalysis. M.HhaI, from *Haemophilus haemolyticus*, is an AdoMet dependent C₅-cytosine methyltransferase which methylates the central cytosine (C) in the recognition sequence 5’-GC₅GC-3’ after stabilizing the target base in an extrahelical position. Many M.HhaI crystal structures provide structural insights into the mechanisms of DNA methylation and base flipping(8). Functional analysis of the WT M.HhaI has been extensive(9-12), including $K_D^{DNA}$ determination for a variety of DNA substrates(13;14). Many structural components of the M.HhaI mechanism have been examined by mutagenesis including Gln²³⁷ which positions itself into the DNA helix and interacts with the lone guanine(15) and Cys⁸¹ which forms a covalent bond to the target cytosine(16). Other mutational studies have examined protein-phosphate interactions(17) and conserved residues within the AdoMet binding pocket(18). Crystal structures have also been solved for the enzyme bound to its cofactor, enzyme bound to DNA containing modified target bases, and mutant structures have been solved(17;19-21). These studies have revealed a large loop movement involving residues 80-99 which appears to be induced by the enzyme’s binding to the cognate DNA sequence. The binary complex with a non-specific DNA sequence has this loop positioned in the open conformation(22), seen also in the binary enzyme/AdoMet complex(20). Moreover, this loop is only seen in the closed conformation in the co-crystal structure containing the tightly bound cognate DNA(19); thus, the enzyme appears to follow an induced fit mechanism in which tight
DNA binding is coupled to loop movement to the closed conformation and coincidentally, base flipping(14;19).

Extrahelical nucleoside stabilization, or base flipping, is used by enzymes to assist in chemical modification of DNA substrates. Enzyme-DNA interactions mediate processes which allow for destabilization of the DNA helix and extrahelical stabilization of the target base. This shift in DNA conformation is utilized in a variety of biological processes including DNA methylation and DNA repair(23-26), by providing access to buried functionalities on DNA bases that would be inaccessible in a $B$-form DNA helix. Current proposals on the mechanisms by which enzymes facilitate base flipping include enzymatic manipulations to either 5’ or 3’ phosphates(8;27), “pinch-pull-push” mechanisms(28) and passive mechanisms involving stabilization of transiently flipped nucleotides in $B$-form DNA(29). Studies on M.HhaI aimed at elucidating the base flipping mechanism include crystallographic studies(19), theoretical calculations(30), NMR studies(31), mutagenesis(32;33), and kinetic characterization(14;34). Many fundamental aspects of base flipping remain highly debated, including which DNA groove the target base moves through as it flips and which protein-DNA contacts facilitate this process. [Insert Figure 1]

We initiated this study to investigate the importance of interactions between Val$^{121}$ and the extrahelical cytosine, and to identify distal protein elements that provide both static and dynamic scaffolding for this interaction. Our motivation came in part from molecular dynamics simulations which identified anticorrelated active site motions(2;6;7). This anticorrelated motion could cause the active site to undergo a compression, which was previously proposed to be important for AdoMet-dependent
methyltransfer reactions(35). Moreover, we hypothesized that such motions could be disrupted by amino acid substitutions in critical peptide elements. Residues 121-132 originate at the active site with Val\textsuperscript{121} and end on the protein surface with Thr\textsuperscript{132} (see Figure 1). In between these two residues, M.HhaI makes an unusual αβ-turn with multiple hydrogen bonds including an internal hydrogen bond between His\textsuperscript{127} and Thr\textsuperscript{132} (see Figure 1C). We used alanine substitutions of residues distal from the active site (His\textsuperscript{127}-Thr\textsuperscript{132}) but connected to a critical residue in the active site (Val\textsuperscript{121}) to probe for correlated motions. Val\textsuperscript{121} is located directly over the target cytosine and we show its positioning is crucial for base flipping and catalysis.
Experimental Procedures

*Site-directed Mutagenesis, Protein Expression, and Purification*-Seven M.HhaI mutants were produced (Val^{121}Ala, His^{127}Ala, Asp^{128}Ala, Asn^{129}Ala, Gly^{130}Ala, Asn^{131}Ala, and Thr^{132}Ala) using the QuikChange PCR mutagenesis kit (Stratagene) with the vector pHSHW-5 (provided by S. Kumar, New England Biolabs) as a template and seven sets of PCR primers (Genset Oligos). PCR products were digested (DpnI) to remove any WT plasmid, transformed via heat shock into competent *Escherichia coli* strain ER1727 and plated onto agar plates supplemented with 60 µg/mL of ampicillin. Sequencing of DNA from individual colonies was done by the UIUC Biotechnology Center (Urbana Illinois). WT and mutant M.HhaI over-expression from pHSHW-5 derived vectors was accomplished with early log phase (OD_{600} = 0.4) induction with 1.0 mM isopropyl β-D-thiogalactopyranoside (IPTG). Cells were centrifugally harvested and protein purified according to previously published methods(36). After purification, enzymes were >95% pure and were flash frozen in 20 µL aliquots and stored at -70° C. WT protein concentration was determined by active site titration and mutant protein concentration was by Bradford Staining based on a standard curve of the WT protein(37).

*Confirmation of Mutant M.HhaI Sequence by Mass Spectrometry*-Nano-electrospray ionization was used to confirm the substitution of alanine at selected positions in M.HhaI. Both mutant and WT enzymes were run on SDS-PAGE gels and visualized by silver-staining (Invitrogen Silver-Quest). Protein bands were excised from the gel and subsequently destained in 1.5 ml siliconized eppendorf tubes. To enhance diffusion of protease into the gel matrix, gel pieces were dried by speed-vac (Savant) and incubated with 100 µL of 12.5 ng/µl sequencing-grade modified trypsin (Promega) for
20-30 minutes on ice after which excess protease was removed and replaced with 50 mM NH₄HCO₃ for overnight digestion at 37°C. The resulting peptides were extracted several times with a 50% acetonitrile/5% formic acid solution, lyophilized, and resuspended in 5% formic acid. Digested peptides were desalted and concentrated using POROS R2 resin (Perseptive Biosystems) that had been loaded into a nanospray capillary (Proxeon Biosystems). Peptides were loaded onto the resin, washed with 5% formic acid, and eluted into a borosilicate capillary (Proxeon Biosystems) with 50% methanol/5% formic acid. The capillary containing the eluted peptides was loaded onto an Applied Biosystems/MDS Sciex Q-STAR Quadrupole/time-of-flight (Q/TOF) mass spectrometer and data was collected over a mass range m/z 300-1500 in positive-ion mode. Peptide peaks containing the desired substituted amino acid were observed for all mutants but not for the WT protein, and similarly, the WT peptide was observed in WT protein and not in the mutants. Both WT and mutant peptide was analyzed further by tandem mass spectrometry (MS/MS) for its amino acid sequence. Data (not shown) was collected over a mass range of m/z 50-2000 in positive-ion mode.

*Circular Dichroism*-Circular dichroism was performed on WT and mutant M.HhaI in 50 mM sodium phosphate, pH 7.0 at room temperature. Data was collected on an Aviv 202 Circular Dichroism Spectrophotometer using a 500 µL quartz fluorescence cuvette with a 0.2 cm slit width (Starna). Data was collected between 190 nm and 265 nm.

*Oligonucleotide Synthesis and Purification*-Oligonucleotides used for kinetic analysis required the use of multiple DNA substrates with a single recognition site, but with variations at the target base. The variations are shown below with the recognition
sequence underlined and the target bases in bold. C is cytosine, M is 5-methylcytosine, F is 5-florocytosine, and B is an abasic nucleotide.

$A_{box}$:

5'-GGGAATTCT\text{\underline{C}}G\text{\underline{G}}CAGTGG\text{\underline{G}}G\text{\underline{G}}G\text{\underline{G}}G\text{\underline{A}}TCC\text{\underline{A}}G-3'$

3'-CCCT\text{\underline{A}}AGTACC\text{\underline{G}}C\text{\underline{G}}\text{\underline{T}}\text{\underline{A}}CC\text{\underline{A}}\text{\underline{C}}\text{\underline{C}}\text{\underline{T}}\text{\underline{A}}\text{\underline{G}}\text{\underline{G}}\text{\underline{T}}\text{\underline{C}}-5'$

$F_{AM\_box}$:

5'-GGGAATTCT\text{\underline{F}}G\text{\underline{F}}G\text{\underline{C}}AGTGG\text{\underline{G}}G\text{\underline{G}}G\text{\underline{G}}G\text{\underline{A}}TCC\text{\underline{A}}G-3'$

3'-CCCT\text{\underline{A}}AGTAC\text{\underline{C}}CG\text{\underline{M}}\text{\underline{G}}T\text{\underline{C}}\text{\underline{A}}\text{\underline{C}}\text{\underline{C}}\text{\underline{C}}\text{\underline{A}}\text{\underline{C}}\text{\underline{T}}\text{\underline{A}}\text{\underline{G}}\text{\underline{G}}\text{\underline{T}}\text{\underline{C}}-5'$

$A_{BM\_box}$:

5'-GGGAATTCT\text{\underline{B}}G\text{\underline{B}}CAGTGG\text{\underline{G}}G\text{\underline{G}}G\text{\underline{G}}G\text{\underline{A}}TCC\text{\underline{A}}G-3'$

3'-CCCT\text{\underline{A}}AGTAC\text{\underline{C}}CG\text{\underline{M}}\text{\underline{G}}T\text{\underline{C}}\text{\underline{A}}\text{\underline{C}}\text{\underline{C}}\text{\underline{C}}\text{\underline{A}}\text{\underline{C}}\text{\underline{T}}\text{\underline{A}}\text{\underline{G}}\text{\underline{G}}\text{\underline{T}}\text{\underline{C}}-5'$

Substrate oligonucleotides were synthesized by Midlands DNA (Midland, TX) and purified on a Dynamax PureDNA HPLC (High pressure liquid chromatography) column (Rainin Instrument Co.) according to the manufacturer’s specifications. Oligonucleotides were stored in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). Concentrations were photometrically determined using calculated extinction coefficients(9). Oligonucleotide duplexes were formed by annealing the single strands in TE buffer with 10 mM NaCl and heated to 95° C for 5 minutes before being cooled slowly to room temperature. Ratios of single strands were optimized to provide the most duplex with the least amount of
residual single strands. For gel mobility shift assays, DNA substrates were radio labeled using $[^{32}\text{P}]$ ATP (Amersham Pharmacia Biotech) and T4 polynucleotide kinase (New England Biolabs).

*Cofactors*- AdoMet and AdoHcy were purchased from Sigma and stored in 0.1N HCl.

*Steady State Assays*- $k_{\text{cat}}$ values for WT and mutant enzymes were determined as previously reported using filter binding assays with a few variations\(^{(9)}\). This turnover constant, and all other reported $k_{\text{cat}}$ values, is actually an apparent constant since it was determined at a single concentration of one or both of the two substrates. Reactions were initiated by the addition of enzyme (10 nM and 20 nM) to AdoMet (1.2 µM) and Abox DNA (1 µM) in MR buffer (100 mM Tris, pH 8.0, 10 mM EDTA, 10 mM DTT (dithreitol), 0.2 mg/mL BSA (bovine serum albumin)) incubated at 37° C. Six time points were taken at ten seconds intervals, quenched on DE-81 filters, and processed as previously described\(^{(37)}\). Samples were analyzed with a Beckman Coulter LS6500 Multi-Purpose Scintillation Counter and linear graphs were fit using Kaleidagraph.

$k_{\text{cat}}$ values were derived for WT and Val\(^{121}\)Ala M.HhaI by another filter binding, steady state assay. Reactions were initiated by the addition of enzyme (8 µM for Val\(^{121}\)Ala and 100 pM for WT) to AdoMet (125 nM for WT and 10 µM for Val\(^{121}\)Ala) and Abox DNA (100 nM for WT and 10 µM for Val\(^{121}\)Ala) in PD buffer (20 mM potassium phosphate, pH 7.5, 200 mM NaCl, 0.2 mM EDTA, 0.2 mg/mL BSA, 2 mM DTT, 10% glycerol) incubated at 37° C. Time points were taken every 10 minutes for 1 hour.
AdoMet Equilibrium Dissociation Constant-$K_D^{\text{AdoMet}}$ values were determined for WT and mutant enzymes using native protein fluorescence as previously described(9). Briefly, a Perkin-Elmer LS50B luminescence spectrometer was used for fluorescence measurements at room temperature. Excitation slit width was 5 mm, and emission slit width was 7.5 mm. A xenon lamp was used to excite at a wavelength of 280 nm. Emission spectra were recorded from 320 nm to 430 nm at a scan speed of 100 nm/min. The samples contained enzyme (1 µM), 100 mM Tris pH 8.0, 10 mM EDTA, 10 mM DTT, and varying AdoMet concentrations (0 µM to 200 µM). For obtaining $K_D^{\text{AdoMet}}$ values, areas of the spectral curves were determined using Origin and curves were fit using SigmaPlot. The equation was fit to a rectangular hyperbola.

Single Turnover Assays-Single turnover assays were conducted as previously reported with two different DNA substrates, Abox DNA and FAMbox DNA(9). For Abox DNA single turnover assays, AdoMet (3 µM) and Abox DNA (100 nM) were mixed in MR buffer and incubated at 6° C. Reactions were initiated by the addition of excess enzyme (0.4 µM to 3 µM) and time points taken at 4, 8, 12, 16, 25, 40, and 60 seconds, quenched in a final concentration of 0.5% SDS and spotted on DE-81 filters for analysis. For FAMbox DNA single turnover assays, AdoMet (800 nM) and enzyme (750 nM) in PD buffer (20 mM potassium phosphate, pH 7.5, 200 mM NaCl, 0.2 mM EDTA, 0.2 mg/mL BSA, 2 mM DTT, 10% glycerol) was incubated at 37° C. Reactions were initiated by FAMbox DNA addition (900 nM). Time points taken at 2, 4, 10, 30, 70, and 120 minutes, were spotted on DE-81 filters for quenching. SigmaPlot was used to fit all single turnover exponential rise to maximum curves.
**DNA Equilibrium Dissociation Constant**-$K_D^{DNA}$ values were determined as previously reported in the presence of the cofactor AdoHcy for two different DNA substrates, Abox and ABMbox(9). For $K_D^{Abox-DNA}$, AdoHcy (25 µM), radio labeled Abox DNA (50 pM), and enzyme (10 pM to 1 nM) were used. For the Val$^{121}$Ala M.HhaI Abox DNA gel shift, enzyme concentrations up to 4 µM were used. For $K_D^{ABMbox-DNA}$, AdoHcy (25 µM), radio labeled ABMbox DNA (200 pM), and enzyme (1 pM to 5 nM) were used. Samples were incubated at room temperature for 10 minutes in MR buffer, than loaded onto a pre-run, 12% non-denaturing polyacrylamide gel. Gels were run at 300 V for 150 minutes at room temperature. Gels were exposed to image plates and analyzed using a Storm 840 densitometer (Molecular Dynamics, Inc). Densitometry was performed using ImageQuant (Molecular Dynamics, Inc). SigmaPlot was used to fit the data to a rectangular hyperbola curve and obtain $K_D^{DNA}$ values.

**Superimpositions and Computer Generated Graphics**-Images of M.HhaI were generated using SYBYL and InsightII on Silicon Graphics computers. PDB (Protein data bank) structures of M.HhaI, M.HaeIII, M.TaqI, and DNA glycosylases include: 1MHT-9MHT, 1HMY, 1DCT, 1G38, 1BNK, 1EBM, 1DIZ, and 1EMH. Superimpositions for Figure 10C were done on InsightII using the Transpose function with six sets of defining atoms: C3’ and C4’ carbon atoms of the flipped sugar, two phosphate atoms flanking the flipped base, and side chain $\alpha$-carbons for Val$^{121}$ and Arg$^{165}$ (Val$^{111}$ and Arg$^{155}$ for M.HaeIII). Superimpositions which had no DNA atoms, 1HMY, used just the protein side chain $\alpha$-carbons. All structures were superimposed onto 3MHT.
Results

Mutant Design—We used alanine replacements in our study because it is the most common amino acid, is relatively unrestricted in conformation, and has a small and inert side chain (38). The mutated residues are not directly implicated in catalysis and with the exception of Val\textsuperscript{121} (4 Å), are distal (13-20 Å) from the active site. Within the region selected, Lys\textsuperscript{122}, Asn\textsuperscript{123}, Phe\textsuperscript{124}, and Ser\textsuperscript{126} were omitted due to likely DNA interactions, based on inspection of the 3MHT co-crystal structure. Ala\textsuperscript{125} was also omitted since it is already alanine. The selected region presents a stretch of peptide that molecular dynamic simulations have predicted to make anti-correlated motions to regions of peptide on the opposite side of the active site and these motions are believed to be critical to catalysis (7). Anti-correlated motions involve coupled spatial displacements of two elements towards and away from each other in time.

Purification and Characterization—Purified proteins were initially characterized to confirm that appropriate mutations had been made, which included DNA sequencing of the encoding plasmid, peptide sequencing by mass spectrometry, and circular dichroism. Circular dichroism spectra for WT and mutant M.HhaI proteins all showed a high degree of similarity; representative data for Val\textsuperscript{121}Ala and WT M.HhaI are shown in Figure 2. DNA sequencing confirmed no secondary mutations had been made while mass spectrometry data of M.HhaI peptide fragments confirmed that the alanine mutations were appropriately made. Tryptic digested M.HhaI peptide fragment 115-122 was observed at 483.27 (483.26 theoretical) in all WT samples, and was shifted in each mutant spectrum due to the alanine mutation altering the fragment’s molecular mass. The Val\textsuperscript{121}Ala fragment was observed at 469.31 (469.24 theoretical) and is representative of
other mutant proteins. The mass spectrometric analysis also revealed that Asn$^{129}$ is partially deaminated to Asp$^{129}$ in all M.HhaI mutants and WT (data not shown). Inspection of peak clusters from MS/MS data of a peptide fragment (residues 123-137) showed heterogeneity in all peaks for amino acids from 130 to 137 and not for amino acids 123 to 129. In addition, MS/MS peak values revealed the contaminant peptide fragment had a mass one unit higher than the WT fragment which disappeared when Asn$^{129}$ was removed. This mass difference is accountable only by an asparagine that was deamidated to an aspartic acid(39). Although not previously reported for M.HhaI, such post translational modifications are well characterized and can potentially effect an enzyme’s function(40). We believe the deamidations at position 129 are unlikely to alter the function of any of the WT or mutant M.HhaI proteins. The Asn$^{129}$Ala mutant has functional parameters that are indistinguishable from the WT protein. The other M.HhaI mutants have similar levels of Asn$^{129}$ deamidation, yet other than the Val$^{121}$Ala mutant, have WT functional parameters. Finally, Asn$^{129}$ is a considerable distance from the active site. In summary, it is highly unlikely that the large effect on DNA binding observed for the Val$^{121}$Ala mutant is derived from the double mutation as opposed to just the valine substitution. [Insert Figure 2]

*AdoMet Equilibrium Dissociation Constant*—M.HhaI has a single tryptophan residue (Trp$^{41}$) located in the AdoMet binding pocket; AdoMet binding results in fluorescence quenching. Steady state native protein fluorescence can be used to determine $K_D^{AdoMet}$, which provides a direct measure of the mutant’s functional integrity and affinity for AdoMet(9). Upon addition of AdoMet, a sharp decrease in native protein fluorescence of M.HhaI is seen with an emission maximum blue shift, further confirming
that Trp$^{41}$ is being shielded from the aqueous solvent by AdoMet. AdoMet titrations provide fluorescence data which yield $K_D^{AdoMet}$ values for mutant and WT proteins (see Table 1 and Figure 3). Since all the mutants show near WT values, including Val$^{121}$Ala, we conclude that the AdoMet binding pocket, an intimate component of the active site, is structurally unperturbed for all our mutants. [Insert Table 1 and Figure 3 and 4]

**Steady State Assays**—Catalytic turnover constants ($k_{cat}$) were obtained by a burst assay and the data fit to a linear equation. Figure 4 shows burst data for Val$^{121}$Ala, Gly$^{130}$Ala, and WT enzymes. The burst magnitude for each assay obtained by extrapolating to zero time was used to determine the concentration of active enzyme used in the assay(37). The burst is caused by the fast processes leading up to and including DNA methylation ($k_{methyltransfer}$) followed by a slow and final step of DNA release, which causes the amount of initial product formed at time zero to appear greater than zero(9). $k_{cat}$ values were obtained from the linear portion of the product versus time profile. The $k_{cat}$ values for all mutants other than Val$^{121}$Ala were similar and close to values previously published for the WT enzyme (0.14 ± 0.02 (s$^{-1}$) (see Table 2))(9). Val$^{121}$Ala showed no detectable activity in this assay (see Figure 4). [Insert Figure 5]

We used an independent measure of $k_{cat}$ to assess the Val$^{121}$Ala mutant (see Figure 5). This assay uses much higher concentrations of mutant enzyme and requires multiple turnovers. $k_{cat}$ is ~$10^5$ fold lower for Val$^{121}$Ala than WT based on this assay. This drastic change of $k_{cat}$ reveals that Val$^{121}$Ala can catalyze methylation, but is much worse than the WT enzyme. [Insert Figure 6, 7, and 8]

**Single Turnover Assays**—Single turnover experiments probe specific catalytic steps of M.HhaI that are not revealed by steady state kinetics(9;34). We used Abox and
FA_{M}box DNA substrates to determine $k_{\text{methylation}}$ and $k_{\text{inactivation}}$ values respectively. The observed methylation of the Abox DNA substrate formally includes all steps through $k_{\text{methylation}}$ (see Figure 6 and Figure 7, Equation A). Recent work form our group has indicated that $k_{\text{methylation}}$ is the rate limiting step under single turnover conditions (Reich and Svedruzic submitted). Thus, $k_{\text{methylation}}$ determined under single turnover conditions with Abox DNA substrates, reflects the transition involving actual methyltransfer. The single turnover constant measured with FA_{M}box DNA is also dominated by methyltransfer kinetics, as previously predicted(41). FA_{M}box DNA inactivates M.HhaI by preventing the $\beta$-elimination step and the enzyme is unable to remove the C$^5$ fluorine atom(11). This produces a stable covalent complex between Cys$^{81}$ of the enzyme and the C$^4$ carbon of the target base (see Figure 7 Equation B). $k_{\text{inactivation}}$ was previously shown to be $\sim$400 fold slower than $k_{\text{methylation}}$(10), and we find a similar relationship here (see Figure 8 and Table 2). Because the catalytic process is slowed down so dramatically, kinetic studies with the mechanism-based inhibitor, FA_{M}box, provide a more sensitive measure of how M.HhaI mutants are impacted in catalysis. This is seen in Table 2 and Figure 8 which show that the His$^{127}$Ala mutant is $\sim$10 fold slower for the FA_{M}box DNA substrate and has near WT rates for the Abox DNA substrate. [Insert Figure 9]

**DNA Equilibrium Dissociation Constants**- DNA dissociation constants ($K_{D}^{\text{DNA}}$) were determined by gel mobility shift assay for multiple DNA substrates (see Table 1 and Figure 9). All mutants except Val$^{121}$Ala show near WT dissociation constants with the Abox DNA substrate ($K_{D}^{\text{DNA-Abox}}$). In order to quantify the impact the Val$^{121}$Ala mutation had on DNA binding of the Abox DNA substrate, a large excess of enzyme (5 $\mu$M) was used. Densitometry of bands of shifted enzyme/DNA complexes within gels between WT
and the Val^{121}Ala mutant at high concentrations revealed at least a $10^5$ fold loss in DNA affinity; this is shown in the $K_D^{DNA-Abox}$ values seen in Table 1 and Figure 9.

WT M.HhaI binds mismatch, abasic, and nicked DNA substrates with stronger affinity than to natural cytosine target sites(13); this is confirmed in the two-fold tighter binding of $AB_{Mbox}$ DNA versus Abox DNA by the WT enzyme (see Table 1 and Figure 9). Interestingly, the Val^{121}Ala gel shift with $AB_{Mbox}$ DNA generated a dissociation constant ($K_D^{DNA-AB_{Mbox}}$) near WT values. Thus, removal of the target cytosine base results in at least a $10^5$ increase in affinity of the mutant for its DNA substrate, suggesting that Val^{121} plays a critical role in extrahelical base stabilization.

Superimpositions- RMSD values were calculated for each superimposition shown in Figure 10C and the values all show a small deviation ($\pm 0.079$ Å) from the average value of 0.292 Å. RMSD values reflect the similarity in the structures seen in Figure 10C, which also reveals how the two co-crystallized cytosine methyltransferases, M.HhaI and M.HaeIII, have almost identical active sites (see green and blue structures respectively in Figure 10C). Superimpositions of the M.HhaI structures shows that Arg^{165} occupies a position distal from the 5’ phosphate for the binary structure with AdoMet and the tertiary structure with an abasic target base (see red and black structures respectively in Figure 10C).
Discussion

Dynamics studies on M.HhaI show that the extrahelical cytosine within the enzyme’s active site has very low B-factors (7). This rigidity is in part due to restricted $\chi$ angles of the target base which is critical for the nucleophilic attack by Cys$^{81}$, transfer of the methyl group from the AdoMet cofactor, and hydrogen bonding interactions with Arg$^{165}$ and Glu$^{119}$ (see Figure 10A and 10B). Val$^{121}$ and other nearby residues appear to be important for active site compression and stabilization of the extrahelical cytosine by determining the appropriate $\chi$ angle(7). We sought to investigate the importance of Val$^{121}$ and the adjacent peptide element (residues 127-132) on the active site compression and extrahelical cytosine positioning (7;19). The adjacent peptide element includes an unusual $\alpha\beta$-turn with a hydrogen bond between His$^{127}$ and Thr$^{132}$ (see Figure 1C) which could help stabilize and maintain active site interactions involving Val$^{121}$.

Tables 1 and 2 summarize our structural and functional analyses of Val$^{121}$Ala and six additional M.HhaI mutants, all involving alanine substitutions. All mutants have near WT secondary structure, as determined by circular dichroism (see Figure 2). All mutants except for Val$^{121}$Ala have near WT values for $k_{\text{cat}}$ and $k_{\text{methylation}}$ while $k_{\text{inactivation}}$ shows a ~10-fold decrease for the His$^{127}$Ala mutant and no detectable signal for the Val$^{121}$Ala mutant (see Table 2). All mutants have near WT affinity for AdoMet and, except for Val$^{121}$Ala, have near WT affinity for Abox DNA (see Table 1), also supporting the idea that these alanine replacements left the tertiary protein structures unperturbed. The Val$^{121}$Ala mutant was dramatically altered in both DNA binding and catalysis, showing losses in activity comparable to the glycine mutant of the nucleophilic Cys$^{81}$ in M.HhaI(16), and the alanine mutant of Cys$^{186}$ in EcoRII(42).
Our mutant analysis probed the importance of the putative hydrogen bond between His$^{127}$ and Thr$^{132}$ in M.HhaI that was suspected to be necessary for positioning Val$^{121}$ above the extrahelical cytosine (see Figure 1C). Neither His$^{127}$Ala nor Thr$^{132}$Ala show a significant change in $k_{\text{cat}}$ or the pre-steady state parameter, $k_{\text{methylation}}$, measured with the Abox DNA substrate (see Table 2). Only in the case of $k_{\text{inactivation}}$, measured with the mechanism-based inhibitor (5-fluorocytosine at the target base, FA$_M$box DNA), is the His$^{127}$Ala mutant ~10-fold slower than the WT enzyme (see Figure 8 and Table 2). FA$_M$box DNA decreases the observed rates for M.HhaI methylation by ~400 fold (see Figure 7 Equation B)(10). Thus, only when the energetics of the methylation reaction are made significantly more difficult do we detect subtle differences in specific catalytic steps for mutants relative to WT M.HhaI. In sum, the alanine substitutions distal from the active site (residues 127 to 132) appear to have minimal impacts on catalysis. We found this somewhat surprising given the importance of Val$^{121}$ to both DNA binding and catalysis (see below).

The Val$^{121}$Ala mutant showed no detectable activity in the single turnover experiments with either Abox or FA$_M$box DNA, and a ~10$^5$ lower steady state turnover rate ($k_{\text{cat}}$) than WT M.HhaI (see Figures 5, 6, and 8 and Table 2). Inspection of several M.HhaI/DNA co-crystal structures suggested that Val$^{121}$ may be important for maintaining protein interactions with the target base. Moreover, we hypothesized that the Val$^{121}$Ala mutant might preclude such interactions and that removal of the target base could recover some DNA binding since such putative negative interactions would be removed. Surprisingly, the mutant binds abasic DNA lacking the target cytosine base (AB$_M$box DNA) with near WT affinity(12); hence, the removal of this cytosine base
recovers at least $10^5$ fold binding energy (see Table 1 and Figure 9). While a definitive mechanistic understanding will require a co-crystal structure of the mutant enzyme/DNA complex, these results have direct bearing on the induced fit mechanisms of DNA binding, base flipping, and loop motions in M.HhaI. [Insert Figure 10]

An interesting conclusion from our kinetic and thermodynamic data is that prevention of the complete base flipping process is detrimental to DNA binding. Furthermore, this loss of DNA affinity can be accomplished by perturbing interactions with the base of the target nucleotide, even though the WT enzyme is highly accommodating for mismatched nucleotides. One obvious interaction previously suggested to contribute to the base flipping process involves protein/phosphate interactions (8;27;31;43-45); for M.HhaI, this includes the phosphate 5’ to the flipped cytosine and Arg$^{165}$. Below we discuss this in detail in the context of a conserved motif, but we propose that any perturbation in this interaction is unlikely to contribute significantly to the mutant’s loss in cognate DNA binding affinity. The tertiary co-crystal structure of WT M.HhaI with an abasic DNA substrate and AdoHcy (black structure, Figure 10C; 9MHT)(21) and in the binary M.HhaI/AdoMet co-crystal structure (red structure, Figure 10C; 3MHT)(20), shows that Arg$^{165}$ moves away from the DNA phosphate and into an active site cavity left vacant by the missing base. Arg$^{165}$ moves 2.3Å away from the 5’ phosphate between the co-crystal structure of the cognate cytosine target site (3MHT) and the abasic target site (9MHT) and this net distance (5.3 Å) is too great to allow any hydrogen bonding between Arg$^{165}$ and the 5’ phosphate (see Figure 10C). Thus, Arg$^{165}$ does not appear to make critical interactions to stabilize the 5’ phosphate with an abasic DNA substrate which has a tighter affinity than cognate DNA
(see Table 1). Furthermore, whatever interactions are critical for the tight binding of abasic DNA are retained in the Val$^{121}$ Ala mutant since it shows nearly the same affinity for this site as the WT enzyme.

The dramatic loss of cognate DNA binding affinity by the Val$^{121}$ Ala mutant may derive partially from the disruption of direct interactions between Val$^{121}$ and the extrahelical base. Although our motif identification (discussed below), the MD simulations(6;7), and NMR studies(31), lend some credence to this hypothesis, it seems unlikely that direct interactions between an active site hydrophobic residue and the extrahelical base would account for a $\sim 10^5$ loss of DNA binding when perturbed(46). Furthermore, the fact that WT M.HhaI binds abasic and mismatched DNA with tight affinity argues against the quantitative significance of such interactions(12). A related explanation is that Val$^{121}$ may be required for the correct assembly of other active site residues, such as Glu$^{119}$, Arg$^{165}$, and Cys$^{81}$ and improper assembly leads to detrimental interactions with the target base (see Figure 10A and 10B). Disruption of critical interactions involving the cytosine base with Glu$^{119}$, Arg$^{165}$, and Cys$^{81}$ could account for the loss in DNA affinity. The $\sim 10^5$ decrease in cognate DNA binding affinity ($\sim 7$ Kcal) corresponds to a loss of two or three hydrogen bonds(46), which could reasonably be assigned to Glu$^{119}$, Arg$^{165}$, and the thioether bond made by Cys$^{81}$ (Figure 10A)(19). The covalent enzyme/DNA adduct involving Cys$^{81}$ could also contribute significant binding energy (Svedruzic and Reich, submitted). The disruption of these interactions could reasonably reposition residues within the active site in the region normally occupied by the extrahelical cytosine base. The fundamental problems with these explanations are that
they fail to account for the lack of tight binding of the cognate DNA site by the Val121Ala mutant, and the tight binding of abasic and mismatched sites by the WT M.HhaI.

We therefore propose a mechanism in which tight DNA binding and base flipping are coupled. This induced fit mechanism involves utilization of the binding energy provided by the enzyme’s transition from the initial complex with non-specific DNA to that involving the cognate site to drive both base flipping and protein loop movement. The most compelling evidence in support of this mechanism at least contributing to our observations with the Val121Ala mutant is that removal of the target cytosine allows the mutant to bind DNA with tight affinity, presumably because base flipping or base stabilization and DNA binding have been uncoupled. Other data supports such a coupling, albeit from a different perspective. The WT enzyme binds mismatched and abasic substrates more tightly than the cognate site, in spite of the loss of specific interactions between the extrahelical base and the active site(13). This observation is somewhat surprising because the extrahelical base is not as well-accommodated within the active site as the cognate cytosine. However, the equilibrium between the stacked and flipped state of DNA bases, which is typically ~10^5 in favor of the stacked state(47), is significantly disturbed with mismatched bases and abasic DNA(48). Thus, M.HhaI’s binding preference for mismatched DNA most likely derives extensively from the increase in concentration of this normally unstable extrahelical conformation. This supports the coupling of tight binding and base flipping since lowering the energy to flip a base increases the available binding energy.

M.HhaI appears to utilize an induced fit mechanism in another context, which we suggest is further coupled to the transitions proposed here. The peptide loop defined by
residues 80-99 undergoes a large motion (25Å) upon binding cognate DNA. More precisely, the enzyme/AdoMet complex(20), and the enzyme/DNA/AdoMet complex with non-specific DNA(22), both show the loop to be positioned in the open conformation. Furthermore, only in the tight ternary complex with specific DNA containing cytosine, abasic, or mismatches at the target base, is the loop rearranged in the closed conformation. Interestingly, this loop motion does not appear to require the cofactor, as M.HhaI catalyzes efficient nucleophilic attack and exchange of the C5 hydrogen on the target cytosine in the absence of the cofactor ((11); Reich and Svedruzic submitted). This loop was proposed to remain in the open conformation when the furanose ring in the target base is prevented from undergoing sugar pucker transitions thought to be important for base flipping(14). Using abasic DNA molecules with sugar analogs in MD simulations and gel shift experiments, both base flipping and tight DNA binding were shown to be decreased when the sugar torsional angles are restricted(14). Our results and interpretation complement these findings. In both cases, perturbing base flipping impacts DNA affinity, and based on prior structural studies(20;22), this transition may be coupled to loop movement.

The closed loop makes extensive contacts with the DNA, AdoMet, and most importantly, Val121(19). We suggest the overall coupling of tight binding, loop motion, and base flipping to be components of an induced fit process and the Val121Ala mutant has drastically impacted the base flipping component of this process. The two other DNA methyltransferases for which co-crystal structures are available (M.HaeIII and M.TaqI), show very similar loop motions and are thought to share an induced fit/loop motion mechanism(49;50). The final tightly bound complex is only achievable following loop
motion, which requires stabilization of the extrahelical base and/or the correct positioning of active site residues that are normally involved in that stabilization(14). The structural mechanism of how Val$^{121}$ contributes to the correct assembly of the active site or catalytic loop is being investigated through the study of the Val$^{121}$ Ala M.HhaI/AdoMet/DNA (abasic) co-crystal structure (Shieh et al., in progress).

Evidence for the widespread presence and evolutionarily ancient origins of enzymes which stabilize extrahelical bases, including both DNA methyltransferases and repair enzymes, was previously described(8). Considering the importance of Val$^{121}$ in M.HhaI, we hypothesized that it may form part of a motif involved in base flipping and stabilization. The structures shown in Figure 11 provide evidence for such a motif involving hydrophobic and charged amino acid residues positioned adjacent to both the extrahelical target base and a proximal phosphate. For M.HhaI, the Val$^{121}$ side chain is 4.1Å from the flipped cytosine, 3.5Å from the 5’ phosphate oxygen of the extrahelical cytosine (O2P), and 3.7Å from the central guanidinium carbon of Arg$^{165}$, while one nitrogen from the same guanidinium is 3.0Å from the other 5’ phosphate oxygen (O1P) (see Figures 10A and 11A). This motif of a positively charged side chain (Arg, Lys, or His), a proximal hydrophobic residue (Ala, Val, Leu, Ile, Pro, or Phe) positioned above the extrahelical base, and adjacent to either 5’ or 3’ phosphate appears in all three methyltransferase co-crystal structures and five of eight DNA repair co-crystal structures (see Figure 11) that we examined(19;45;49-54). Figure 11D shows the polar Gln$^{144}$ side chain in place of a positively charged residue in our motif. Although positively charged side chains appear frequently at protein-nucleic acid interfaces, the conserved placement in bacterial and human enzymes of hydrophobic and charged residues adjacent to
extrahelical bases and phosphates is compelling. Interestingly, the motif includes interactions with phosphates which are 5’ and 3’ to the target base, implying that no unique interaction to either phosphate is critical to this motif’s function. While the mechanism whereby the motif described here functions in either base flipping or stabilization awaits further experiments, it provides a structural signature which supports the previous evolutionary hypothesis(8).

Acknowledgments

We thank Dr. John Perona for structural insights and article review, Dr. Tom Bruice and Dr. Jia Luo for discussions related to MD analysis of M.HhaI, Miguel del Rios and Dr. Blake Gillespie for CD assistance, Dr. James Pavlovich for mass spectrometry assistance, and Dr. Vyas Sharma for technical and kinetic insights.
Reference List


Figure Legends

Figure 1A: M.HhaI (grey) is shown complexed to AdoHcy (yellow) and a DNA substrate (blue). The flipped cytosine (dark blue) can be seen and red regions mark locations of alanine mutants. Figure 1B is the same figure as 1A but with a 90° rotation to look down the axis of the DNA. Figure 1C shows various contacts (dotted black lines) within the active site of the M.HhaI/DNA complex. DNA is blue; Val^{121}, His^{127}, and Thr^{132} are shown in red while Asp^{128}-Asn^{131} backbones are in orange. Distances are shown from the Val^{121} side chain to the extrahelical cytosine and 5’ phosphate and from Thr^{132} to His^{127}.

Figure 2: Circular diochroism spectra were collected for WT M.HhaI (dotted line) and the Val^{121}Ala mutant (solid line). The spectra of alanine mutants 127-132 have spectra similar to WT also.

Figure 3: AdoMet affinity determination by native protein fluorescence of M.HhaI with AdoMet titrations at room temperature. WT data is shown as filled squares (■), the Val^{121}Ala mutant as open circles (O), and Asn^{129}Ala mutant as open diamonds (◊). Error bars are shown.

Figure 4: Burst product formation over 60 seconds with 1.2 μM AdoMet and 1 μM Abox DNA in MR buffer (100 mM Tris, pH 8.0, 10 mM EDTA, 10 mM DTT, 0.2 mg/mL BSA) incubated at 37° C. Enzyme addition initiated the reaction. Concentrations were 10 nM (open symbols, □, △, O) and 20 nM (filled symbols, ■, ▲, ●), with WT M.HhaI (□),
Val^{121}\text{Ala} (O), and Gly^{130}\text{Ala} (∆). Time points were taken at 10 second intervals. Both symbols for Val^{121}\text{Ala} (O, ●) are shown but overlap. Error bars are shown.

Figure 5: Steady state product formation over 60 minutes with 125 nM AdoMet and 100 nM Abox DNA for WT and 10 µM AdoMet and 10 µM Abox DNA for the Val^{121}\text{Ala} mutant in PD buffer (20 mM potassium phosphate, pH 7.5, 200 mM NaCl, 0.2 mM EDTA, 0.2 mg/mL BSA, 2 mM DTT, 10% glycerol) incubated at 37° C. Enzyme addition initiated the reaction and enzyme concentrations were 8 µM for Val^{121}\text{Ala} (□) and 100 pM for WT (O). Error bars are shown.

Figure 6: Single turnover product formation over 60 seconds with 3 µM AdoMet and 100 nM Abox DNA in MR buffer (100 mM Tris, pH 8.0, 10 mM EDTA, 10 mM DTT, 0.2 mg/mL BSA) incubated at 6° C. Enzyme addition to 0.4 µM (3.0 µM for Val^{121}\text{Ala}) initiated the reaction and time points were taken at 4, 8, 12, 16, 25, 40, and 60 seconds. WT is shown as filled squares (■), the Val^{121}\text{Ala} mutant as open circles (O), and the Asn^{129}\text{Ala} mutant as open triangles (∆). Error bars are shown.

Figure 7: Chemical mechanisms of M.HhaI are shown for the WT reaction with a cognate target base, Abox DNA, and for the 5-fluorocytosine as the target base, FAMbox DNA. Equations A and B show kinetic steps of M.HhaI which correlate to the chemical steps shown below the equations. These rates were examined in this study. These equations are an approximation since several steps are in fact reversible, which is not reflected in these equations.
Figure 8: Single turnover product formation over 120 minutes with 800 nM AdoMet and 750 nM enzyme in PD buffer (20 mM potassium phosphate, pH 7.5, 200 mM NaCl, 0.2 mM EDTA, 0.2 mg/mL BSA, 2 mM DTT, 10% glycerol) at 37° C. Reactions were initiated by FAMbox DNA addition to 900 nM and time points were taken at 2, 4, 10, 30, 70, and 120 minutes. WT (■), Val^{121}Ala (O), and His^{127}Ala (Δ) are shown. Error bars are shown.

Figure 9: DNA affinity determination by gel mobility analysis. PAGE electrophoresis with WT and the Val^{121}Ala mutant M.HhaI and two different DNA substrates, one with a cognate target base, Abox DNA, and a second with an abasic target base ABMbox DNA. Enzyme concentrations are shown in each lane. $K_D^{DNA}$ values obtained from this analysis are reported in Table 1.

Figure 10A: The active site of M.HhaI is shown with Arg^{165}, Cys^{81}, Glu^{119}, flipped cytosine, and AdoHcy. The image was generated on InsightII from 3MHT.pdb and hydrogen bonds are shown (dotted line) with distances. The Cys^{81} thiolate interaction with C6 of the cytosine and the cytosine’s C5 interaction with the co-factor are also shown. Figure 10B was constructed on Isis Draw and shows the AdoMet cofactor, many active site interactions (dotted lines), and the χ angle, or propeller twist which rotates around the glycosidic bond. Improper χ angles can disrupt many of the interactions shown in Figure 10A and 10B. Figure 10C shows superimpositions of Val^{121}, Arg^{165}, and flipped residues for various WT M.HhaI structures and one M.HaeIII structure. M.HaeIII
residues, Val^{111} and Arg^{155}, are shown in parenthesis. Red is the AdoMet co-crystal and has no DNA (1HMY.pdb), green is with a cytosine target base (3MHT.pdb), yellow is with an adenine target base (7MHT.pdb), pink is with uracil as the target base (8MHT.pdb), black has an abasic target base (9MHT.pdb), and blue is M.HaeIII with a cytosine target base (1DCT.pdb). Although the DNA component of the abasic structure (black) is difficult to see due to the superimpositions, it can be seen upon careful inspection.

Figure 11: Six active site structures of base flipping enzymes are shown. All these enzymes have a positive side chain positioned adjacent to a 5’ phosphate and the target base in addition to a hydrophobic residue and distances are shown. The conservation of positioning for these enzyme active sites argues that this is an important motif used by base flipping enzymes to facilitate catalysis, and quite possibly base flipping. A is M.HhaI from 3MHT.pdb, B is M.TaqI from 1G38.pdb, C is E.Coli 3-Methyladenine DNA Glycosylase from 1DIZ.pfb, D is Human Uracil-DNA Glycosylase from 1EMH.pdb, E is Human 3-Methyladenine DNA Glycosylase from 1BNK.pdb, and F is Human 8-Oxoguanine Glycosylase from 1EBM.pdb.
Tables
Table 1. Thermodynamic constants for WT and mutant M.HhaI. $K_D^{\text{DNA-Abox}}$ for Val$^{121}$Ala M.HhaI is $\sim 10^5$ fold down versus WT while $K_D^{\text{AdoMet}}$ and $K_D^{\text{DNA-ABMbox}}$ have near WT values. $K_D^{\text{AdoMet}}$ values reflect affinities for the AdoMet substrate while $K_D^{\text{DNA}}$ values reflect affinities for two different DNA substrates, Abox DNA (unmethylated) and ABMbox DNA (hemimethylated). Errors were calculated using standard deviation analysis.

<table>
<thead>
<tr>
<th>M.HhaI</th>
<th>$K_D^{\text{AdoMet}}$ ($\mu$M)</th>
<th>$K_D^{\text{DNA-Abox}}$ (pM)</th>
<th>$K_D^{\text{DNA-ABMbox}}$ (pM)</th>
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<tbody>
<tr>
<td>Val$^{121}$Ala</td>
<td>7.8 +/- 2.34</td>
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<td>848 +/- 253</td>
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<tr>
<td>His$^{127}$Ala</td>
<td>16 +/- 3.31</td>
<td>350 +/- 100</td>
<td></td>
</tr>
<tr>
<td>Asp$^{128}$Ala</td>
<td>14 +/- 2.89</td>
<td>400 +/- 35</td>
<td></td>
</tr>
<tr>
<td>Asn$^{129}$Ala</td>
<td>10 +/- 2.11</td>
<td>390 +/- 240</td>
<td></td>
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<tr>
<td>Gly$^{130}$Ala</td>
<td>8.9 +/- 0.59</td>
<td>500 +/- 100</td>
<td></td>
</tr>
<tr>
<td>Asn$^{131}$Ala</td>
<td>11 +/- 1.26</td>
<td>315 +/- 125</td>
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<tr>
<td>Thr$^{132}$Ala</td>
<td>19 +/- 2.65</td>
<td>240 +/- 90</td>
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<tr>
<td>WT</td>
<td>14 +/- 0.62</td>
<td>110 +/- 50</td>
<td>247 +/- 48</td>
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Table 2. Kinetic analysis of WT and mutant M.HhaI enzymes. $k_{\text{cat}}$ for Val$^{121}$Ala is $\sim 10^5$ fold down versus WT and $k_{\text{methylation}}$ and $k_{\text{inactivation}}$ values were not detectable using this assay. His$^{127}$Ala also shows a 10-fold decrease in $k_{\text{inactivation}}$ from WT values. $k_{\text{methylation}}$ is the methyltransfer rate constant measured with Abox DNA ( unmethylated), $k_{\text{inactivation}}$ is the inactivation constant measured with FA$\text{Mbox}$ DNA ( hemimethylated), and $k_{\text{cat}}$ is the catalytic turnover constant. Errors standard deviations.

<table>
<thead>
<tr>
<th>M.HhaI</th>
<th>$k_{\text{methyl}}$ (sec$^{-1}$)</th>
<th>$k_{\text{inactivation}}$ (min$^{-1}$)</th>
<th>$k_{\text{cat}}$ (sec$^{-1}$)</th>
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<td>Val$^{121}$Ala</td>
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<td>Non Detectable</td>
<td>7.46 +/- 0.7 x 10$^{-7}$</td>
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<td>His$^{127}$Ala</td>
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<td>0.002 +/- 0.0001</td>
<td>0.036 +/- 0.0141</td>
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<tr>
<td>Asp$^{128}$Ala</td>
<td>0.27 +/- 0.054</td>
<td>0.025 +/- 0.002</td>
<td>0.024 +/- 0.0032</td>
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<td>Asn$^{129}$Ala</td>
<td>0.21 +/- 0.037</td>
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<td>0.084 +/- 0.0159</td>
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<tr>
<td>Gly$^{130}$Ala</td>
<td>0.12 +/- 0.013</td>
<td>0.011 +/- 0.004</td>
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<tr>
<td>Asn$^{131}$Ala</td>
<td>0.11 +/- 0.043</td>
<td>0.020 +/- 0.002</td>
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<tr>
<td>Thr$^{132}$Ala</td>
<td>0.10 +/- 0.012</td>
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<tr>
<td>WT</td>
<td>0.12 +/- 0.040</td>
<td>0.020 +/- 0.001</td>
<td>0.046 +/- 0.0265</td>
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</tbody>
</table>
Figures

Figure 1
Figure 2

Ellipticity vs. wavelength (nm)

- Ellipticity values range from -8 to 4.
- Wavelength range is from 200 to 260 nm.
Figure 3
Figure 4

![Graph showing the relationship between time (sec) and pmol Product.](image-url)
Figure 5

[Graph showing enzymatic activity over time with different conditions labeled.]

- 100 pM WT
- 8 µM Val^{127}Ala

Product (fmole) vs. Time (min)
Figure 6
Figure 7.

Equation A

\[ \frac{1}{k_{\text{methylation}}} = \frac{1}{k_{\text{attack}}} + \frac{1}{k_{\text{methyltransfer}}} + \frac{1}{k_{\text{elimination}}} \]

Equation B

\[ \frac{1}{k_{\text{inactivation}}} = \frac{1}{k_{\text{attack}}} + \frac{1}{k_{\text{methyltransfer}}} \]
Figure 8
Figure 9

Val^{121}Ala
AB\_box

WT
AB\_box

Val^{121}Ala
Abox

Complex

Free DNA
Flipped residues: abasic, adenine, uracil, and two cytosines