Kinetic and catalytic properties of M.HpyAXVII, a phase-variable DNA methyltransferase from Helicobacter pylori

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The bacterium Helicobacter pylori is one of the most common infectious agents found in the human stomach. H. pylori has an unusually large number of DNA methyltransferases (MTases), prompting speculation that they may be involved in the carcinization of epithelial cells. The mod-4a/4b locus, consisting of the hp1369 and hp1370 ORFs, encodes for a truncated and inactive MTase in H. pylori strain 26695. However, slipped-strand synthesis within the phase-variable polyguanine tract in hp1369 results in expression of an active HP1369–1370 fusion thesis within the phase-variable polyguanine tract in H. pylori. The MTase in hp1370, designated M.HpyAXVII. Sequence analysis of the mod-4a/4b locus across 74 H. pylori strain genomes has provided insights into the regulation of M.HpyAXVII expression. To better understand the role of M.HpyAXVII in the H. pylori biology, here we cloned and overexpressed the hp1369-70 fusion construct in Escherichia coli BL21(DE3) cells. Results from size-exclusion chromatography and multi-angle light scattering (MALS) analyses suggested that M.HpyAXVII exists as a dimer in solution. Kinetic studies, including product and substrate inhibition analyses, initial velocity dependence between substrates, and isotope partitioning, suggested that M.HpyAXVII catalyzes DNA methylation in an ordered Bi Bi mechanism in which the AdoMet binding precedes DNA binding and AdoMet’s methyl group is then transferred to an adenine within the DNA recognition sequence. Altering the highly conserved catalytic motif (DPP(Y/F)) as well as the AdoMet-binding motif (FXGXG) by site-directed mutagenesis abolished the catalytic activity of M.HpyAXVII. These results provide insights into the enzyme kinetic mechanism of M.HpyAXVII. We propose that AdoMet binding conformationally “primes” the enzyme for DNA binding.

Restriction endonucleases cleave DNA at a particular recognition sequence, and the cognate MTases methylate a base(s) in this sequence, thereby making it refractory to cleavage. R-M systems have evolved to protect bacteria from bacteriophages. The MTase of an R-M system ensures that the host DNA is methylated, thereby identified as “self,” whereas the nuclease cleaves the phage DNA, which is unmethylated (1). DNA MTases can be broadly classified into exocyclic or aminomethyltransferases and endocyclic or ring methyltransferases. Exocyclic methylation gives rise to N6-adenine as well as N4-cytosine methylation. Ring methylation gives rise to C5-cytosine methylation (2). MTases could also be involved in epigenetic regulation of gene expression by differential methylation of specific sites within a genome (3). N6-Adenine methylation has been identified as the predominant epigenetic signal in prokaryotic cells (4), whereas C5-cytosine methylation is thought to be mainly associated with R-M system–based protection of bacteria against bacteriophages. However, we have previously reported that M.HpyAVIB, a phase-variable C5-cytosine MTase, is involved in the modulation of gene expression in Helicobacter pylori, which results in a hypermutator phenotype (5). We have also reported that H. pylori uses the C5-cytosine methylation signal to regulate gene expression, which plays an important role in motility, adhesion, and virulence (6). N4-Cytosine methylation is extant in bacteria but occurs at a lower frequency compared with N6-adenine and C5-cytosine methylation. N4-Cytosine methylation is much more common in thermophilic and extreme thermophilic archaebacteria (7). It has been reported that DNA methylation by an α-class N4-cytosine MTase in the thermophilic bacteria, Caldicellulosiruptor bescii, is obligatory for DNA transformation (8). We have reported that N4-cytosine methylation by M2.HpyAll plays an important role in global transcriptional regulation in H. pylori (9).

According to the linear arrangement of the AdoMet-binding motif, the target recognition domain (TRD) and the catalytic motif exocyclic DNA MTases can be classified into α, β, γ, δ, ε, and ζ subgroups. The TRD is defined as the relatively nonconserved, large stretch of amino acids that imparts sequence specificity to the MTase. The low conservation in the TRD explains the myriad of DNA sequence specificities exhibited by different MTases (2).

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This article contains Figs. S1–S6.

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4 The abbreviations used are: MTase, methyltransferase; AdoHcy, S-adenosyl-L-homocysteine; TRD, target recognition domain; Me-DNA, hemimethylated DNA; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; MALS, multi-angle light scattering; ssDNA, single-stranded DNA; ITC, isothermal titration calorimetry; SPR, surface plasmon resonance; IPTG, isopropyl β-D-thiogalactopyranoside.
A phase-variable DNA methyltransferase

*H. pylori* is a Gram-negative, spiral-shaped, microaerophilic bacteria, which is the only formally recognized definitive bacterial carcinogen in humans (10). The naturally competent organism *H. pylori* encodes a large number of R-M systems (11). It has been speculated that the uncharacteristically large number of DNA MTases present in *H. pylori* may be involved in the cancerization of epithelial cells in the gastric environment (12). The existence of solitary MTases with no associated restriction enzyme paved the way for a number of studies that elucidated the roles of DNA methylation beyond genome protection (13).

M.HpyAXVII belongs to the β subgroup according to the domain architecture of conserved DNA MTase motifs (14). M.HpyAXVII is peculiar in a way, because the catalytic motif (DPPY), TRD, and AdoMet-binding motif (FXGXG) are positioned on the C-terminal half of M.HpyAXVII. However, the entire N-terminal half of the protein is highly conserved across homologues of M.HpyAXVII in other *H. pylori* strains. Attempts at making N-terminal truncations of the protein proved fractious because the truncated versions were extremely unstable and underwent rapid degradation during the purification process (data not shown).

M.HpyAXVII exists as two distinct ORFs in *H. pylori* 26695, viz. *hp1369* and *hp1370*. It was observed that *hp1369* contains a polyguanine tract (G10) that could exhibit phase variation. It was discovered that the basal state of the poly-G tract (G10) results in a stop codon toward the end of *hp1369* ORF, which likely results in a truncated protein. The addition of a single nucleotide to the poly-G tract brings *hp1369* and *hp1370* in frame, giving rise to a fully functional fusion HP1369-HP1370 protein, which was named M.HpyAXVII (15).

In this study, we look at the variation in poly-G length at the phase-variable locus in *hpaxvii* across different strains of *H. pylori* for which full genome data are available. Additionally, we present the results of a reporter assay with an *hp1369-LacZ* fusion construct in *Escherichia coli* DH5α cells, which show that this locus, in *H. pylori* 26695, undergoes phase-variable switching, resulting in the expression of a fully functional N6-adenine DNA MTase. To carry out biochemical characterization of M.HpyAXVII, a modified fusion construct was generated where the poly-G tract was disrupted. Our aim was to investigate the biochemical properties of M.HpyAXVII, which include its oligomeric status, DNA and AdoMet binding, and kinetics of DNA methylation.

Results and discussion

**hpaxvii is highly conserved among H. pylori strains**

Analysis of *hpaxvii* nucleotide sequence homology across the genomes of 74 fully sequenced *H. pylori* strains yielded multiple-sequence alignment that exhibits striking identity between the homologues. However, the nucleotide sequence corresponding to the TRD, which is responsible for DNA recognition, had much less homology compared with the rest of the gene. The poly-G tract in some strains (36 of 74) (Fig. 1A) renders M.HpyAXVII truncated toward the C-terminal half, switching the gene phase variably “OFF” in these strains. To demonstrate the high sequence homology between M.HpyAXVII across *H. pylori* strains, the phase-variably OFF genes were turned “ON” by adding or removing a single guanine nucleotide within the poly-G tract. The alterations to poly-G length are shown in Table 1. The amino acid sequences thus obtained from 58 strains were aligned using MUSCLE (16), and a sequence logo (17) was generated using the WebLogo server (18). M.HpyAXVII is highly conserved among all of the strains except for the TRDs (Fig. S1). It has been shown before that the TRDs of Type III MTase genes (*mods*) exhibit domain movement via homologous recombination due to the conserved non-TRD sequences flanking the TRDs. This domain movement and subsequent DNA recognition site variability are speculated to be important for epigenetics-driven adaptive evolution of *H. pylori* (19). M.HpyAXVII is most likely an important methylome-dictating DNA MTase in *H. pylori*. This observation is further strengthened by the fact that *hpaxvii* from a large majority of strains contains a polyguanine tract, which could result in phase variation at this locus.

**The poly-G tract length is variable across H. pylori strains**

The multiple-sequence alignment obtained from the above analysis provided more information about the phase-variable nature of the *mod 4a/4b* loci across *H. pylori* strains. The role of homopolymeric base repeats in phase-variable gene regulation is well-known at the transcriptional level (20) as well as the translational level (21). Here, we have analyzed the status of
poly-G repeats within *hpyaxvii* across 74 fully sequenced strains of *H. pylori*. Across the genomes of these strains, the number of guanines in the poly-G tract varied between 7 and 17 (Fig. 1B). For the purpose of this particular analysis, only poly-G tract lengths of 7 or above have been identified, because repeats of 6 or fewer are likely to be encountered at other positions within the gene too. The homopolymeric poly-G tract is located at the 1.4-kb mark within the *hpyaxvii* gene in 54 of 74 strains. In about 30% of the strains (21 of 74), both *hp1369* and *hp1370* ORFs within the *hpyaxvii* gene are in-frame and could potentially code for a functional M.HpyAXVII protein (Fig. 1A). There are no poly-G tracts in the *hpyaxvii* homologues in 12 of the above 21 strains, which implies that this locus is “ALWAYS ON” in these strains (Fig. 1A). In other words, the *hpyaxvii* gene is not phase-variably regulated in these 11 strains. In strain Hp238, there is no poly-G tract at the usual 1.4-kb mark; however, there is an 8G tract at the 2.15-kb mark, which truncates the protein. This locus is potentially phase-variable, because the addition of a guanine (8G to 9G) brings the entire gene in-frame. Hp238 is included in the “outlier” fraction in the pie chart in Fig. 1A. Details regarding the outliers are explained in Table 1. Interestingly, in about half of the strains (36 of 74) examined, the *hpyaxvii* gene would result in expression of a truncated, and therefore inactive, M.HpyAXVII. However, the addition or removal of a single guanine within the poly-G tract will result in the expression of a full-length M.HpyAXVII (Fig. 1A). In the case of *H. pylori* 26695, this alteration is 10G to 11G. The exact alterations in the poly-G repeat length to bring about the expression of full-length M.HpyAXVII in each strain are delineated in Table 1. In nine strains, the poly-G tract is basally found to contain the ideal number of repeats for the expression of full-length M.HpyAXVII (Fig. 1A). One could speculate that in these nine strains, M.HpyAXVII is phase-variably ON, whereas in the 36 strains mentioned above, the MTase is phase-variably OFF. In 15 strains, the *hpyaxvii* homologues contain frameshifts, at a position other than immediately downstream of the 1.4-kb mark, which render the MTase truncated, regardless of the status of the poly-G tract at the 1.4-kb mark. *H. pylori* J99 has a shorter variant of *hpyaxvii*, with no poly-G tract, which probably codes for an N-terminal truncated version of M.HpyAXVII. Taken together, the data from the *hpyaxvii* gene in 74 *H. pylori* strains suggest that the poly-G tract at the 1.4-kb mark adds an extra layer of regulation of M.HpyAXVII expression via phase variation.

### Table 1

| Strain               | Repeats | Frame | Alteration in repeats for hp1369-hp1370 fusion |
|----------------------|---------|-------|-----------------------------------------------|
| *H. pylori* pyl 26695| 10G     | -     | 10G (However, protein truncated at 2726th amino acid position) |
| *H. pylori* pyl 238  | 8G      | -     | 8G (Different locus, @ 2158th base pair) |

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**A phase-variable DNA methyltransferase**

The mod 4a/4b locus in *H. pylori* 26695 consists of the ORFs *hp1369* and *hp1370*. However, individually, these ORFs do not code for a single polypeptide that contains all of the conserved amino acid motifs necessary for DNA MTase activity. The *hp1369* locus has 10 guanines (10G) in a poly-G tract at the 3'-end. Interestingly, the 10 guanines result in the positioning of a premature stop codon toward the end of *hp1369*, which results in *hp1370* being out of frame and thereby expressing a truncated protein. However, this locus was identified as potentially phase-variable, and slipped-strand mispairing could bring the entire *hp1369-hp1370* loci (hereafter designated *hpyaxvii*) into frame to code for a full-length protein, M.HpyAXVII (22).

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*hpavaxxii*, in its native form (10G), was cloned into an expression vector (pGEX4T2). The number of guanines in the poly-G tract was confirmed as 10 by Sanger sequencing using a primer 100 bp upstream of this locus. The pGEX4T2-*hpavaxxii* plasmid construct was transformed in *E. coli* expression strain BL21(DE3) to test whether phase-variable expression of full-length protein was taking place. Cloning in pGEX4T2 vector results in a GST tag at the N-terminal end of the protein. Five single colonies were picked and checked for the induction of the truncated protein (82 kDa). Interestingly, one out of the five samples showed the presence of the full-length protein (120 kDa). This suggests that a change in the number of Gs in the poly-G tract could be responsible for the phase variation resulting in full-length protein expression. The identities of the expressed proteins were confirmed by Western blotting with antibodies against GST tag (Fig. 2A). Equal number of cells were taken from each sample (uninduced and induced) by normalizing for cell density via $A_{600}$ measurements. Prior to probing with antibody, Ponceau S staining of the blot was performed to ensure that equal amounts of lysates were loaded on each well (Fig. S6). Phase-variable, reversible, switching ON and OFF of genes has been implicated to play an important role in host-adapted pathogenic bacteria. Furthermore, phase-variable regulation of Type III DNA MTases has been demonstrated as a critical mechanism in pathogenic bacteria, which enables them to switch between distinct cell types with variable methylation patterns and hence variable gene expression profiles (23).

**Changes in poly-G repeat frequency could be responsible for phase variation in *hpavaxxii* gene**

To assess the role of poly-G tract in phase variation in *H. pylori* 26695, an *hp1369-LacZ* fusion construct was made in pETBlue-1 vector. If the number of guanines in the tract remains 10, *hp1369-LacZ* will be in-frame and will result in expression of functional LacZ enzyme, which in turn imparts blue color to the *E. coli* colonies grown in the presence of X-Gal. On the other hand, deviation in the number of repeats from 10 (to 9 or 11) can result in an out-of-frame composition for the *hp1369-LacZ* construct, which will yield a white colony. A similar strategy was employed to visualize in vivo phase variation at MOD525 locus in *H. pylori* recombinant clones (24). When the construct was transformed in DH5α strain of *E. coli* and plated on medium containing X-Gal, blue-white sectored colonies were observed along with blue colonies (Fig. 2B). The sectored colonies represent two subpopulations with the random switching ON/OFF of the gene due to phase variation. As the plasmid multiplies in the bacterial cell because of slipped-strand mispairing, a change in the poly-G repeat length may take place. This change will place the LacZ in the OFF condition, thus creating two subpopulations with ON and OFF LacZ, giving rise to sectored colonies (Fig. 2C). A 1.7-kb DNA fragment lacking G repeats was cloned in pETBlue-1 and used as the negative control (Fig. 2B).

**Preliminary characterization of DNA M.HpyAXVII**

*N*-Adenine DNA MTases catalyze the transfer of a methyl group from AdoMet to an adenine moiety within a specific recognition sequence. The conserved amino acid motif that is involved in catalysis (DPPY) is encoded within *hp1369*, whereas the TRD and the AdoMet-binding motif (FXGXX) are encoded within *hp1370*. Therefore, only in the event of phase-variable switching ON to a full-length protein will there be a functional M.HpyAXVII MTase, which can carry out adenine methylation. To express and purify only the full-length version of M.HpyAXVII, site-directed mutagenesis was performed to disrupt the polyguanine tract. The *hpavaxxii* fusion construct obtained was cloned and expressed using pET28a expression system in *E. coli* BL21(DE3) cells (Fig. S2, A–C). The protein was purified to high homogeneity, albeit with some degradation, which showed up as faint, lower-molecular weight bands in SDS-PAGE. The degraded fragments were detected when probed with an antibody specific for the full-length protein (Fig. S2, D and E). Furthermore, the degradation products were isolated from the SDS-polyacrylamide gel and analyzed via MALDI-TOF peptide mass fingerprinting (data not shown). These results confirm that the lower-molecular weight bands are indeed degraded M.HpyAXVII. The purified full-length enzyme showed optimal methylation activity at pH 7.5 and 37 °C in the presence of 50 mM NaCl. In this study, the kinetic properties of the enzyme have been investigated in detail.
Determination of molecular mass and oligomeric status

Molecular size and oligomeric status were determined by performing size-exclusion chromatography. A Superdex 200 column was calibrated with Bio-Rad gel filtration standard, which consists of globular proteins of known molecular masses ranging from 1.35 to 670 kDa. Different concentrations of M.HpyAXVII were injected, and the elution volumes were recorded. M.HpyAXVII eluted as a predominant symmetric peak at an elution volume corresponding to 219.3 kDa, as extrapolated from the standard curve (Fig. 3A and B). However, a minor peak was also observed at a lower elution volume. To further clarify this observation, the gel filtration experiment was repeated, coupled with multi-angle light scattering (MALS) detection, under identical conditions. MALS calibration was performed by loading BSA onto the Superdex 200 column. The calibration yielded two distinct peaks, which matched the well-documented monomeric and dimeric species of BSA in solution (data not shown). M.HpyAXVII eluted as a predominant symmetric peak at an elution volume corresponding to 199.8 ± 6.6 kDa, according to the best fit obtained through BSA light-scattering calibration (Fig. 3C). MALS data allow for accurate measurement of the root mean square radius ($R_g$) of a protein in solution. By comparing the $R_g$ of the protein in question with the $R_g$ of BSA, the molecular weight of the protein of interest can be arrived at (25). The peak fraction was reinjected into the column, which yielded a peak at exactly the same elution volume (data not shown). Based on the primary sequence, the molecular mass of M.HpyAXVII is ~95 kDa. SDS-PAGE of M.HpyAXVII showed an approximate molecular mass of 95 kDa. Taken together, the above data suggest that M.HpyAXVII predominantly exists as a dimer in solution. Most DNA MTases reported in the literature are monomeric in solution (26, 27). However, dimeric MTases have also been reported, such as CcrM methylase (28), DpnII MTase (29), and KpnI MTase (30). MTases belonging to Type III R-M systems, such as EcoP15I MTase (31) and EcoPI MTase (2), are known to be dimeric in solution.

Determination of methylation site

For the determination of site recognized and methylated by M.HpyAXVII, different duplexes (duplexes 5–14 in Table 2) were used as DNA substrates for an in vitro methylation assay. M.HpyAXVII methylated duplexes 1, 2, 3, 8, and 10, but not duplexes 4, 5, 6, 7, and 9 (data not shown). Comparison between the sequences of duplexes 1–10 identified 5′-TCAGC-3′ as a possible recognition sequence. The recognition site contains one adenine residue on each strand (5′-TCAGC-3′/5′-GCTGA-3′). To find out the target adenine, duplex 11 (Table 2) was methylated with M.HpyAXVII, in the presence of [3H]AdoMet. An MnlI site was designed into the duplex in such a way that digestion with MnlI restriction enzyme separates the two adenosines into different fragments. Methylated duplex 11 was purified and then digested by MnlI. The digestion products were separated on 20% PAGE and then analyzed for incorporation of radiolabel. It was observed that the [3H]radiolabeled methyl group was incorporated onto the adenine on the top strand (Fig. S3A). The recognition site was thus determined as 5′-TCAGC-3′. It was previously reported that SMRT sequencing of the frame-corrected M.HpyAXVII methylated the adenine within the recognition site 5′-TCAG-3′ (15). To test whether M.HpyAXVII has any preference for the 5th nucleotide in a recognition sequence 5′-TCAGN-3′, where N can be A, G, T, or C, methylation assays were performed with each DNA duplex substrate. All four duplexes were methylated by M.HpyAXVII; however, there seems to be a slight but signifi-
**Table 2**

| Duplex | 5′–3′ sequence (double-stranded) |
|--------|----------------------------------|
| 1      | CTTCGTCGTCTCCATGCAGCTAGCCAGAG   |
| 2      | GAATCGCTGAAAGGCGGCTGCTGGTTC     |
| 3      | CTTCGTCGTCGTTCCGTGCTGCTGCTGGTTC|
| 4      | GAATCGCTGAAAGGCGGCTGCTGCTGCTGGTTC|
| 5      | GAATCGCTGAAAGGCGGCTGCTGCTGCTGGTTC|
| 6      | GAATCGCTGAAAGGCGGCTGCTGCTGCTGGTTC|
| 7      | GAATCGCTGAAAGGCGGCTGCTGCTGCTGGTTC|
| 8      | GAATCGCTGAAAGGCGGCTGCTGCTGCTGGTTC|
| 9      | GAATCGCTGAAAGGCGGCTGCTGCTGCTGGTTC|
| 10     | GAATCGCTGAAAGGCGGCTGCTGCTGCTGGTTC|
| 11     | GAATCGCTGAAAGGCGGCTGCTGCTGCTGGTTC|

**A phase-variable DNA methyltransferase**

The specificity constant was determined as \( k_{cat}/K_m \). M.HpyAXVII methylated circular supercoiled as well as linear pUC19 with approximately similar affinities and efficiencies. However, there seems to be a significant preference for the 26-bp duplex DNA containing a single recognition site (Fig. 5). It should be noted that the pUC19 substrates contain several recognition sites, and the concentrations denoted in the results are all normalized for number of sites. No methylation was observed with single-stranded oligonucleotide substrate (Fig. S5). The specificity constant for 26-mer duplex DNA was about 11-fold higher than for supercoiled DNA and about 8-fold higher than for linear plasmid DNA. Characterized DNA MTases generally tend to have low turnover (rate of methylation) coupled with high substrate affinity (low \( K_m \)); thus, \( k_{cat}/K_m \) remains high (2), and they tend to exhibit sufficiently high specificity for methylation of their target sequence. The kinetic parameters calculated using the different DNA substrates are given in Table 3.

**Determination of kinetic parameters (AdoMet)**

For the determination of \( K_m \) and \( V_{max} \) for the determination of \( K_m \), a series of reactions containing M.HpyAXVII (200 nM), DNA (2 μM), and increasing concentrations of [3H]AdoMet (0.2–15 μM) were performed. Increasing AdoMet concentration led to a progressive stimulation of methylation reaction. Whereas the initial portion of the curve shows a hyperbolic curve (Fig. 6, inset), saturation was never achieved (Fig. 6). Similar concentration dependence has been observed previously in the case of T4 Dam (32) and HpyAVIAM, an orphan adenine methyltransferase from H. pylori (33). The velocity dependence to AdoMet could be explained if M.HpyAXVII were to contain two binding sites for AdoMet, a high-affinity as well as a low-affinity site. However, the stoichiometry results obtained from isothermal titration calorimetry (ITC) experiments with AdoMet showed that M.HpyAXVII contains only one binding site per monomer (Fig. 10A). Lineweaver–Burk plot of the initial portion of the curve gave a \( K_m \) of 4.2 μM, \( k_{cat} \) of \( 1.5 \times 10^{-3} \) s\(^{-1} \), and a specificity constant of 357 M\(^{-1}\) s\(^{-1} \).

**Initial velocity studies with DNA and AdoMet**

In a bisubstrate reaction, the initial velocity dependence between the substrates can be examined to decipher whether the reaction follows an ordered or a Ping Pong mechanism. The product formation was monitored at various concentrations of DNA, keeping the AdoMet constant and vice versa. The experiments were carried out using 26-bp duplex DNA and tritiated AdoMet. The linear regressions of the double-reciprocal plots of 1/\( v \) versus 1/[DNA] (Fig. 7A) and 1/\( v \) versus 1/[AdoMet] (Fig. 7B) intersected beyond the y axis, within quadrant II. This pattern supports a ternary complex formation during catalysis, which could be a random or an ordered Bi Bi mechanism. A Ping Pong mechanism can be ruled out because the lines are not parallel to each other. The slope and y intercept replots of all of the double-reciprocal plots had a linear relationship (data not shown), which rules out a steady-state random kinetic mechanism.
Product inhibition kinetics

To investigate the specific order in which the DNA and AdoMet bind the enzyme during an ordered Bi Bi ternary complex formation, product inhibition studies were performed. Because M.HpyAXVII methylates only a single strand in a duplex DNA, hemimethylated DNA (Me-DNA) substrate was used as one of the products, the other being S-adenosyl-L-homocysteine (AdoHcy). Oligonucleotide 1 listed in Table 4 was modified to produce Me-DNA substrate. The adenine within the recognition sequence 5'-TCAGC-3' was premethylated and procured from Sigma-Aldrich. This modified oligonucleotide was annealed with oligonucleotide 2 to obtain the Me-DNA substrate. Product inhibition experiments were carried out by varying one substrate while keeping the other constant (and in excess) in the presence of one of the products. The experiment was repeated in a range of product concentrations to delineate the inhibitory effect of each individual product on each individual substrate. The double reciprocal plots yield four distinct patterns (Fig. 8), which can fall under different inhibitory profiles (viz. competitive inhibition, noncompetitive inhibition, or uncompetitive inhibition). AdoHcy showed competitive inhibition with respect to AdoMet (Fig. 8A), whereas the pattern with DNA matched uncompetitive inhibition (Fig. 8B). This indicates that AdoMet as well as AdoHcy compete for the same active site and therefore can bind to the free enzyme. Product inhibition by Me-DNA was noncompetitive with respect to AdoMet (Fig. 8C) and noncompetitive/mixed with respect to DNA (Fig. 8D). An ordered mechanism with DNA binding first would have resulted in a competitive inhibition pattern between Me-DNA and DNA at constant AdoMet, which was not the case (Fig. 8D). Because a competitive inhibition pattern is observed only between AdoHcy and AdoMet (and not between Me-DNA and DNA), a random order of binding can be ruled out. Thus, the results of product inhibition studies indicate a likely mechanism where AdoMet binds to the enzyme first, followed by DNA binding and subsequent catalysis. A summary of inhibition patterns obtained with respect to each product and substrate combination is shown in Table 5.

The kinetic mechanism among DNA MTases are varied. An ordered Bi Bi mechanism with AdoMet binding first to the enzyme is seen in EcoRI MTase, T4 Dam, EcoRV MTase, and KpnI MTase. EcaI MTase and EcoP15I exhibit a random order of substrate binding. Hhal MTase, Mspl MTase, and mammalian DNMT3a exhibit an ordered Bi Bi kinetic mechanism where DNA binding to the enzyme precedes AdoMet binding and subsequent catalysis (2).
Substrate inhibition studies

The order of binding indicated by product inhibition kinetics was further confirmed by substrate inhibition analyses. In an ordered Bi Bi mechanism, a relatively high amount of the substrate that binds to the enzyme second can lead to the formation of nonproductive binary complexes, thereby causing inhibition of the forward reaction. Substrate inhibition analyses have been successfully used to reconfirm kinetic mechanisms exhibited by mammalian DNMT3a MTase (34), T4 Dam (32), EcoP15I MTase (35), and KpnI MTase (30). Substrate inhibition was studied by adding high amounts of one substrate to the reaction while the concentrations of the other substrate and enzyme were kept constant. An AdoMet gradient from 0.2 to 15 μM, in the presence of 200 nM M.HpyAXVII and 2 μM single-site duplex, did not elicit any substrate inhibition. In fact, as observed earlier, the reaction velocity values did not reach an asymptote even at 15 μM AdoMet (Fig. 6). On the other hand, titration of DNA substrates across a broad range of concentrations (up to 10 μM single-site duplex) elicited very clear substrate-dependent inhibition of catalysis (Fig. 9, A (single-site duplex DNA) and B (pUC19)). To clarify that this observation is not due to AdoMet being limited, a similar DNA gradient was tested at various AdoMet concentrations, yielding identical inhibitory patterns with respect to DNA concentration (data not shown). At high concentrations of the second substrate, which in this case is DNA, the enzyme is likely forming a non-productive binary complex. No such inhibition will be observed even at very high concentrations of the first substrate, which is AdoMet (Fig. 6). In the presence of high concentrations of ssDNA (oligonucleotide 1, Table 4) the methylation of duplex DNA seems to be getting inhibited, but no methylation is detected when ssDNA alone is used as substrate (Fig. S5). This could mean that M.HpyAXVII binds to ssDNA, but methylation will take place only on duplex DNA. The substrate inhibition patterns point toward a mechanism where M.HpyAXVII binds to AdoMet first and forms a catalytically competent complex, which is then acted upon by the second substrate, DNA. These results are consistent with that of the product inhibition analyses.

Isotope partitioning analysis with [3H]AdoMet

Isotope partitioning analysis was performed to assess the catalytic competency of the M.HpyAXVII–AdoMet complex. Product inhibition and substrate inhibition analyses indicate that the enzyme follows an ordered Bi Bi reaction mechanism.
where the AdoMet binding precedes DNA binding and cationysis. The enzyme is presaturated with radiolabeled AdoMet in standard buffer at 37 °C for 3 min. The reaction is then initiated by diluting the presaturated complex into buffer containing DNA as well as AdoMet. When the initiation was performed in radiolabeled AdoMet, the product formation happened as a burst followed by a constant rate of methylation. In an identical setup, when the initiation of reaction was performed in DNA and unlabeled AdoMet, a similar product formation was observed, albeit with a lower burst amplitude (Fig. 10A). The y intercept of the linear region yields the burst magnitude. A positive burst magnitude was observed in the case of M.HpyAXVII, which indicates that the enzyme–AdoMet complex is catalytically competent (Fig. 10B). This confirms the proposed kinetic mechanism where M.HpyAXVII binds AdoMet first, followed by DNA during methyl transfer. Isotope partitioning analyses have been used previously to demonstrate the AdoMet-first order of binding in EcoRI MTase (36), Rsrl MTase (37), and Kpnl MTase (30). Isotope partitioning study with labeled DNA has also been performed to delineate the order of substrate binding by MspI MTase (38).

**Table 4**

| Serial no. | Description | 5′–3′ sequence |
|------------|-------------|----------------|
| 1          | Substrate duplex component (forward) | CTCAGCGGGATCC |
| 2          | Substrate duplex component (reverse) | CCTGAGTGTGAGATCTCCGCTCGAAG |
| 3          | hpyaxvii forward primer | GGATCCATTAAGAAACGCGAAACG |
| 4          | hpyaxvii reverse primer | GCTTCATTAAGAAACGCGAAACG |
| 5          | F658C SDM forward primer | CTCAGGGGATCC |
| 6          | F658C SDM reverse primer | CCTGAGTGTGAGATCTCCGCTCGAAG |
| 7          | Y407V SDM forward primer | GCGTACGGTTAACAGCTTAAAGAAACGCGAAACG |
| 8          | Y407V SDM reverse primer | GCTTCATTAAGAAACGCGAAACG |
| 9          | Methyalted duplex component (forward) | CTCAGCGGGATCC |
| 10         | hpl1369 forward primer | ATGAAAACGCGAAACG |
| 11         | hpl1369 reverse primer | CATTGTAAAGAAACGCGAAACG |
| 12         | hpyaxvii fusion (MI) | GCTTCATTAAGAAACGCGAAACG |
| 13         | hpyaxvii fusion (MII) | GCTTCATTAAGAAACGCGAAACG |
| 14         | Biotinylated oligonucleotide | Bt-CTCGAGCTCATCCTCAGCAGATCAAG |

**Figure 8. Product inhibition analysis.** Methylation reactions were performed at the indicated concentrations of reaction products. Lineweaver–Burk plots are shown of rates of methylation versus AdoMet concentrations at various constant concentrations of AdoHcy (A), rates of methylation versus DNA concentrations at various constant concentrations of AdoHcy (B), rates of methylation versus AdoMet concentrations at various constant concentrations of Me-DNA (C), and rates of methylation versus DNA concentrations at various constant concentrations of Me-DNA (D). Error bars, S.D.

**Table 5**

| Product inhibition of M.HpyAXVII | Variable substrate | Fixed substrate | Type of inhibition |
|----------------------------------|-------------------|-----------------|-------------------|
| AdoHcy                           | AdoMet            | DNA             | Competitive       |
| AdoHcy                           | DNA               | AdoMet          | Uncompetitive     |
| Me-DNA                           | AdoMet            | DNA             | Noncompetitive    |
| Me-DNA                           | DNA               | AdoMet          | Noncompetitive/mixed |

Purification and characterization of AdoMet-binding motif (F658C) and catalytic motif (Y407V) mutants of M.HpyAXVII

The catalytic activity of DNA methyltransferases is contingent on a highly conserved AdoMet-binding (FXXG) as well as a catalytic (DPPY) site. Mutations within these motifs reduce or abrogate catalytic activity (2, 4). It has been demonstrated that substitution of the tyrosine within the DPPY motif to alanine or glycine (39) or leucine (33) results in reduced methylation activity. Similarly, replacement of the phenylalanine in the AdoMet-binding motif (FXXG) to alanine was shown to abrogate AdoMet binding and therefore the catalytic activity in EcoRV MTase (40). Substitution of the FXGXG phenylalanine to serine resulted in inactivation of M1.HpyAVI, an orphan N6-adenine DNA methyltransferase from H. pylori (33). Site-directed mutagenesis at the conserved motifs (F658C and Y407V) was performed as explained under “Experimental procedures.” The overexpression and purification protocol was identical to the one employed for the WT protein. The far-UV CD spectra of both the mutant proteins did not reveal any significant dif-
ferences from that of the WT protein (Fig. S4, inset), confirming that the mutations do not lead to any gross structural alterations. The catalytic activity of the mutants was assayed along with the WT M.HpyAXVII. DNA methyl transfer was measured as a function of increasing enzyme concentration (Fig. S4). Both of the mutants were catalytically inactive.

Isothermal titration calorimetry analysis of AdoMet binding

Site-directed mutagenesis of the highly conserved phenylalanine in the FXGXG abrogated methylation activity in M.HpyAXVII F658C protein (Fig. S4). The inability of the mutant to bind AdoMet would explain the inactivation. To assess to what extent the AdoMet-binding ability is compromised as a result of this point mutation, ITC was employed to calculate $K_d$ values of M.HpyAXVII WT as well as mutant proteins. ITC is a very accurate method for calculating binding affinities between proteins and small ligands, such as AdoMet and AdoHcy (41). It measures the heat changes associated with the interactions between protein and ligands in a concentration-dependent manner (42). In conjunction with computational tools, the heat absorbed or released during a particular interaction can be correlated with the binding kinetics. ITC experiments were performed as described under “Experimental procedures.” The data were fitted to a “one-set-of-site” binding model to obtain the binding constant ($K$) and stoichiometry ($n$). $K_d$ was calculated as $1/K$. M.HpyAXVII WT bound AdoMet with a $K_d$ value of 3.85 μM and a stoichiometry of ∼2 (Fig. 11A). Because M.HpyAXVII exists as a dimer in solution and methylates DNA as a dimer, this translates to a single AdoMet binding site per monomer. M.HpyAXVII F658C showed very weak binding with AdoMet (Fig. 11B). It should be noted that the ITC titrations with F658C mutant were repeated several times with various excess concentrations of AdoMet in the syringe (up to 1.5 mM), whereas optimum binding was observed with the WT as well as Y407V mutant protein at an AdoMet concentration of 500 μM (protein in the sample cell being 20 μM in all cases). A “one-set-of-site” binding model fit on the data obtained with F658C resulted in a calculated $K_d$ value of 353 μM, which depicts very weak binding. However, the reliability of the mathematical fit is questionable because the $c$-value ($K \times [\text{receptor}]$ in molarity × $N$ (stoichiometry)) is too low ($c = 0.166$). Thus, in practical terms, the inference is that the F658C substitution has compromised the enzyme’s ability to bind AdoMet. The catalytic mutant (M.HpyAXVII Y407V) was able to bind AdoMet with a $K_d$ value similar to that of WT protein ($K_d = 2.17$ μM; $N \sim 2$) (Fig. 11C). This shows that the loss of enzymatic activity due to the Y407V mutation is due to the inability of the enzyme...
to catalyze methyl transfer and is independent of AdoMet binding. All $K_d$ values reported in this study are an average of results obtained from three independent experiments (Table 6).

**Surface plasmon resonance (SPR) analysis to quantify DNA binding**

DNA binding by M.HpyAXVII was studied using SPR. SPR analysis of protein–ligand binding is based on the fundamental assumption that the binding kinetics in solution is comparable with or at least can be correlated to binding between either partner of the complex on an immobile phase and the other in the mobile phase. In this particular experiment, biotinylated 26-bp dsDNA molecules, containing a single 5'-TCAG-3' site each, were immobilized onto streptavidin chips via biotin–streptavidin interaction on the surface of the flow cell. Different concentrations of M.HpyAXVII in suitable buffer were passed over this surface. Binding of protein to the immobilized ligand causes changes in the refractive index at the interface. Binding at each concentration of enzyme is measured for 100 s followed by a 300-s buffer wash. The association and dissociation curves are fit using a one-set binding model, as elaborated under Experimental procedures.

Table 6

| Receptor (protein) | Ligand (AdoMet) | $K_d$ $\mu$M | Stoichiometry |
|--------------------|----------------|-------------|--------------|
| M.HpyAXVII WT      | $\text{ND}^a$ | $2.23$      | $1.89$       |
| M.HpyAXVII F658C   | $\text{ND}^b$ | $2.23$      | $1.89$       |
| M.HpyAXVII Y407V   | $3.85$         | $1.96$      | $1.96$       |

$^a$ Not detected.  
$^b$ Not applicable.

The calorimetric titration of AdoMet with M.HpyAXVII WT (A), AdoMet with M.HpyAXVII F658C (B), and AdoMet with M.HpyAXVII Y407V (C) is depicted. The top panels in A–C represent the raw heat change measured during 15–20 injections of 500 $\mu$M AdoMet (AdoMet = 1.5 mM in B) into 280 $\mu$l of 20 $\mu$M protein. Bottom panels, integrated heat values derived from raw data after factoring in dilution enthalpy of protein. Curves were fitted using a “one-set binding model,” as elaborated under Experimental procedures.

**SPR analysis to determine order of binding**

It has been shown previously that the presence of AdoMet or its analogues can enhance the specificity with which MTases bind to their recognition sequences (43). SPR-based DNA binding analysis was repeated in the absence and presence of sinefungin, a noncatalyzable structural analogue of AdoMet. The product inhibition and substrate inhibition data suggest that M.HpyAXVII binds to AdoMet first to form a catalytically competent complex, which then binds to DNA, followed by methyl transfer. Thus, AdoMet-bound M.HpyAXVII (or, in this case, sinefungin-bound) should have higher specificity for single-site duplex DNA. M.HpyAXVII preincubated with 40 $\mu$M sinefungin for 5 min prior to injection showed a $K_d$ value of 0.29 $\mu$M (Fig. 12B), whereas the enzyme preincubated with only the buffer bound DNA with a $K_d$ value of 1.6 $\mu$M (Fig. 12A). The experiment was repeated with an AdoMet-binding point mutant of the MTase (M.HpyAXVII-F658C). The $K_d$ values, in this case, in the absence and presence of sinefungin were 2.2 $\mu$M (Fig. 12C) and 2 $\mu$M (Fig. 12D), respectively. The results indicate that the binding of sinefungin (and, by extension, AdoMet) makes the M.HpyAXVII more conducive to subsequent binding to DNA, a characteristic that is not observed in the AdoMet-binding mutant (F658C).

**Summary**

_H. pylori_ 26695 _hpasyxvii_ locus remains phase-variably OFF. In this study, the poly-G tract was modified in such a way that M.HpyAXVII was expressed as a full-length DNA MTase, and specific binding and enzyme bulk effect were subtracted by initiating flow over a surface that lacks DNA. The average $K_d$ value calculated from the binding isotherms (Fig. 12A) was 1.6 $\mu$M. Typical $K_d$ values for DNA MTases lie within a range of $10^{-8}$ to $10^{-6}$ M (2).
the phase-variable nature of the poly-G tract was nullified using synonymous codons for glycine (i.e. GGG GGG GGG GGT to GGA GGC GGT GGA). Compared with other well characterized Type III DNA MTases, M.HpyAXVII is a much larger protein. Incidentally, the conserved sequence motifs identified by Malone et al. (viz. motifs IV (DPPY), V, VI, TRD, X (FXGXD), I, and II) are located on the C-terminal half of the protein. Nevertheless, the N-terminal half of the protein is highly conserved across all *H. pylori* strains analyzed in this study. This prompted us to investigate the biochemical as well as kinetic properties of M.HpyAXVII.

On account of the following lines of evidence, we arrive at a proposed kinetic mechanism for M.HpyAXVII. 1) Lineweaver–Burk plots of the initial velocity dependence between DNA and AdoMet yielded a set of lines that converged on quadrant II, which points toward a ternary complex formation which follows neither a random Bi Bi nor a Ping Pong mechanism. 2) Product inhibition analysis with AdoHcy shows a competitive inhibition pattern with AdoMet, which indicates that AdoMet and AdoHcy competes for the free enzyme form and AdoMet is most likely the first substrate to bind. 3) Substrate inhibition data indicate that M.HpyAXVII forms a nonproductive binary complex at very high concentrations of DNA, whereas no such inhibition is observed at very high concentrations of AdoMet. 4) Isotope partitioning analysis demonstrates that M.HpyAXVII–AdoMet binary complex is catalytically competent. 5) SPR-based DNA-binding analysis shows that M.HpyAXVII WT binds DNA with 5-fold higher affinity in the presence of an AdoMet analogue (Sinefungin). A similar increase in affinity is not observed with the AdoMet-binding mutant (F658C) of M.HpyAXVII (Table 7). We propose that the binding of AdoMet makes the enzyme conformationally “primed” for the subsequent binding of DNA.

The high variability at the TRD of M.HpyAXVII could be a tool with which the epigenetic N6-adenine methylation signature of an individual bacterium can be switched via a homologous recombination event. It is well-known that *H. pylori* has a surprisingly low number of global transcriptional regulators compared with other bacteria, pathogenic or nonpathogenic (44). DNA MTases like M.HpyAXVII may act as transcriptional regulators that can regulate the gene expression of multiple genes by methylating a particular DNA recognition site. Given this context, it is pertinent to investigate whether M.HpyAXVII contributes to the phenotype of *H. pylori*.

Recombination events at the TRD would lead to variation within a subpopulation, with respect to methylome status, thereby providing adaptive fitness in a highly dynamic environment like the human stomach. The TRD switching explained by Furuta et al. (19) might allow *H. pylori* to employ M.HpyAXVII as a substitute transcriptional regulator that can alter the methylome status and regulate the expression of multiple genes. The poly-G tract, on the other hand, might serve as a reversible ON/OFF switch, which can fine-tune this regulatory aspect by creating a subpopulation of cells where M.HpyAXVII is either ON or OFF.

### Experimental procedures

#### Strains and plasmids

*H. pylori* 26695 strain (cagA+ iceA1 vacAs1am1) genomic DNA was obtained as a gift from New England Biolabs. *E. coli* strain DH5α (F′ end A1 hsdR17 [r′K− mK−] glnV44 thi1 recA1 gyrA59)
relA1 Δ(lacIZYA – argF) U169 deoR (Φ80dlac Δ (LacZ)M15)) was used as a host for preparation of plasmid DNA. pET-28a expression vector was purchased from Novagen. E. coli strain BL21(DE3) (F’ompT gal dcmlonhsdS3884 (rpsL::Tn10) DE3 [lacI lacI57 T7 gene 1 indI sam7 ninS]) was used as a host for over-expression and purification of protein.

Reagents

Restriction endonucleases, AdoMet, and Q5 high-fidelity DNA polymerase were purchased from New England Biolabs. T4 DNA ligase, DpnI restriction endonuclease, DNA ladders, and protein molecular weight markers were obtained from Fermentas Life Sciences. Coomassie Brilliant Blue R-250, proteinase K, Tris, heparin-Sepharose, protease inhibitor mixture, AdoHcy, and Isopropyl β-D-1-thiogalactopyranoside (IPTG) were procured from Sigma. Ni²⁺-nitrilotriacetic acid–agarose and other reagents used were of analytical or ultrapure grade.

DNA substrates

All oligonucleotides used in this study were synthesized by Sigma. The concentrations of oligonucleotides were determined by UV absorbance at 260 nm. The extinction coefficient of oligonucleotides was calculated using the sum of the extinction coefficients of the individual bases. Duplex dsDNA was formed by annealing oligonucleotide 1 with excess of oligonucleotide 2 (Table 4) in the presence of 1× saline sodium citrate buffer. pUC19 DNA used in assays was prepared by the alkaline lysis method from DH5α cells and quantified by determining the UV absorbance at 260 nm. Hemimethylated DNA, oligonucleotide 9 (Table 4), for product inhibition studies was designed by replacing the adenine at the 16th position with methyladene (5'-TC_A^mG-3') and was synthesized by Genosys-Sigma and purified through HPLC. The annealing protocol was identical to the one followed to make regular DNA substrate.

PCR amplification and cloning of hpyaxvii truncation (hp1369) and hpyaxvii fusion construct (hp1369-hp1370)

The 1422-bp-long hp1369 gene was amplified using primers 10 and 11, whereas the hp1369-hp1370 fusion construct was amplified using primers 3 and 4 (Table 4). The primers were designed with the help of the annotated complete genome sequence of Helicobacter pylori 26695, considering the putative gene sequences of hp1369 and hp1370 obtained from TIGR. The amplified product corresponding to hp1369-hp1370 fusion was cloned at the EcoRV site in pETBlue-1 (Novagen) via blunt end cloning followed by directional subcloning into pET28a and pGEX4T2 expression vectors between the BamHI and XhoI sites.

Site-directed mutagenesis

hp1369-hp1370 fusion construct was synthesized by implementing the megaprimer strategy (45). Primers 12 and 14 were used for construction of MI fusion, where the polyguanine tract is disrupted in such a way that the amino acid composition at this region is not perturbed. The full-length PCR product was obtained in the second round PCR by extension of the megaprimer using pET28a-hp1369-hp1370 as template. The PCR product was then purified, digested with DpnI restriction enzyme to cleave the methylated template DNA, transformed into E. coli DH5α strain, and plated on lysogeny broth agar medium containing kanamycin (50 µg/ml). The mutants were confirmed by DNA sequencing. M.HpyAXVII point mutations at the AdoMet-binding motif (phenylalanine to cysteine, F658C) and catalytic motif (aspartic acid to valine, Y407V) were created by implementing a one-step overlapping mismatch primer method (46). The changes in nucleotide sequence were designed in such a way in each case that a unique restriction endonuclease site was created in the event of a successful integration of the mutation. This allowed quick and easy screening of mutation-incorporated clones.

Overexpression and purification of His₆-tagged M.HpyAXVII WT and mutants

An overnight-grown culture of E. coli BL21(DE3), containing pET-28a with WT hp1369-70, was inoculated into 1 liter of lysogeny broth containing 50 µg/ml kanamycin. At a cell density corresponding to 0.8 A₆₀₀, the cells were induced with a 0.2 mM final concentration of IPTG and further grown at 18 °C overnight. The entire protein purification procedure was performed in buffer A (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 5 mM β-mercaptoethanol, and 5% glycerol). The cells were pelleted and resuspended in sonication buffer (buffer A along with 100 µg of lysozyme, 0.05% Triton X-100, 100 µg of phenylmethylsulfonyl fluoride, and 5 mM imidazole) and lysed by pulsed sonication. The lysed cells were centrifuged, and the supernatant was passed through an Ni²⁺-nitrilotriacetic acid affinity column for binding (47). The column was washed with 100 column volumes of wash buffer (buffer A containing 30 mM imidazole), and elution was performed in an imidazole gradient mixture (30 – 450 mM imidazole) prepared in the same buffer. Samples of elution fractions were run on a 10% SDS-PAGE (48) to check the presence of purified protein. The elution fraction containing the protein was pooled and passed through a heparin-Sepharose column for further purification. The bound protein was eluted using a salt gradient. Elution fractions containing protein of the expected size were pooled and dialyzed against buffer containing 50% glycerol (buffer A except for NaCl concentration, which was maintained at 100 mM). An additional round of purification through gel filtration chromatography with Sephadex 100 column was also performed. The point mutants F658C and Y407V were also purified using the exact same protocol as mentioned above. Protein concentration was estimated using Bradford’s method using known amounts of BSA to build a standard curve (49). Additionally, the protein concentrations were recomibrated by measuring UV absorbance at 280 nm and equating the A₂₈₀ value to the theoretical extinction coefficient of M.HpyAXVII.

Molecular mass determination

The molecular mass of purified M.HpyAXVII was determined using gel filtration chromatography (30). 500 µg of purified M.HpyAXVII was injected onto the column to obtain the
elution profile. The fractions at which the protein eluted were pooled and re-injected to confirm the observations. MALS was used in conjunction with gel filtration chromatography for a more precise estimation of oligomeric status. Gel filtration analysis was repeated, albeit with refractive index and MALS detection in conjunction with conventional A280 detection. MALS calibration was performed using BSA standards.

**In vitro methylation assay**

Methylation assays were done to monitor the incorporation of tritiated methyl groups onto DNA by using a modified ion-exchange filter-binding assay (26). Methylation assays were carried out at 37 °C in a reaction mixture containing DNA, [3H]AdoMet, and purified protein in standardized buffer (50 mM Tris–HCl, pH 7.4, 5 mM β-mercaptoethanol). The reaction mixture was then transferred onto Whatman DE81 filter paper and washed, and tritium content was determined, in scintillation fluid, using a liquid scintillation counter. Background counts were measured and subsequently subtracted from the reaction data.

**Determination of kinetic parameters**

Kinetic studies were performed using pUC19 plasmid DNA as well as single-site 26-mer duplex DNA (oligonucleotide 1 and 2 annealed). Methylation assays were carried out, as described earlier, for 30 min to determine initial velocity dependence. In a series of similar reactions containing M.HpyAXVII (200 nM) and [3H]AdoMet (2 μM), the concentration of DNA was varied in the range of 0.1–10 μM. Initial velocities versus DNA concentration were plotted and fitted with the Michaelis–Menten equation using GraphPad Prism software. Nonlinear regression with Michaelis–Menten correlation provided \( K_{m}^{DNA} \) and \( V_{max} \), values for each type of DNA substrate. All subsequent kinetic experiments were performed using the single-site 26-mer duplex DNA as substrate. Similarly, initial velocity experiments were carried out by varying the concentration of [3H]AdoMet in the range of 0.5–10 μM while keeping the DNA concentration fixed at 2 μM and keeping other reaction conditions identical. The turnover number (\( k_{cat} \)) was calculated as the ratio of \( V_{max} \) to the total enzyme concentration used per reaction. Unless otherwise indicated, all enzyme activity data were the average of at least duplicate determinations.

**Product inhibition studies**

Product inhibition studies were done under identical conditions as described for initial velocity dependence experiments. Inhibition by AdoHcy was studied using 1 μM single-site 26-mer duplex DNA (fixed concentration) while keeping the AdoHcy concentrations fixed (0, 5, 15, and 50 μM) and varying the concentration of [3H]AdoMet from 0.5 to 3 μM for each of the fixed concentrations of AdoHcy. Similarly, another series of identical reactions included 2 μM [3H]AdoMet (fixed concentration); AdoHcy concentrations fixed at 0, 5, 25, and 50 μM and DNA concentration varied in the range of 0.25–2 μM for each of the fixed concentrations of AdoHcy. Double reciprocal plots of initial velocity versus [3H]AdoMet or DNA concentrations were obtained at each concentration of the AdoHcy. \( K_{i}^{AdoHcy} \) was determined from these double-reciprocal plots of initial velocity versus AdoMet concentration followed by secondary plots with their slopes (data not shown). Product inhibition studies were performed with Me-DNA in a similar fashion.

**Isotope-partitioning studies**

Isotope-partitioning analysis helps to identify the competent complex in a bisubstrate reaction (41). [3H]AdoMet is preincubated with the enzyme in standard buffer in ice. The reaction is initiated by the addition of DNA and AdoMet (either radiolabeled or unlabeled). The product formation is monitored as a time course. The partitioning of radiolabeled versus unlabeled substrate indicates the competency of the enzyme–substrate complex.

**Surface plasmon resonance analysis**

A biotinylated 26-bp dsDNA was prepared by annealing oligonucleotide 15 and oligonucleotide 2 (Table 4). Oligonucleotide 15 is an exact replica of oligonucleotide 1, which was used to make the duplex substrate used for all kinetic analyses, except for a biotin tag at the 5'-end. The annealed DNA duplex substrate was immobilized on the surface of a streptavidin-coated sensor chip to a final concentration of ~1200 response units/flow cell. The binding reactions were carried out at 25 °C in a continuous flow of standard buffer containing 10 mM HEPES buffer (pH 7.4), 150 mM NaCl, 3 mM EDTA, and 0.05% surfactant P-20 at a flow rate of 20 μl/min. Increasing concentrations of M.HpyAXVII were injected onto the surface of the biosensor chip for 100 s at a flow rate of 20 μl/min, followed by a dissociation run of 300 s. The surface was regenerated using running buffer containing MgCl2. Each experiment was repeated twice to ensure reproducibility of results. Standard Langmuir binding equation resulted in a good fit for the association as well as dissociation phase. The dissociation constant \( K_d \) was calculated according to the equation, \( k_d = k_{off}/k_{on} \).

**Isothermal titration calorimetry analysis of AdoMet binding**

ITC was used to determine the affinity of M.HpyAXVII to AdoMet. The WT protein, AdoMet-binding mutant (F658C), and catalytic mutant (Y607V) were assayed with AdoMet as the ligand. M.HpyAXVII WT and the mutants purified as mentioned before were dialyzed against buffer containing 25 mM Tris–HCl, pH 7.4, and 100 mM NaCl. AdoMet from New England Biolabs was used for ligand titrations. Fresh dilutions of AdoMet were prepared in the same buffer as the enzyme just before the start of the experiment. The sample cell was filled with 280 μl of 20 μM protein, and the syringe was filled with 500 μM AdoMet. When AdoMet binding of M.HpyAXVII F658C mutant was assayed, up to 1.5 mM AdoMet was used in the syringe for injection. 20 injections of 2 μl of AdoMet were added to the protein at intervals of 4 min with stirring at 400 rpm at a temperature of 25 °C. The heat of dilution was determined each time by titrating the AdoMet into the buffer. AdoMet titrations were performed on 50 μM lysozyme in the sample cell to rule out any nonspecific heat changes. Microcal Origin version 7 was used to analyze the data. The data were fitted to a “one-set-of-site” binding model to obtain the binding constant (K) and stoichiometry (n). The c values (K × protein concentration × n) of all of the ITC data, where binding was
significant, were in the acceptable range of 5–1000. The dissociation constant ($K_d$) was calculated as $1/K$. All of the experiments were performed in triplicates.

**Data representation and statistical analysis**

All enzyme kinetic experiments were performed in duplicates. The cpm values obtained from $[^3]H$]methyl groups catalytically transferred onto DNA were normalized by calculating the specific activity of AdoMet used per study. The product formation data were represented as $xy$ scatter plots with corresponding S.D. values. All enzyme kinetic data representation and linear regressions were performed with GraphPad Prism version 6.01. Lineweaver–Burk plot linear regressions for all enzyme kinetic data had a $p$ value < 0.005. ITC data were analyzed, and curves were fitted using Origin software.

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