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# Distal Structural Elements Coordinate a Conserved Base Flipping Network

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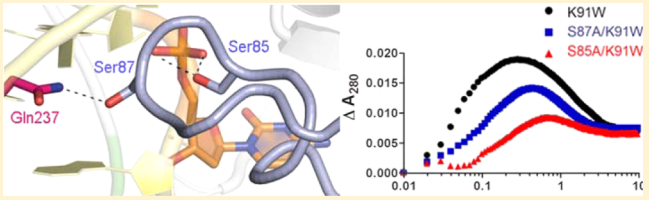
**ABSTRACT:** One of the most dramatic illustrations of enzymatic promotion of a high-energy intermediate is observed in DNA modification and repair enzymes where an individual base is rotated (flipped) 180° around the deoxyribose−phosphate backbone and into the active site. While the end states have been extensively characterized, experimental techniques have yet to yield a full description of the base flipping process and the role played by the enzyme. The C5 cytosine methyltransferase M.HhaI coordinates an ensemble of reciprocal DNA and enzyme rearrangements to efficiently flip the target cytosine from the DNA helix. We sought to understand the role of individual amino acids during base flipping. Our results demonstrate that M.HhaI initiates base flipping before closure of the catalytic loop and utilizes the conserved serine 85 in the catalytic loop to accelerate flipping and maintain distortion of the DNA backbone. Serine 87, which forms specific contacts within the DNA helix after base flipping, is not involved in the flipping process or in maintaining the catalytically competent complex. At the base of the catalytic loop, glycine 98 acts as a hinge to allow conformational dynamism of the loop and mutation to alanine inhibits stabilization of the closed loop. Our results illustrate how an enzyme utilizes numerous, distal residues in concert to transform substrate recognition into catalysis.




To access nucleoside targets buried within the double helix, DNA-modifying and repair enzymes utilize a conserved mechanism termed base flipping.[1,2] This mechanism was first discovered in the site specific cytosine C5 methyltransferase M.HhaI, which has played a pivotal role in our understanding of how DNA-modifying enzymes locate, access, and catalyze covalent modification of specific sites within DNA.[3,4] M.HhaI methylates the central (underlined) cytosine of the palindromic GCGC sequence as part of the restriction−modification system in *Haemophilus haemolyticus*. Cytosine methylation in bacteria is used for self-recognition as the basis of a primitive immune system, while in eukaryotes, it is an epigenetic signal with critical roles in transcriptional regulation.[5,6] The catalytic domain of the C5 methyltransferases is highly conserved across the three domains of life, defined by the central Rossmann fold, the cofactor *S*-adeonsylmethionine (AdoMet) binding pocket, and a flexible catalytic loop (Figure 1). The catalytic domain is flanked by the variable recognition domain on the opposite side of the catalytic loop, forming a bilobal structure with the DNA binding groove along the juncture of the domains. Molecular dynamics simulations describe a complex web of interactions throughout the enzyme that are coordinated to generate the conformational rearrangements that are necessary for catalysis,[1,2] but characterization of the role of individual residues in the dynamic network has awaited our ability to discretely monitor these rearrangements in real time. Understanding how an enzyme utilizes individual amino acids to generate the chemomechanical forces necessary to achieve the transition state is expected to have immediate value in biomolecular catalyst design and engineering.[3,4]

Binding of M.HhaI to the cognate substrate is proposed to draw the recognition domain toward the catalytic domain,[5,6] causing subtle distortion of the native DNA structure to destabilize the helical position of the target base and allowing rotation into the active site. Once destabilized, the target cytosine is flipped into the active site by one of three proposed mechanisms: (1) direct displacement of the target base through the minor groove by Gln237 of the recognition domain,[7] (2) subtle distortion of the ribophosphate backbone that induces steric strain on the base and makes the flipped form favorable,[8,9] and (3) closure of the catalytic loop pushing the base through the major groove by residues Ser85, Ile86, and Ser87.[2] Uncertainty about the mechanism of base flipping has arisen, in part, because the crystal structures show both Gln237 and Ser87 occupying the interhelical space vacated by the target cytosine. When the loop is in the open form, Ser87 and Gln237 are separated by 35 Å, with compression of the domains and closure of the loop bringing them into direct contact across the helix.[5,6,10] Closing of the catalytic loop also assembles the active site for catalysis and is the final checkpoint in ensuring sequence specificity via direct contacts to bases in the cognate sequence.[11]

We have simultaneously tracked cytosine flipping[12] and the repositioning of the catalytic loop[13,14] to probe the role of individual amino acids in coordinating enzyme motions leading to catalysis. Our results are consistent with the closure of the catalytic loop occurring only after the initiation of base flipping,

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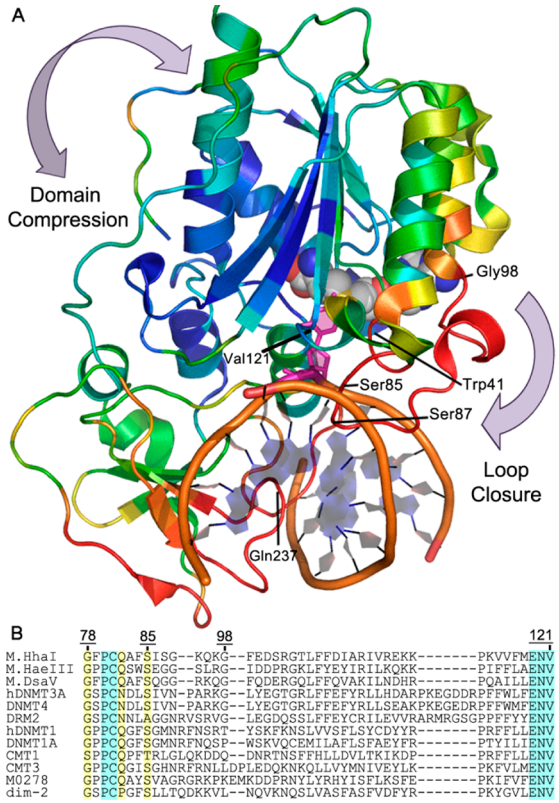


**Figure 1.** Conservation of a dynamic network. (A) 2HR1 ternary crystal structure[15] of M.HhaI with backbone atoms colored by C$\alpha$ root-mean-square deviation between the ternary and binary (2HMY[39]) forms. The spectrum from blue (0 displacement) to red (>2 displacement) shows the largely static $\beta$ sheets comprising the catalytic core, while the recognition and catalytic loops at the perimeter of the enzyme are significantly more mobile, with Ser87 of the catalytic loop moving 20 Å upon recognition of the cognate sequence. (B) Alignment of C5 methyltransferases from bacteria and eukaryotes shows a high degree of conservation of residues involved in the base flipping network in a highly divergent background. Enzymes with the M. prefix are part of restriction–modification systems, including the structurally characterized M.HhaI and M.HaeIII as well as M.DsaV from the cyanobacteria *Dunaliella salina*. hDNMT3A (DNA nucleotide methyltransferase, *Homo sapiens*), DNMT4 (*Danio rerio*), and DRM (domain-reorganized methylase, *Arabidopsis thaliana*) are de novo methyltransferases that prefer unmethylated DNA, while hDNMT1, DNMT1A (*Zea mays*), CMT3 (chromo-methyltransferase, *A. thaliana*), M0278 (*Methanosarcina mazei*), and dim (defective in methylation, *Neurosporra crassa*) are all maintenance methyltransferases that prefer hemimethylated substrates.

but loop residues accelerate flipping by distorting the phosphate backbone. One conserved serine residue, Ser85, was found to have a defined role in the generation and maintenance of the extrahelical cytosine, while the poorly conserved but structurally remarkable Ser87 makes a minimal contribution to flipping. Gly98, which acts as a hinge of the catalytic loop, is necessary for rapid attack by Cys81 and stabilization of the loop in the closed position. Our results detail the discrete contributions of these residues within a network of coordinated motions leading to catalysis by M.HhaI with unprecedented insight into the process of cytosine flipping.

## MATERIALS AND METHODS

Oligodeoxynucleotides (12 bp) were used as substrates to minimize the effects of nonspecific binding and translocation prior to specific binding. The rates of base flipping and nucleophilic attack were measured by fitting the $A_{280}$ traces upon mixing solutions of enzyme and AdoHcy with substrate in a stopped-flow apparatus using a three-step equilibrium model as described below.

Unlabeled AdoHcy and AdoMet were purchased from Sigma-Aldrich. Labeled AdoMet was purchased from Perkin-Elmer. All other reagents were purchased from Fisher Scientific unless noted. All kinetic and spectroscopic experiments were conducted in fluorescence buffer (FB) comprised of 25 mM potassium phosphate (pH 7.1), 100 mM sodium chloride, 1 mM EDTA, and 0.02% sodium azide. $\phi$ and $\psi$ angle analysis of the 2HR1 and 2HMY crystal structures was conducted using the MolProbity server. Single-turnover ($k_{methyltransfer}$) analysis was conducted using an RQF-3 rapid quench-flow instrument (KinTek) as described previously;[13] $k_{cat}$ was determined as previously described.[14]

**DNA Substrate Preparation.** Deoxyoligonucleotide substrates used in this study (Table 1) were purchased in desalted form from IDT DNA, dissolved in FB, and annealed with the complementary strand by being heated to 65 °C and then slowly cooled to room temperature. DNA concentrations were determined from a calculated molar extinction coefficient at 260 nm using the Cavaluzzi–Borer correction.[15]

**Table 1. Conformational and Catalytic Rate Constants of M.HhaI and Its Mutants[a]**

| | $k_{(flip+stack)}$[b] | $k_{attack}$ | $k_{release}$ | $k_{methyltransfer}$ | $k_{cat}$ |
|---|---|---|---|---|---|
| wild type | 18 | 0.9 | 0.4 | 0.19 | 0.023 |
| K91W | 18 | 0.9 | 0.4 | 0.17 | 0.022 |
| W41F/K91W | 3.0 | 0.8 | 0.4 | 0.15 | 0.025 |
| S85A/K91W | 3.2 | 0.9 | 0.5 | 0.06 | 0.007 |
| S87A/K91W | 12 | 0.9 | 0.4 | 0.15 | 0.023 |
| G98A/K91W | 15 | 0.2 | 0.2 | 0.06 | 0.010 |

[a]All rates are given in inverse seconds. All values had standard errors of <8% across at least five replicates. [b]Because of our inability to determine how the distribution between the stacked and flipped states varies with these mutants, we are unable to assign the individual flipping and stacking rate constants.

**Enzyme Mutagenesis, Expression, and Purification.** Appropriate deoxyoligonucleotides (IDT DNA) were used to introduce the desired mutations into a pET-28 plasmid containing the wild-type or K91W M.HhaI gene by the QuikChange protocol (Stratagene). Mutant enzymes were expressed in T7Iq *Escherichia coli* cells (NEB) in 2×YT medium via IPTG induction for 4–6 h at 25 °C once $OD_{600}$ had reached 1.0.

Purification of the protein was conducted essentially as described previously[13] except that the P-11 phosphocellulose column (Whatman) was run prior to the nickel affinity column, which was found to give a more stable enzyme. DNAase and Triton X-100 were omitted without consequence with regard to yield or purity. All proteins were found to be >95% pure via densitometry analysis of sodium dodecyl sulfate–polyacrylamide gels of the purified protein fractions. Following dialysis into storage buffer,[13] purified enzyme was concentrated via an Amicon Ultracel 10k filtration membrane. The enzyme


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**Scheme 1. Kinetic Mechanism of M.HhaI**[a]

$$E^oS_M + D^h \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E^oS_MD^d \underset{k_{stack}}{\overset{k_{flip}}{\rightleftharpoons}} E^oS_MD^f \underset{k_{open}}{\overset{k_{close}}{\rightleftharpoons}} E^cS_MD^f \underset{k_{release}}{\overset{k_{attack}}{\rightleftharpoons}} E^cS_MD^a \xrightarrow{k_{methylation}} E^cS_HD^{5mC}$$

[a]The ternary complex is assembled with the enzyme in the loop-open form ($E^o$), cofactor AdoMet ($S_M$), and DNA substrate with a helical target base ($D^h$). The development of complementary interactions between the enzyme and the cognate site serves to subtly distort the target cytosine ($D^d$), after which the enzyme initiates flipping of the target base into the active site ($D^f$). The catalytic loop closes ($E^C$) to infiltrate the helix and initiate nucleophilic attack at C6 of the target cytosine ($D^a$), followed by the transfer of a methyl from cofactor AdoMet to generate 5-methylcytosine (5mC).

concentration was determined using the previously calculated molar extinction coefficient of 25500 $M^{-1}$ $s^{-1}$ at 280 nm.[13]

**Observation of Base Flipping.** Time-resolved base flipping was monitored on an Applied Photophysics SX.18MV stopped-flow reaction analyzer operating in absorbance mode following the recently published protocol.[12] The source and detector were oriented to give a 10 mm path length for maximal absorbance at 280 nm. An enzyme and an AdoHcy solution in FB were mixed against a solution of DNA in FB to give final concentrations of 2 $\mu$M enzyme, 50 $\mu$M AdoHcy, and 1.5 $\mu$M DNA. Changes in $A_{280}$ were monitored by 1000 data collection points over 1, 10, or 100 s, and the resulting data fit as described in the kinetic modeling section for the wild-type enzyme. The C81A traces were fit to the equation $Y = Y_0 + (Y_{Max} - Y_0)(1 - e^{-kt})$ using Prism 5 (GraphPad).

**Fluorescence Tracking of Loop Position.** Measurement of the degree of loop closure at equilibrium was conducted using an LB55 fluorimeter as described previously.[13,14] In the course of this research, it was discovered that the K91W reporter mutant exhibits changes in the magnitude and rates of observed loop closure in response to cognate and noncognate DNA identical to those of the previously described W41F/K91W enzyme when the enzyme is saturated with AdoHcy. Therefore, the single mutant was used exclusively for the fluorescence experiments described in these studies. Time-resolved fluorescence experiments and analysis were conducted essentially as described previously.[13,14]

**Kinetic Modeling.** Assignment of the microscopic rate constants associated with base flipping was conducted by fitting of the $A_{280}$ traces using KinTekSim using a three-step reversible model comprising the binding−displacement, flipping, and nucleophilic attack steps. For the wild-type enzyme, fitting was possible when the rate of assembly of the ternary complex, $k_{on}$, was approximately $4 \times 10^9$ $M^{-1}$ $s^{-1}$ based upon a $k_{off}$ of 0.045 $s^{-1}$ [13,14] and a $K_D$ of 10 nM,[16] as there is no spectroscopic signature visible prior to the increase in absorbance characteristic of the base flipping step. Inclusion of the binding step was necessary to accurately fit the flipping and attack portions of traces for the W41F/K91W, S85A/K91W, and G98A/K91W enzymes because of the polyphasic nature of these traces prior to initiation of base flipping. Overall, binding is still found to be essentially diffusion-controlled with these mutants, and the observed lag in the $A_{280}$ signal indicative of a reduction in the base flipping rate with these mutants after they are bound at the cognate site. Fitting allowed assignment of the two apparent kinetic first-order rate constants for flipping and for nucleophilic attack with some assumptions. Although the value is not empirically determinable in the system, our model assumed that the maximal increase in $A_{280}$ for the wild-type enzyme is representative of 100% of the base being flipped from the helix with effectively no extrahelical base at time zero, based upon the $10^7$ equilibrium favoring the helical form of the target base in the absence of M.HhaI.[17] On the basis of the electron density and atomic distances observed in the ternary crystal structures, our model assumed ~50% of the target base is covalently bound to Cys81 at equilibrium.[8,18] In our hands, the observed forward rates with the wild-type enzyme are in good agreement with the previously published values using the wild-type enzyme (Table 1).[13,14]

## RESULTS AND DISCUSSION

**Kinetic Resolution of Base Flipping and Loop Closure.** The precatalytic mechanism of M.HhaI is shown in Scheme 1. After the cognate sequence is located, the target cytosine is expelled from the helix with concomitant loss of hypochromicity and the $A_{280}$ increases proportionally with the amount of base flipped until nucleophilic attack by Cys81 dearomatizes the ring, decreasing the absorbance.[12] The black circles in Figure 2 show a representative trace for the absorbance at 280 nm during this process. The rate of catalytic loop closure ($k_{close}$) is measured using the observed increase in quantum yield of a tryptophan residue engineered into the loop at position 91 (a Trp reporter), the use of which has been described previously.[13,14] A prior study that used the W41F/I86W Trp reporter to concurrently measure base flipping and loop closure found that this mutant closes the loop at the same rate as base flipping.[12] The simultaneity of flipping and loop closure was interpreted as evidence that cytosine flipping is driven by the closure of the catalytic loop as it approaches from the minor groove. This mechanism has also been suggested by MD studies that have attempted to map the trajectory between

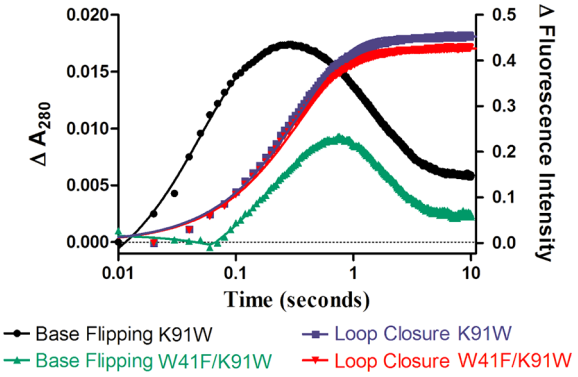


**Figure 2.** Base flipping and loop closure are not coupled in M.HhaI. The black circles are the base flipping trace with the K91W mutant, and green triangles show the W41F/K91W base flipping trace to be significantly slower (left axis). Blue squares and red triangles show the fluorescence signal from catalytic loop repositioning (right axix). Base flipping traces were fit to a three-step binding, flipping, and nucleophilic attack model using KinTekSim, while the loop closure traces were fit to a single exponential.


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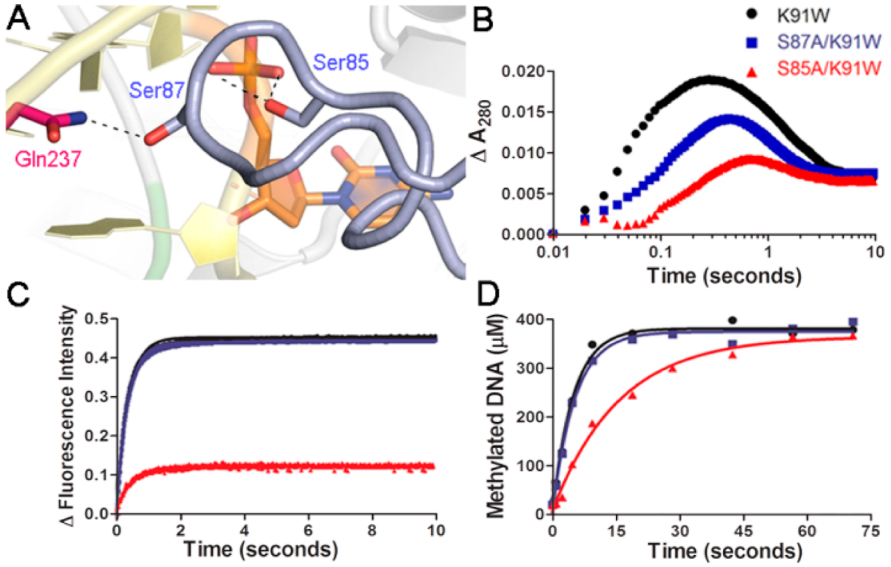


**Figure 3.** Role of Ser85 and Ser87 in base flipping. (A) In the ternary complex, residues Ser85 and Ser87 at the tip of the catalytic loop enter the DNA helix directly below the target base and in the space vacated by the flipped base, respectively. (B) Base flipping and nucleophilic attack by the mutant enzymes on GCGC DNA showing the greatly reduced rate of flipping by the S85A mutant. (C) Closure of the catalytic loop to initiate nucleophilic attack showing the S85A mutant is unable to maintain the loop-closed form when bound to the cognate substrate. (D) Single-turnover assays reveal that slowing base flipping results in a slowing of the single-turnover rate for only the S85A mutant.

the enzyme–DNA binary complex and the catalytically poised ternary complex.[19,20]

Alternatively, several lines of evidence suggest the loop does not act as a piston to drive the base from the helix, instead serving to prevent re-entry of the flipped base, or simply as a specificity filter through the development of sequence specific contacts. A deletion mutant lacking the entire catalytic loop remains capable of flipping the fluorescent surrogate 2-aminopurine, albeit with greatly reduced binding affinity and little catalytic activity.[7] When the cytosine base is removed while leaving the deoxyribose and phosphate moieties, the enzyme still distorts the phosphate backbone while the sugar ring puckers into the position seen when the base is present.[8,18] In addition, the reported rate of base flipping by wild-type M.HhaI (19 $s^{-1}$) by Gerasimaite et al.[12] is significantly faster than the rate of loop closure measured with three different Trp reporters that also contained the W41F mutation.[13,14] The side chain of Trp41 is critical for formation of $\pi-\pi$ interactions with the adenosyl moiety of the cofactor, and mutation to Phe results in an approximately 13-fold increase in $K_D^{AdoHcy}$.[21] Cofactor binding is highly correlated with base flipping and loop closure in M.HhaI and has been shown to stabilize the ternary complex approximately 5 kcal/mol over the binary complex.[21] Weakening of this interaction would be expected to have pleiotropic effects throughout the approach to the catalytically competent complex that we predicted to be observable as perturbations in one or several of the conformational transitions on path to catalysis.

A Trp reporter (K91W) was generated without the W41F mutation to evaluate the effects of the W41F mutation on base flipping and loop closure. Figure 2 shows an overlay of the spectroscopic traces of base flipping and loop closure from the W41F/K91W and K91W mutants in response to mixing with the cognate substrate. The observed $k_{flip}$ value of the W41F/K91W mutant is 6-fold slower than the wild-type (wt) and K91W values, clearly demonstrating a correlation between cofactor binding and base flipping (Table 1). The magnitude of the increase in $A_{280}$ associated with base flipping is also greatly reduced relative to that of the K91W reporter, which has flipping and attack rates essentially identical to that of the wt enzyme (Table 1). The decrease in $k_{flip}$ was sufficient to make $k_{loop}^{obs}$ (2.2 $s^{-1}$) essentially coincidental with $k_{flip}^{obs}$ (3.0 $s^{-1}$), giving the appearance of kinetic coupling between these steps. Importantly, $k_{close}$ was found to be essentially the same between the two mutants, further demonstrating that the rate-limiting transition to loop closure is base flipping, as previously suggested.[13,14,16] Unlike the W41F/K91W mutant, K91W has $k_{methyltransfer}$, $k_{cat}$, and $K_D^{AdoHcy}$ values nearly identical to those of wt M.HhaI (Table 1),[13,14,16] making it an ideal platform for evaluating the microscopic transitions in M.HhaI. The solution heteronuclear single-quantum coherence nuclear magnetic resonance (NMR) spectrum of the K91W mutant is also nearly identical to that of the wt enzyme in the enzyme–AdoHcy binary and ternary forms. The exception is the resonance corresponding to residue 91 (DOI: 10.1021/bi3012912), supporting the idea that the K91W mutant is structurally very similar to the wt.

Figure 2 clearly shows that loop closure follows the initiation of base flipping with K91W and only slightly precedes nucleophilic attack by Cys81. While we do not know precisely at which point during the loop closure that fluorescence changes occur, these results are inconsistent with any mechanism requiring that the loop undergo such changes prior to base flipping. We note that our prior demonstration that two loop reporters (K91W and E94W) provide nearly identical kinetics of loop closure[13,14,22] is most consistent with these reported changes reflecting the positioning of the loop in a state at or near its final, closed position. Thus, we suggest that the loop does not act to directly eject the base from the helix but instead closes once the base has begun to vacate the DNA helix. Our recent work using miscognate sites[23] (sites with a single-base pair substitution) suggests that upon location of the target sequence, compression of the protein domains around the DNA destabilizes the target base pair, in agreement with


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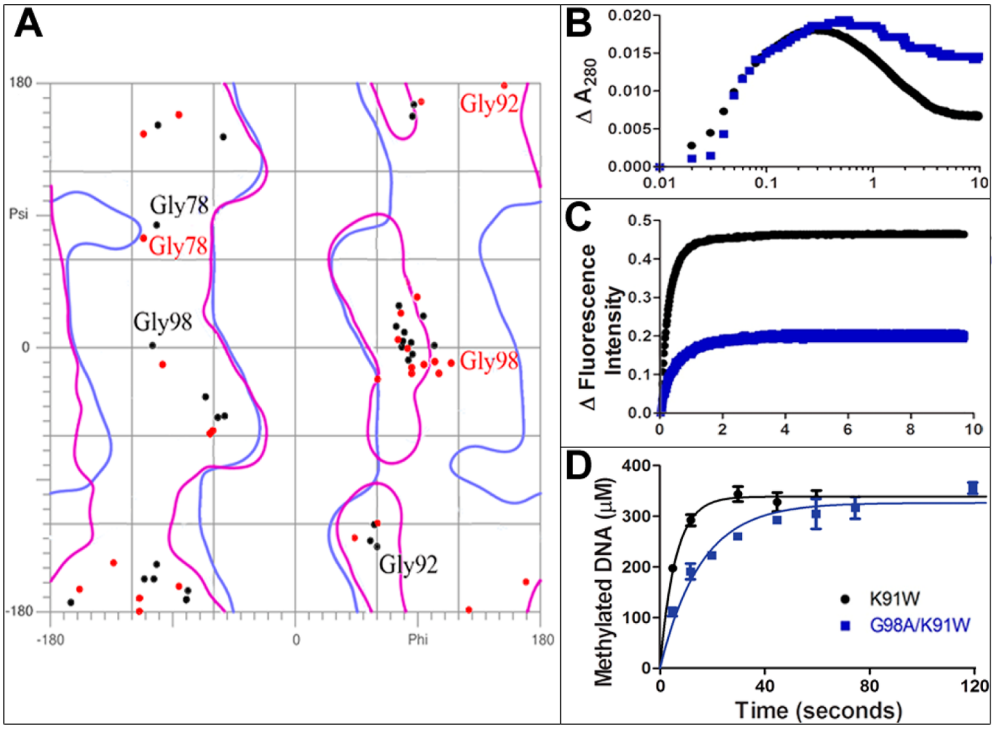


**Figure 4.** Gly98 stabilizes the closed form of the catalytic loop. (A) Analysis of $\phi$ and $\psi$ of the loop-open (red circles) and loop-closed (black circles) crystal forms of M.HhaI. Generally accessible angles are encompassed by the purple lines, and glycine accessible regions are colored blue. Gly98 adopts glycine specific angles in the open form with a 180° change in $\phi$ upon closure. (B) Observing the base flipping and nucleophilic attack processes reveals a normal flipping rate but a reduced rate of nucleophilic attack by Cys81. (C) The Gly98Ala mutant is unable to stabilize the closed form of the loop when bound to cognate DNA. (D) Single-turnover assays showing that the level of G98A/K91W is decreased in methyl transfer kinetics.

several molecular dynamics models of the flipping process.[2,19,20] The observed decrease in the flipping rate with the W41F mutant validates the computational predictions that cofactor binding facilitates compression of the domains to destabilize the target cytosine. It is likely this destabilized state is captured by closing of the catalytic loop via distortion of the backbone toward the fully flipped position using Ser85, as discussed below.

**A Network of Interactions Stabilizes the Catalytically Competent Complex.** The results with W41F/K91W demonstrate that single-amino acid mutations can manifest perturbations to the precatalytic conformational rearrangements in M.HhaI that can be dissected via our spectroscopic assays. We sought to further determine if this approach can discern the role of individual residues predicted to be involved in base flipping. All mutants subsequently described in this paper were created within the K91W background and compared to the K91W enzyme serving as the wild type.

In the ternary complex with the cognate substrate, the side chain oxygen of Ser85 is positioned 2.6 Å from the O1P atom of the phosphate 5′ to the flipped cytosine and 3 Å from an ordered water molecule located between O1P and O2P of this phosphate (Figure 3A). The O2P atom is also contacted by the highly conserved Thr250, as well as Arg165, which is essential for the proper positioning of the flipped base in the active site (Figure 3A).[6,10] The only significant distortion to the DNA structure engendered by M.HhaI involves the sugar−phosphate dihedral angles at this position. Mutation of Ser85 to alanine results in a much decreased amplitude of $A_{280}$ in the base flipping assay. Modeling of this trace shows a 6-fold reduction in the flipping rate. Nucleophilic attack is not affected, and the enzyme is able to capture the flipped base in the active site, resulting in an apparent equilibrium distribution of flipped, attacked target cytosine equal to that of the wild-type enzyme (Figure 3B). However, the catalytic loop appears to be perturbed, as evidenced by the reduction in the magnitude of the fluorescence signal relative to that of the wild type or S87A enzyme at equilibrium (Figure 3C). These results hint at a dynamic equilibrium between the open and closed states of the loop even after nucleophilic attack with this mutant that awaits further characterization. The effect of disrupting the flipping process and/or loop equilibrium is manifested as a 3-fold reduction in $k_{methyltransfer}$.

These results describe dual roles for the Ser85 residue and offer insight into its conservation across the DNA cytosine methyltransferase family (Figure 1B). The reduction in the rate of base flipping is consistent with an active role of this residue in the base flipping process (although see below). While the part of the catalytic loop involving the Trp reporter (K91W) does not begin closing until after the initiation of base flipping (Figure 2), $^{19}$F NMR and MD studies have suggested the existence of a transient, destabilized state of the target base at $\chi \sim 285°$, the last energetic barrier to exit of the target cytosine from the helix.[20,24] Both of these studies also found that binding of cofactor and/or cofactor product drives the transition from the destabilized state to the fully flipped state, in agreement with the results presented herein and results observed with the T4 DNA adenine methyltransferases.[25] A similar, early intermediate state was captured crystallographically along the flipping trajectory for uracil DNA glycosylase (UDG).[26] Our results support the proposal that compression of the two domains is sufficient to achieve this transient,


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intermediately flipped state and initiate closure of the catalytic loop. Thereafter, Ser85 assists in distortion of the DNA backbone to provide the final energetic push for base flipping. Lying along the only structurally conserved elements in the recognition domain, Thr250 and Ser252 also approach the backbone just below the target cytosine. The side chains may act in a manner analogous to that of the serine triad of UDG that acts to stabilize the extrahelical form of the target base in this enzyme.[26−29]

Ser87 has been proposed to act, along with Ile86 and Ser85, as the piston that drives the target cytosine from the DNA helix[2] and, alternatively, to play only a passive role by preventing the base from re-entering the helix.[30] In the ternary structure, the Ser87 side chain hydroxyl is within hydrogen bonding distance of the terminal amide of Gln237 from the recognition domain, the pair occupying the space left by bringing the target base into the active site (Figure 3A). However, the lack of conservation at this position among C5 methyltransferases (Figure 1B) and the absence of any homologous interaction in the M.HaeIII−DNA cocrystal and DNMT1 and DNMT3A holo structures preclude assumptions about the importance of this residue in base flipping.[31−34]

The base flipping and loop closure kinetics of the S87A/K91W mutant (S87A) are listed in Table 1. This mutation has minimal effects on the rate of base flipping, loop closure, nucleophilic attack, single turnover, and steady state turnover. The base flipping trace in Figure 3B shows a slight reduction in the flipping rate with the intermediate apex occurring at 0.42 s compared with a value of 0.19 s for the K91W mutant. Figure 3C shows that closure of the catalytic loop occurs at a nearly identical rate and an increase in magnitude, demonstrating the enzyme is bound to the substrate in the loop-closed form at equilibrium. The lack of effect seen in the single-turnover rate [$k_{methyltransfer}$ (Table 1)] indicates there is no significant effect on assembly of the active site with this mutation. Despite the unique and intriguing structural aspects of this residue observed in the M.HhaI ternary complex, the minimal role this side chain plays in catalysis explains the lack of conservation across the C5 methyltransferase family.

**Glycine 98 Regulates Loop Positioning.** Gly78 and Gly98 frame the catalytic loop of M.HhaI, functioning as the hinges about which the loop flexes (Figure 1A). Gly78 is highly conserved across C5 methyltransferases and immediately precedes the catalytic Pro-Cys motif (Figure 1B). Analysis of the $\phi$ and $\psi$ angles of these residues reveals that the backbone of Gly98 undergoes a nearly 180° change in $\phi$ between the open and closed states, while Gly78 has only a small change in conformation (Figure 4A). The angles of Gly98 in the open state are glycine specific and not favorable for amino acids with a $\beta$ carbon. Mutation of this residue was expected to disrupt the equilibrium between the open and closed forms of the loop without disrupting the catalytic pocket, offering further insight into the role of the loop in base flipping and catalysis.

Figure 4B shows that mutation of Gly98 to alanine does not disrupt formation of the ternary complex or the rate of base flipping (Figure 4B). These results imply that the catalytic loop is still largely open, or highly dynamic in the holo/binary form, because DNA is able to access the binding pocket at the same rate as the wt enzyme. However, the extent of formation of the covalent adduct with Cys81, at the opposite end of the catalytic loop, is reduced. The spectroscopic traces of the target cytosine clearly show the rapid base flipping step followed by slow, incomplete attack on the target base (Figure 4B). Figure 4C shows that the loop of G98A closes at a rate comparable to that of K91W, but with only ∼40% of the signal change of the native K91W reporter. This inability to completely stabilize the closed form is translated to a 3-fold reduction in $k_{methyltransfer}$ and a 2-fold reduction in $k_{cat}$ (Figure 4D). As expected, formation of the covalent linkage between Cys81 and the target cytosine significantly contributes to the overall stabilization of the flipped base.

While there is relatively little displacement of Gly98 between the open and closed forms, the flexibility of glycine at this position plays an important role in allowing rapid exchange between the two forms of the catalytic loop in M.HhaI and maintaining a stable ternary complex. Our results also show that it facilitates nucleophilic attack at C6 within the active site, despite being 12 Å away. Similar "hinge" roles for glycine in regulating loop positioning,[35] including involvement in the regulation of substrate specificity during DNA polymerization,[36] and transiently populated enzyme states[37] have been observed for analogous glycines in helix−loop junctions. As loop closure is the last specificity checkpoint during catalysis [13] and loop mutants have been shown to modulate sequence fidelity,[38] characterization of loop closure with the G98A mutant on miscognate substrates may yield important details about what induces closure of the loop and methylation patterning by mammalian methyltransferases, as discussed below.

The segregation of conservation at the hinge position between the bacterial and de novo methyltransferases compared to the maintenance methyltransferases (Figure 1B) is particularly interesting. The murine Dnmt3a−Dnmt3L cocrystal structure shows Gly739 at a position structurally identical to that of Gly98 in M.HhaI and Gly88 in M.HaeIII.[31,32] In contrast, recently published structures of both human and mouse DNMT1 show a serine (positions 1246 and 1249, respectively) serving as the hinge for the catalytic loop,[33,34] although all available DNMT1 structures lack a catalytically bound DNA substrate. These structures show occlusion of the active site via distal structural elements, preventing complete closure of the catalytic loop. The observation that the interactions of DNMT3A accessory factor DNMT3L and the catalytically essential CXXC subdomain of DNMT1 stabilize the catalytic loop in the closed position demonstrates control of the loop position is a likely mechanism for the regulation of DNMT activity in the development of epigenetic patterning.[39] A complete description of how the cytosine methyltransferase catalytic loop is regulated will be invaluable in elucidating how cytosine methylation is established within a mammalian genome, for which M.HhaI serves as an excellent model. Our results demonstrate that the base flipping, loop closure, and nucleophilic attack steps can be decoupled, and while highly coordinated, each conformational transition is independently concerted via a dynamic network of interactions throughout the enzyme.

## ■ CONCLUSIONS

We have demonstrated that the catalytic loop of M.HhaI is not directly responsible for physically pushing the target base from the DNA helix. The base flipping and loop closure steps are discrete, and the catalytic loop is able to close only once the hydrogen bonds keeping the base helical have been broken. The motive force behind base flipping is derived initially by destabilization of the target cytosine, followed by closure of the catalytic loop to contort the phosphate backbone into a

conformation favoring the fully flipped form of the target base. A similar mechanism involving a late conformational change was reported for another base flipping enzyme (uracil DNA glycosylase).[26−28] The role of Ser85 is to bend the DNA backbone to accelerate flipping and maintain the extrahelical state via continued distortion of the backbone. Ser87 appears to play only a minimal role in the maintenance of the extrahelical conformation, despite the side chain occupying the interhelical space of the target base. Mutation of Gly98 to alanine confirms the role of this residue as a hinge for the dynamic catalytic loop, shown by the apparent inability of the mutant to generate a fully closed loop and the ∼5-fold reduction in the rate of nucleophilic attack. Our functional dissection of these residues illuminates how the enzyme coordinates an induced fit mechanism across regions proximal and distal to the active site to achieve the transition state.

We have addressed the three mechanisms proposed for active participation of the enzyme in base flipping, in other words, whether the enzyme accelerates the base flipping process. It remains formally plausible that the substitutions investigated here act by altering the active site environment surrounding the flipped cytosine and thus the reverse (stacking) kinetics. In other words, M.HhaI is not actively involved in base flipping and functions largely by slowing the restacking of the flipped cytosine. This seems very unlikely because the flipping rates involving M.HhaI are uniformly slower than the rates for the spontaneous base pair opening reported by others, and the residues investigated here are spatially separated and functionally distinct. Finally, our demonstration that changes in DNA sequence also alter the base flipping kinetics is most easily accommodated by an active base flipping mechanism (DOI: 10.1021/bi3012912).

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### Notes

The authors declare no competing financial interest.

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