Structure and mutagenesis of the DNA modification-dependent restriction endonuclease AspBHI

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The modification-dependent restriction endonuclease AspBHI recognizes 5-methylcytosine (5mC) in the double-strand DNA sequence context of (C/T)(C/G)(5mC)N(C/G) (N = any nucleotide) and cleaves the two strands a fixed distance (N12/N16)3 to the modified cytosine. We determined the crystal structure of the homo-tetrameric AspBHI. Each subunit of the protein comprises two domains: an N-terminal DNA-recognition domain and a C-terminal DNA cleavage domain. The N-terminal domain is structurally similar to the eukaryotic SET and RING-associated (SRA) domain, which is known to bind to a hemi-methylated CpG dinucleotide. The C-terminal domain is structurally similar to classic Type II restriction enzymes and contains the endonuclease catalytic-site motif of DX20EAK. To understand how specific amino acids affect AspBHI recognition preference, we generated a homology model of the AspBHI-DNA complex, and probed the importance of individual amino acids by mutagenesis. Ser41 and Arg42 are predicted to be located in the DNA minor groove 5 to the modified cytosine. Substitution of Ser41 with alanine (S41A) and cysteine (S41C) resulted in mutants with altered cleavage activity. All 19 Arg42 variants resulted in loss of endonuclease activity.

Mammalian DNA cytosine methylation is an important epigenetic modification1. It remains unclear how cytosine methylation within particular sequences is initiated, maintained and particularly, recognized. Epigenetic DNA modification is dynamic, and differences are found in the epigenomes of cells during normal development2, aging and mental health, and during pathologic processes such as cancer, among many others3. To learn more about the role of epigenetic modification in development and disease, and to understand the mechanisms that control its locations and levels in the human genome, the genomic locations of modified cytosines must be mapped with accuracy, to single-base resolution. Newly identified ‘modification-dependent’ restriction endonucleases are proving useful for this purpose4,5 and for understanding how specific recognition of modified cytosine occurs.

AspBHI from Azoarcus sp. BH72 belongs to a family of modification-dependent restriction endonucleases that recognize 5-methylcytosine (5mC) in the context of specific DNA sequences and cleave N12/N16 3' downstream of the modified cytosine4,6. These proteins vary in length from 388 amino acids (AspBHI) to 456 (MspJI), and include a conserved core region of ~390 amino acids (Fig. 1a). FspEI has an additional amino-terminal 50 amino acids not present in other family members, whereas MspJI has insertions in multiple locations7. Besides MspJI, the other family members share sequence conservation throughout the entire region, with invariant (~26%) or conservatively substituted positions (~30%) scattered throughout the conserved core (Fig. 1b). Only one insertion of six residues was found in the conserved core of LpnPI (residues 316–321).

Previously we reported the tetrameric structure of MspJI which recognizes (5mC)NN(G/A)7. Here we report the structure of AspBHI which recognizes (C/T)(C/G)(5mC)N(C/G)8 and we confirm that it also forms a tetramer. To understand how specific amino acids of AspBHI determine its substrate recognition preference, we generated a homology model of the AspBHI-DNA complex, and probed the importance of a number of individual amino acids by mutagenesis.
Results

Tetrameric form of AspBHI. We determined the structure of AspBHI at the resolution of 2.8 Å (Table 1). Like MspJI, AspBHI is assembled into a tetramer, formed by molecules A, B, C, and D (Fig. 2a–b). Molecules A and B form a closed dimer with high quality electron densities observed for all 388 residues. Interestingly, molecules C and D have an intact N-terminal (DNA-recognition) domain up to Pro216, but the entire C-terminal (DNA-cleavage) domain could not be traced due to discontinuous residual densities. We inferred the general location of the C-terminal domains of molecules C and D by comparison with those of MspJI (Fig. 2c), and found them to be in a void along the crystallographic 6-fold axis with a diameter of 100 Å (Fig. 2d). Absence of crystal packing forces may allow the C-terminal domains of molecules C and D to be mobile and thus unobservable. Analytical gel-filtration measurement confirmed that AspBHI exists as a tetramer in solution (Fig. 2e). An “invisible” domain in a protein crystal structure is not a common occurrence, but several examples have been observed. In these structures, as in ours, a large space is found where a domain connected to another by a linker can move as a rigid body owing to the absence of any intra-molecular or inter-molecular crystal-packing interactions.

Monomeric AspBHI structure. Focusing on molecules A and B, the monomeric AspBHI contains two domains, connected by a 10-residue linker (residues 212 to 221) including residue Pro216 packing forces may allow the C-terminal domains of molecules C and D to be mobile and thus unobservable. Analytical gel-filtration measurement confirmed that AspBHI exists as a tetramer in solution (Fig. 2e). An “invisible” domain in a protein crystal structure is not a common occurrence, but several examples have been observed. In these structures, as in ours, a large space is found where a domain connected to another by a linker can move as a rigid body owing to the absence of any intra-molecular or inter-molecular crystal-packing interactions.
the N-terminal DNA binding domain, mostly in the loops, and a 15-residue insertion revealed that MspJI has seven insertions of five to eight residues in the coordinates of the mouse SRA–DNA complex (Fig. 1a). Superimposing the AspBHI and MspJI structures length (388 residues), while MspJI is the largest (456 residues) (Fig. 2f). Among the family members, AspBHI is the smallest in (a), Superimposing the protein components, the bound DNA was positioned over the mostly basic surface of AspBHI except for an apparent acidic pocket. An equivalent pocket is present in the SRA–DNA complex with DNA, where Asp474 of mouse UHRF1 interacts with the flipped 5mC nucleotide, for subsequent endonuclease catalysis, or for both.

A model of the N-terminal SRA-like DNA-binding domain in complex with DNA. Like MsplI, the N-terminal domain of AspBHI is structurally similar to the eukaryotic SET and RING-associated (SRA) domain of UHRF1 (Fig. 3a–b), which binds to hemi-methylated 5mCpG dinucleotide sequences. The C-terminal domain of AspBHI is structurally similar to several prokaryotic Type II endonucleases (Fig. 3c–d). We created a model of the AspBHI N-terminal SRA-like domain bound to DNA, using the coordinates of the mouse SRA–DNA complex. After superimposing the protein components, the bound DNA was positioned over the mostly basic surface of AspBHI except for an apparent acidic pocket. An equivalent pocket is present in the SRA–DNA complex where Asp474 of mouse UHRF1 interacts with the flipped 5mC nucleotide, for subsequent endonuclease catalysis, or for both.

In order to hydrogen bond with the ring atom N3 and the exocyclic amino group N4 (NH₂) of the flipped 5mC (Fig. 3b), the methyl group of 5mC interacts with the Cx and Cβ atoms of Ser486 in UHRF1 (Fig. 3b). This is likely because the binding pocket glutamate of motif V (‘ENV’) of the 5mC-methyltransferases, which...
Figure 2 | Structure of AspBHI. (a) Four AspBHI monomers, A, B, C and D, form a tetramer. Molecules C and D have mobile C-terminal domains (indicated by a circle). (b) AspBHI tetramer, rotated ~90° from the view of panel (a). (c) For comparison, MspJI has an intact tetramer showing in a similar orientation of panel (a). (d) The disordered C-terminal domains of molecules C and D of AspBHI tetramer were located in the void space along the crystallographic 6-fold axis with a diameter of 100 Å. (e) Elution profile of AspBHI on Superdex 200™10/300 GL (GE Healthcare). The column buffer was 20 mM Tris-HCl (pH 7.5), 300 mM NaCl and 1 mM DTT, and 150 ng of AspBHI was loaded onto the column. The inset shows the standardization of the size exclusion column using a Gel Filtration Markers Kit for Protein Molecular Weights (SIGMA-ALDRICH, Cat. No. MWGF1000) at the time AspBHI was profiled using the same buffer. (f) Monomeric AspBHI contains two domains connected by a linker. (g) AspBHI has a discontinuity in strand β8 owing to the insertion of a 310 helix (right panel), whereas MspJI has a corresponding 20-residue-long curved strand β8 (left panel). Pairwise sequence alignment is shown above the panels. (h) The 310 helix of molecule A is involved in the dimer interface with the C-terminal helix αL of molecule B. The amino end of the 310 helix (Ala149 of molecule A) interacts with the carboxyl end of helix βL (Ser368 of molecule B). Arrows indicate helical dipoles.
likewise hydrogen bonds with the flipped substrate cytosine preparatory to methyl transfer.

Our model of the AspBHI N-terminal domain bound to DNA, derived from the UHRF1 SRA-DNA complex, suggests that three loops (Loops 2B, B3 and 6C) might intrude into the DNA minor or major grooves (Fig. 3a and 3e) and provide the interactions needed for AspBHI to recognize its DNA substrate sequence. Loop-2B (residues 23–31 between strand b2 and helix aB) could make base-specific contacts in the minor groove on the 3′ side of the flipped 5mC, where N(C/G) is recognized, and Loop-B3 (residues 39–43 between helix aB and strand β3) could make base-specific contacts in the minor groove on the 5′ side where (T/C)(C/G) is recognized. Loop-2B is unique to AspBHI in sequence among the family members (Fig. 1b) as well as in length compared with UHRF1. The corresponding loop in UHRF1 is a one-residue sharp turn. Alanine mutations of potential contact residues within Loop-2B were constructed and tested. K24A and R27A cleaved phage DNA similarly to WT AspBHI (Fig. 4, lanes 2 and 4), but plasmid digestion was somewhat reduced, especially for K24A. T25A and D32A [Asp32 is an invariant residue within the family, Fig. 1b] abolished cleavage activity altogether (Fig. 4, lanes 3 and 5).

Loop-B3 contains Ser41 and Arg42 that are unique to AspBHI (Fig. 1b). The corresponding loop in UHRF1 also approaches the DNA from the minor groove and contains Val451, which occupies the space left behind by the flipped 5mC, and His450, which interacts with the 5′ base pair. To examine the effects of Loop-B3 mutations, we changed Ser41 and Arg42 to all 19 other amino acids (the results are discussed below). The third loop, Loop-6C is between strand b6 and helix aC (residues 84–99). The corresponding loop in UHRF1 contains Arg496, which hydrogen bonds from the major groove with the intra-helical orphaned guanine (Fig. 3a). Loop-6C is six-residue shorter than its UHRF1 counterpart, and it adopts a different conformation due perhaps to the absence of DNA (Fig. 3a), making it too short to reach the DNA major groove in the current model.
Nevertheless, Loop-6C is a prime candidate for making base specific interaction in the major groove if the substrate DNA and/or protein undergo structural rearrangement during binding.

S41A and S41C variants have altered cleavage activities. Substitutions of Ser41 by other amino acids drastically reduced enzyme activity (data not shown) except for the alanine (S41A) and cysteine (S41C) replacements. These two variants showed somewhat different cleavage properties towards modified plasmid or phage DNA compared to the WT enzyme (Fig. 4, lanes 6–7): S41A cleaved phage XP12 DNA similarly to WT enzyme (Fig. 4b, lane 6), but barely cleaved pUC19 DNA, except for converting supercoiled DNA to nicked intermediate (only one strand cut) and linear form (one double-strand cut) (Fig. 4c, lane 6). S41C demonstrated the opposite effect: it cleaved phage XP12 DNA much less efficiently than pUC19. The phage DNA appears to be trapped by the S41C protein precipitation (Fig. 4b, lane 7, the band near the top loading well), although it is not clear whether the bound DNA had been cleaved.

To investigate the specificity of the S41A and S41C variants, we used three 56-bp synthetic duplexes containing the symmetric sequence 5'-NC(5mC)GGN-3' (Fig. 5a), methylated on both strands. If the enzyme recognizes the top strand methylated site, cleavage on the 3' side N12/N16 away will result in two products of 43-bp and 9-bp, both with a 4-bp overhang. We termed these products as P1 and P5 with averaged lengths of 45-bp and 11-bp (Fig. 5b). [The product P5 was not observed probably because it was too small to be stained or the small duplex (9 bp + 4 nt overhang) dissociated at 37°C after cleavage and the two short single-stranded oligonucleotides ran out of the gel.] If the enzyme recognizes the bottom strand methylated site, cleavage will result in two products of 39-bp (P2) and 17-bp (P4). And if the enzyme recognizes both top and bottom strand methylated sites, cleavage on both sides will result in three products of averaged lengths of 28-bp (P3), 17-bp (P4), and 11-bp (P5). The cleavage products were resolved using 20% native PAGE (Fig. 5b). The results indicate that AspBHI is capable of cleaving the substrates having a 5' pyrimidine base (T or C) (lanes 1 and 7) but not a guanine (or adenine): lane 4 of Fig. 5b only shows top strand (with a 5' C) recognition products, P1 and P5 (not visible), but not the bottom strand (with a G) recognition products P2 and P4.

S41A variant showed lower activity in cleaving all three substrates as a significant amount of full-length duplex oligonucleotides remained (Fig. 5b, lanes 2, 5 and 8). However, it appeared to prefer the S9 substrate, with the two 5' most positions being a C on both strands, compared with substrate S7 that has 5' T or 5' C on each strand (comparing lanes 2 and 8). This is in contrast to the WT enzyme that cleaved substrate S7 better (comparing lanes 1 and 7), suggesting a potential change of substrate specificity. On the other hand, an approximately equal amount of P1 and P2 products were generated by S41A on S7 substrate (lane 2), suggesting S7 might be a poor substrate for S41A, regardless of a 5' T or 5' C. The S41C variant had a digestion pattern similar to that of the WT enzyme. However, in addition to the predominant cleavage position at N12/N16 from the modified cytosine, S41C appears to have additional cleavage positions (as marked with asterisk in lanes 6 and 9) – an observation previously observed as wobble cleavage.

Arg42 is essential for activity. A total of 19 variants R42X (natural amino acids other than arginine) were constructed by site-directed mutagenesis. All 19 variants were purified through nickel-chelated and heparin affinity chromatography. All were inactive in cleaving modified plasmid DNA, including the conservative Arg42-to-lysine substitution (data not shown). Arg42 might interact with the target 5mCG base pair (the only unambiguous base pair within the recognition sequence) during the initial protein-DNA encounter or stabilize the flipped 5mC via interaction with the orphaned guanine.
for enhanced recognition and tightening of the protein-DNA complex and thereby promoting cleavage. The precise way in which Arg42 and Ser41 mediate specific DNA recognition awaits the solution of a protein-DNA complex structure.

**Discussion**

The wide diversity of restriction enzymes\(^1\), from the smallest dimeric PvuII\(^2\), to tetrameric Type IIF enzymes\(^3\), and the polymerized SgrAI\(^4\), make them versatile tools for laboratory experimentation, and fascinating subjects for studies of molecular architecture\(^5\). Here we show structurally that the modification-dependent restriction enzyme AspBHI comprises two domains, one typically eukaryotic and the other typically prokaryotic. The N-terminal part of AspBHI (residues 1–211) resembles an SRA-like 5-methylcytosine binding domain in structure and function. It recognizes 5mC within the specific DNA sequence context. The C-terminal part of AspBHI (residues 222–388) resembles a classic Type II restriction endonuclease of the PD-(D/E)XK superfamily\(^6\). It is attached to the N-terminal domain by a 10-residue loop, and cleaves duplex DNA outside of the recognition sequence on one side, \(N_{12}/N_{16}\) downstream of the 5mC, somewhat like a Type II restriction enzyme.

Figure 5 | S41A and S41C activity assays on methylated oligonucleotide substrates. (a) Schematic diagram of the fully methylated oligonucleotide substrates (M = 5mC) used for analyzing possible cleavage products (P1–P5 shown in panel b). (b) Duplex oligonucleotides (20 ng) were incubated at 37 °C for 2 hours with 0.5 μg (0.29 pmoles) of WT, S41A, or S41C. Products were resolved on a 20% TBE native PAGE gel and visualized with Sybr Gold staining. Inserted is a 10–20% gradient SDS-PAGE showing the proteins used for crystallization (Se-Met) and for activity (WT, S41A and S41C). NEB protein ladder was used as molecular weight markers.

FokI, the best-known Type II restriction enzyme, has a similar domain organization comprising an N-terminal recognition domain and a C-terminal catalytic domain. It also recognizes an asymmetric sequence and cleaves downstream \(N_9/N_{13}\), but there the similarities stop. FokI is monomeric in solution and double-strand (ds) cleavage occurs by transient dimerization between the catalytic domains of neighboring molecules at least one of which is bound to a recognition site\(^6,7\). AspBHI (and MspJI\(^7\)), in contrast, assembles into a tetramer, even in the absence of DNA, with two centers for ds DNA cleavage (i.e. two catalytic-domain mediated dimers) and four 5mC-recognition domains. A complex model based on structural and biochemical evidence has been proposed for MspJI\(^7\) and likely also applies to AspBHI - in which three monomers of the tetramer are involved, respectively, in binding modified cytosine, making the first proximal \(N_{12}\) cleavage in the same strand, and then making the second distal \(N_{16}\) cleavage in the opposite strand. In contrast to AspBHI, the N6-methyladenine dependent restriction enzyme DpnI, comprises an N-terminal combined recognition and catalytic domain and a C-terminal non-catalytic DNA-binding domain\(^8\) (opposite of the domain arrangement of AspBHI and MspJI), and is monomeric.
The variety of restriction enzymes also makes them fascinating subjects for studying protein-DNA interactions among enzymes with a common basic function – highly specific DNA recognition and cleavage. Surprisingly, even for very well characterized restriction enzymes such as EcoRV\(^6\)--\(^8\), the mechanistic features that determine specificity and selectivity are difficult to model on the basis of the available structural information\(^9\). Other than requiring a 5mC-G base pair, AspBHI is promiscuous in the bases it recognizes on either side of the modified cytosine: 5’-(C/T)(C/G)(5mC)(N-C/G)-3’. For example, the 5’ most base can be a thymine or cytosine but not a guanine (or adenine) (Fig. 5b). We attempted to relax specificity further on the 5’ side of the 5mC by targeted mutagenesis of Ser41 and Arg42, but we were unsuccessful. Arg42, which is not conserved among family members (Fig. 1b), was found nevertheless to be essential for enzyme activity, and all Arg42 mutants were inactive. Ser41 mutants were likewise inactive except S141A and C. Interestingly, S41A, which loses the ability to make hydrogen bonds, showed some mutant were likewise inactive except S141A and C. Interestingly, S41A, which loses the ability to make hydrogen bonds, showed some DNA cleavage assays using methylated plasmids and phage DNA. Dcm\(^+\) pUC19 (100 μg) was incubated with various methyltransferases (M.AluI, M.SssI, M.HaeIII, M.HpaII, M.HhaI, or M.MspI) overnight at 37°C in the presence of 32 mM AdoMet (160 mM AdoMet for M.SssI) in a total reaction volume of 500 μL. Reactions were treated with 5 μL Proteinase K (10 mg ml\(^{-1}\)) for 1 h at 37°C. Plasmids were then purified by spin column (Qiagen) and the DNA concentration was measured using the Nanodrop.

DNA cleavage assays using methylated plasmids and phage DNA. Dcm\(^+\) pUC19 (100 μg) was incubated with various methyltransferases (M.AluI, M.SssI, M.HaeIII, M.HpaII, M.HhaI, or M.MspI) overnight at 37°C in the presence of 32 mM AdoMet (160 mM AdoMet for M.SssI) in a total reaction volume of 500 μL. Reactions were treated with 5 μL Proteinase K (10 mg ml\(^{-1}\)) for 1 h at 37°C. Plasmids were then purified by spin column (Qiagen) and the DNA concentration was measured using the Nanodrop.

### Methods

All enzymes, plasmids and bacterial strains, if not otherwise specified, were obtained from New England Biolabs (NEB). Escherichia coli codon optimized AspBHI with an N-terminal 6xHis tag was cloned into a pUC19 derivative pZ1 (Z. Zhu, NEB) between Ndel and BamHI sites. Site-directed mutagenesis was carried out by inverse PCR using Vent\(^\text{®}\) DNA polymerase and mutagenic primers designed with NEB in-house software. The entire alleles in AspBHI variants were sequenced to confirm the desired mutation.

### Protein expression and purification

Wild type (WT) and mutant AspBHI with N-terminal 6xHis tags were expressed in a Dcm-deficient E. coli strain T7 Express (C2566). Cells were grown at 30°C in 10 mL (small scale) or 0.5 to 1 L (medium scale) in LB \(\Delta\) Amp to OD\(_{600}\) of 0.3–0.6 and induced with a final concentration of 0.5 mM Isopropyl \(\beta\)-D-1-thiogalactopyranoside (IPTG). Induced cultures were grown overnight at 25°C, harvested and then kept at \(-20^\circ\)C. His-tagged proteins (small scale) were partially purified using Qiagen Ni-NTA spin kit as recommended by the supplier and used in the experiments shown in Fig. 4. For medium-scale production cells were lysed using sonication in 20 mM Tris-HCl, pH 7.5, 400 mM NaCl, 20 mM imidazole. Clarified cell extract was loaded over a gravity column using a Ni-NTA resin (Qiagen). Protein was eluted with 500 mM imidazole. Pooled fractions were then diluted by 10 fold in 20 mM Tris-HCl, pH 7.5, 20 mM NaCl and loaded over a 5 mL Hi-Trap Heparin column using an AKTA FPLC machine (GE Healthcare). The proteins were eluted at \(-250–290\) mM NaCl with a linear gradient of 20 mM to 1 M NaCl. Fractions containing AspBHI were identified on 10–20% gradient Tris-Glycine gels with the protein appearing as the major band (purity approximately 95%; Fig. 5b insert). Proteins were diluted to a working stock of 0.5–1 mg ml\(^{-1}\) and used in the experiments shown in Fig. 5b.

### Crystallography

For crystallization of AspBHI, 12 L of IPTG-induced E. coli culture were harvested and the non-tagged enzyme was purified to homogeneity by Precise\(^\text{®}\) using Vent\(^\text{®}\) DNA polymerase and mutagenic primers designed with NEB in-house software. The entire alleles in AspBHI variants were sequenced to confirm the desired mutation.

Crystallography. For crystallization of AspBHI, 12 L of IPTG-induced E. coli were harvested and the non-tagged enzyme was purified to homogeneity by chromatography through Heparin DM, Bio-Gel HTP hydroxyapatite, Mono Q, and Heparin TSK columns. Alternatively, further purification was performed via tandem HiTrap Q/SP (GE Healthcare) and a sizing column Superdex 200 (GE Healthcare). The position of the protein peak in the Superdex 200 column suggests the protein is a tetramer (Fig. 2e).

Final concentrations of the protein are between 6–20 mg ml\(^{-1}\) in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM dithiothreitol (DTT). Crystallizations were carried out by the hanging-drop vapor-diffusion method at 16°C using equal amounts of protein and well solutions. Conditions giving large and well-diffacting AspBHI crystals were (i) 12% polyethylene glycol 3350 with 0.5 M K\(_2\)HPO\(_4\)/Na\(_2\)HPO\(_4\) (pH 7.4) and (ii) 6–15% polyethylene glycol MME 5000, 5% Tascimate (Hampton Research), and 100 mM HEPES (pH 5.6–6.5). The AspBHI crystal structure was solved using single-wavelength anomalous diffraction methods\(^{20}\) using the following datasets: a native data set, a Se anomalous dataset from a selenium-methionine labeled crystal, three each for molecules A and B, and two each for molecules C and D (228M located in disordered C-terminal domains of molecules C and D were not detected). In the Hg derivative, a total of four Hg\(^{2+}\) atoms were found in the asymmetric unit, two of which reacted to Cys255 and Cys306 of molecules A or B. All the data were processed using the program HKL2000\(^{21}\), which calculated values of Rmerge and |<I>/<I>| (Table 1). Phasing, map production, and model refinement were conducted using the PHENIX software suite.\(^{22}\) The AutoSol Wizard\(^{23}\) of PHENIX used RESOLVE\(^{24}\) to carry out density modification and applied non-crystallographic symmetry (NCS) calculated from positions of heavy atoms\(^25\), resulting in the multi isomorphous replacement with anomalous scattering (MIRAS) electron density map with superior quality compared to either single anomalous diffraction (SAD) map. Maps and model were visualized with COOT\(^{26}\) as well as manual model manipulation during refinement rounds without the disordered C-terminal domains of molecules C and D. Individual thermal B-factors were refined only at the end stages of refinement, with the averaged root-mean-square deviation of 3.7 Å for main chain atoms and 5.1 Å for side chain atoms and did not significantly for any ordered domain of the modeled monomers. Distribution of averaged crystallographic thermal B-factor pre residue for the four monomers is shown in Figure 1C, with the highest B-factors occur in the loops.

### DNA cleavage assays using methylated plasmids and phage DNA. Dcm\(^+\) pUC19 (100 μg) was incubated with various methyltransferases (M.AluI, M.SssI, M.HaeIII, M.HpaII, M.HhaI, or M.MspI) overnight at 37°C in the presence of 32 mM AdoMet (160 mM AdoMet for M.SssI) in a total reaction volume of 500 μL. Reactions were treated with 5 μL Proteinase K (10 mg ml\(^{-1}\)) for 1 h at 37°C. Plasmids were then purified by spin column (Qiagen) and the DNA concentration was measured using the Nanodrop.
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Author contributions J.R.H. performed crystallographic work, R.L.N. and A.L. performed site-directed mutagenesis, R.N.L., A.L. and S.Y.X. purified mutants and performed activity assays, M.Y.M. constructed and assessed all 19 mutants of R42X. A.F. performed over-expression of non-tagged AspBHI used for crystallography, D.C.K. contributed enzyme reagents, R.M.G. and X.Z. performed purification and crystallization trials, G.G.W. performed structural analysis, suggested structure-based mutagenesis and assisted in preparing the manuscript, X.C., Y.Z. and S.Y.X. organized and designed the scope of the study, and all were involved in analyzing data and preparing the manuscript.

Additional information Accession codes The X-ray structure (coordinates and structure factor files) of AspBHI has been submitted to the Protein Data Bank as entry 4OC8.

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