The *Pvu*II DNA (Cytosine-*N4*)-methyltransferase Comprises Two Trypsin-Defined Domains, Each of Which Binds a Molecule of *S*-Adenosyl-L-methionine†

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ABSTRACT: Earlier studies have shown that *Pvu*II methyltransferase is monomeric and transfers a methyl group from *S*-adenosyl-L-methionine (AdoMet) to cytosine, generating *N4*-methylcytosine in duplex 5′-CAGCTG-3′ DNA. This study examines the interactions between *Pvu*II methyltransferase and AdoMet. Trypsin preferentially cleaved the protein into two large fragments, with initial cleavages after Arg^{183} and Lys^{186}. UV-mediated photochemical labeling with [^{3}H-CH_3]AdoMet, followed by trypsin digestion, revealed that both large fragments of the protein were labeled. Rapid gel filtration confirmed that each molecule of the intact enzyme bound two molecules of AdoMet (net K_d = 9.3 μM). When *Pvu*II methyltransferase was preincubated with a range of [^{3}H-CH_3]AdoMet concentrations, bursts of product formation resulted upon DNA addition. These data indicate that *Pvu*II methyltransferase is catalytically competent with one and with two bound molecules of AdoMet. These results, together with those from earlier studies, suggest possible roles for the second molecule of AdoMet.

Bacterial restriction−modification systems involve two enzymatic activities: a DNA-sequence-specific endonuclease that in some cases protects the host cell from bacteriophage infection, and an AdoMet-dependent[1] methyltransferase that prevents suicidal cleavage of host DNA by methylating the same DNA sequence recognized by the endonuclease (Bickle & Kruger, 1993). For a variety of reasons, including their roles in controlling mammalian gene expression [*e.g.*, see Shemer et al. (1991)], there is growing interest in DNA methyltransferases. The genes for many of these restriction−modification systems have been cloned, facilitating detailed study of the catalytic and kinetic mechanisms of the methyltransferases (Flynn et al., 1996; Marzabal et al., 1995; Mashhoon & Reich, 1990; Nardone et al., 1986; Rao et al., 1989; Reich & Mashhoon, 1991; Szilak et al., 1993; Wu & Santi, 1987). Methyltransferases from type II restriction−modification systems require only AdoMet and DNA for catalysis (Cheng, 1995), and methylation of the two DNA strands requires two independent binding events with the monomeric methyltransferase in opposite orientations (Reich & Mashhoon, 1993).

These catalytic and kinetic studies are complemented by an increasing number of DNA methyltransferase structures (Cheng et al., 1993; Labahn et al., 1994; Reinisch et al., 1995) that have yielded two quite surprising results. First, methyltransferases that act on substrates as different as DNA adenine, DNA cytosine, and catechol all have strikingly similar structural elements that are also reflected in conserved sequence motifs (Malone et al., 1995; Schluckebier et al., 1995). Second, DNA methyltransferases rotate the target (methylatable) nucleotide by roughly 180° without breaking any backbone bonds in the DNA, such that the nucleotide projects into a concave catalytic pocket; this process is referred to as "base flipping" (Cheng & Blumenthal, 1996; Klimasauskas et al., 1994; Klimasauskas & Roberts, 1995a; Reinisch et al., 1995; Roberts, 1995). The kinetic implications of base flipping have yet to be explored in detail, though an assay for base flipping has now been developed (Allan & Reich, 1996).

The *Pvu*II methyltransferase (M·*Pvu*II) is part of a type II restriction−modification system and catalyzes methylation of the exocyclic amino group on the internal cytosine in CAGCTG sequences, yielding *N4*-methylcytosine (Blumenthal et al., 1985; Gingeras et al., 1981; Janulaitis et al., 1983a,b). The gene for this enzyme, *pvuIIM*, was isolated from a plasmid of the Gram-negative bacterium *Proteus vulgaris* (Blumenthal et al., 1985). M·*Pvu*II, being monomeric with a molecular mass of approximately 37 kDa (Tao et al., 1989), is typical of methyltransferases from type II restriction−modification systems (Lauster, 1989).

DNA methyltransferases are suggested to have evolved via a gene duplication mechanism (Lauster, 1989; Malone et al., 1995). The evidence for such duplication, if it did indeed occur, has in most cases been obscured by divergence (Guyot & Caudron, 1994). However, the amino acid sequence of M·*Pvu*II reveals an apparent internal duplication [Figure 1 (Tao et al., 1989)], and both homologous regions contain motifs implicated in catalysis by these enzymes. We report here that M·*Pvu*II is split into two large fragments by trypsin digestions, and that each of the large fragments includes a binding site for the substrate AdoMet.

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[1] Abbreviations: AdoHcy, *S*-adenosyl-L-homocysteine; AdoMet, *S*-adenosyl-L-methionine; bp, base pair(s); CAPS, 3-(cyclohexylamino)-1-propanesulfonate; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetate; Lrp, leucine-responsive regulatory protein of *Escherichia coli*; M·, indicating a DNA methyltransferase as in M·*Pvu*II; MOPS, 3-(*N*-morpholino)propanesulfonate; PVDF, poly(vinylidene difluoride); SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet light.

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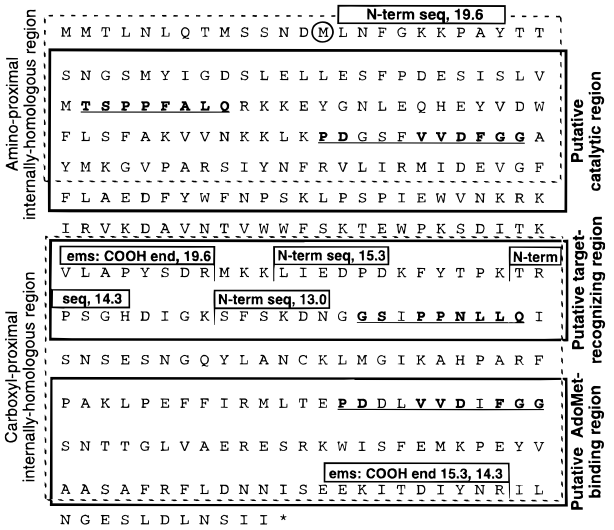

FIGURE 1: Trypsin cleavage points and other features of the M·*Pvu*II amino acid sequence. The amino acid sequence of M·*Pvu*II has an internal duplication (Tao et al., 1989); the internally homologous regions are outlined by the two dashed boxes. The two most highly conserved motifs, named for the amino acid sequence of their consenses (DPPY and FXGXG), are shown as boldface underlined letters. The circled methionine is the internal translational start of the smaller 36 908 kDa enzyme. Vertical lines indicate trypsin cleavage points as determined by N-terminal sequencing (black boxes indicate range sequenced) or by electrospray mass spectroscopy (ems). Functional regions of M·*Pvu*II are taken from predictions presented in Malone et al. (1995) that have since been confirmed by X-ray crystallography (Gong et al., 1997); the approximate borders of the three functional regions are indicated by the solid boxes.

EXPERIMENTAL PROCEDURES

Materials. [^{3}H-CH$_3$]AdoMet (81 or 15 Ci/mmol) and [^{14}C-CH$_3$]AdoMet (56 Ci/mmol) were from Amersham, and unlabeled AdoMet hydrogen sulfate was from Boehringer Mannheim. Whatman produced the DE 81 ion-exchange paper disks and S40 paper disks. Scinti-Safe scintillation fluid was from Curtin-Mathesin Scientific. Sinefungin, trypsin, LysC endoprotease, chymotrypsin, SBTI, TLCK, AdoHcy, Tris-HCl, MOPS, CAPS, NaCl, NaH_2PO_4, EDTA, and DTT were from Sigma Chemical Co. Synthetic oligonucleotides were made on an Applied Biosystems Model 391 DNA synthesizer by Drs. Mats Fernstrom and Ming You (Medical College of Ohio). En^{3}Hance autoradiography enhancer was from NEN/Dupont. Electrophoresis Duplicating Paper (EDP) was from Kodak, and the G-50 Sephadex (cross-linked dextran) "NICK-Spin" columns were from Pharmacia. Bio-Rad was the source of the protein assay kit, PVDF membrane, and the 10−20% acrylamide gradient precast SDS minigels. Software used in data analysis included EnzymeKinetics ver. 1.4.1 (Trinity Software) and KaleidaGraph ver. 3.0.8 (Abelbeck Software).

M·PvuII. M·*Pvu*II is made in two forms due to alternative translation inititators: these two forms differ in size by 13 amino acids (M_r = 38 375 and 36 908), and the shorter form is about 20-fold more abundant than the larger form (Blumenthal et al., 1985). The significance of having two forms is not known, but clones producing only the shorter form are unstable unless their expression is tightly controlled (G.M.A., unpublished data). Our kinetic burst and spun-column experiments made use of the purified shorter form of M·*Pvu*II, kindly provided by James Keller and Drs. John Anderson, Margaret O'Gara, and Xiaodong Cheng (Cold Spring Harbor Laboratory), who purified it from an over-expressing clone constructed in this laboratory (O'Gara et al., 1997). The trypsin time course experiments used M·*Pvu*II preparations containing both forms that were kindly provided by Drs. Lydia Dorner and Ira Schildkraut (New England Biolabs, Inc.).

Protease Digestions. M·*Pvu*II was digested with 1.0% w/w protease/MTase at 25 °C in 25 mM MOPS (pH 7.0), 100 mM KCl, 1 mM DTT, and 30 mM EDTA. At the times indicated, 10 μg of methyltransferase was removed, and digestion was stopped with 5 μL of a 1% solution of either TLCK or SBTI. These samples were boiled for 2 min in SDS sample buffer and loaded onto a 15% SDS−polyacrylamide gel (Laemmli, 1970). The gel was stained with Coomassie blue, and photographed using Kodak Electrophoresis Duplicating Paper or with a Photometrix CCD camera (used courtesy of Dr. John Langmore, University of Michigan). In some cases, the stained gel was analyzed at 573 nm with a Shimadzu CS-930 scanning densitometer.

N-Terminal Sequence Analysis and Electrospray Mass Spectroscopy. For the N-terminal sequence analysis, 165 μg of M·*Pvu*II was digested with 0.3% w/w trypsin at 25 °C for 2 min in 25 mM MOPS (pH 7.0), 100 mM NaCl, and 2 mM DTT. Twenty micrograms of the cleaved protein was electrophoresed on a 10−20% gradient SDS gel and blotted to PVDF membrane in electroblotting buffer containing 10% methanol and 10 mM CAPS. The membrane was stained with Coomassie blue, and the protein fragments were excised for amino acid sequencing. Protein for electrospray mass spectroscopy was cleaved with trypsin as described above, and diluted into water. N-Terminal sequence determination and electrospray mass spectroscopy were carried out at the University of Michigan Protein and Carbohydrate Structure Facility (Ann Arbor, MI).

Effect of Trypsin on M·PvuII Activity. Trypsin digestion was carried out as described above, but with 4% w/w trypsin/methyltransferase. After various digestion times, samples were removed, reactions were terminated with SBTI, and 3.5 μg of digested protein was added to 125 μL methylation reactions; additional trypsin-digested samples were removed for loading onto a 10−20% acrylamide gradient SDS gel. The methylation reactions (Nelson & McClelland, 1987) contained 10 mM Tris (pH 7.5), 50 mM NaCl, 10 mM EDTA, 5 mM DTT, 0.5 μM [^{3}H-CH$_3$]AdoMet (81 Ci/mmol), and 5 μg of bacteriophage λ DNA. At 0, 5, 10, and 15 min, 25 μL from each methylation reaction was mixed with 20% TCA to stop the reaction and spotted onto Whatman S40 paper disks. The disks were washed 3 times for 10 min with gentle shaking in 10% TCA, and once in 2-propanol, then dried for 10 min at 65 °C, and placed in 2.5 mL of Scinti-safe scintillation fluid. C^3H_3 incorporation was measured in a Beckman LS 380 scintillation counter. The SDS−polyacrylamide gel was stained with Coomassie blue and subjected to scanning densitometry.

Photochemically Labeling M·PvuII with [^{3}H-CH$_3$]AdoMet. Ten micrograms of either intact or trypsin-digested M·*Pvu*II was UV-irradiated in the presence of [^{3}H-CH$_3$]AdoMet using a hand-held UV lamp with peak output at 254 nm and a surface intensity of 300 μW/cm^2 (UVP, San Gabriel, CA). The protein was illuminated on ice for 20 min in 25 μL reactions containing 25 mM MOPS (pH 7.0), 100 mM NaCl, 2 mM DTT, 30 mM EDTA, and 1.6 μM [^{3}H-CH$_3$]AdoMet (81 Ci/mmol) (Drummond et al., 1993); longer illumination times resulted in reduced incorporation, presumably due to radiolytic decay of the protein. Compared to the standard

conditions for activity assays (noted in the preceding section), these reactions were carried out at lower pH to stabilize the AdoMet, and at higher ionic strength to minimize possible nonspecific AdoMet−M·*Pvu*II interactions. The reactions were resolved on a 15% acrylamide−SDS gel, stained with Coomassie blue, and treated with En^{3}Hance to allow fluorography. The gel was dried and put onto film for several days to a week. In some control reactions, the concentration of AdoMet was 4 μM during the photolabeling, and 40 or 150 μM AdoHcy was added. To test for saturability of the photochemical labeling, the [^{3}H-CH$_3$]AdoMet was progressively diluted with unlabeled AdoMet, and incorporation was corrected for the changed specific activity. The rate of increase in protein labeling as a function of increasing AdoMet concentration was greatly slowed, though not zero, at the highest AdoMet concentration tested in this way (16 μM; data not shown).

Spun Column Separations. We determined the ratio of protein recovered to AdoMet bound by using spun column chromatography with M·*Pvu*II or, as a control, Lrp (Matthews & Calvo, 1994), a 37.6 kDa DNA-binding protein with a charge and size similar to those of M·*Pvu*II. The purified Lrp was kindly provided by Dr. Rowena Matthews. Lrp or M·*Pvu*II were added at a concentration of 2 μM to 150 μL reaction mixtures containing 25 mM MOPS (pH 6.8), 150 mM NaCl, 2 mM DTT, and 16 μM [^{3}H-CH$_3$]AdoMet (7.5 $\times$ 10^{-3} Ci/mmol), and allowed to reach equilibrium by preincubating for 20 min at room temperature. Protein-bound and unbound AdoMet were separated by centrifugation for 2 min at 500*g* on buffer-equilibrated NICK Spin columns (which contain G-50 Sephadex). In another experiment, the concentration of AdoMet was varied from 2 to 100 μM. Protein recovery was determined by assaying 65 μL of the eluate with the Bio-Rad protein assay kit [based on the Coomassie blue dye-binding assay (Bradford, 1976)], using bovine serum albumin as the standard. Since our observation (Results) that multiple molecules of AdoMet could bind each molecule of M·*Pvu*II might be explained if the amount of protein recovered was artifactually underestimated, we confirmed the stock concentration as measured by dye binding through determination of the A_{280}, using a molar extinction coefficient ϵ_M of 56 540 M^{-1} cm^{-1} for purified M·*Pvu*II (Mach et al., 1992; Pace et al., 1995); dye binding gave a concentration of 590 μM, and absorption gave a concentration of 513 μM. The amount of AdoMet bound was determined by counting an equal volume of eluate in 1 mL of scintillation fluid. As noted above, these reactions were carried out at a lower pH to stabilize the AdoMet, and at a higher ionic strength to minimize possible nonspecific AdoMet−M·*Pvu*II interactions, than the standard conditions for activity assays.

To measure the exchange rates at AdoMet-binding sites, 2 μM M·*Pvu*II was preincubated with 50 μM [^{14}C-CH$_3$]-AdoMet (0.33 Ci/mmol) in the buffer described above. At time zero, an equal volume of 50 μM [^{3}H-CH$_3$]AdoMet (0.4 Ci/mmol) was added, and after various times (10 s−16 min), samples were loaded onto NICK Spin columns to resolve bound from unbound AdoMet. The ^{3}H/^{14}C ratio of the eluate was determined by liquid scintillation. Separately loading equal volumes of the preincubation and ^{3}H mixes onto the same column indicated that no detectable exchange occurs on the column itself.

Design of the DNA Substrate. The DNA substrate used for these experiments was a duplex of two 30-mer complementary oligonucleotides (Pvu33R; Figure 2), based on the sequence surrounding the unique *Pvu*II site in the plasmid pBR322 since this laboratory has already conducted studies with that site (Rice et al., 1995). The oligonucleotides were designed with three-nucleotide 5′-overhangs (so the duplex region is 27 bp) to allow ligation of the duplex into a single, multi-site substrate. However, for the experiments reported here, they were used as a single-site, homogeneous substrate, since DNA-binding enzymes can show altered reaction kinetics based on the length of the DNA molecule and the position of the recognition site relative to the ends of the molecule (Modrich, 1982; Modrich & Zabel, 1976; Nardone et al., 1986; Surby & Reich, 1992, 1996a,b). Note that each mole of this duplex represents 2 mol of methyltransfer substrate, as each strand can be independently methylated. OD_{260} measurements of each single-stranded oligonucleotide were made separately, and equal amounts of the two complementary oligonucleotides were mixed before annealing.

FIGURE 2: DNA substrate used for the kinetic burst analysis of M·*Pvu*II. The Pvu33R duplex was designed by making complementary 30-mer oligonucleotides which contain the sequence surrounding the *Pvu*II site in the plasmid pBR322. The boxed sequence is the substrate site for M·*Pvu*II, with the target bases indicated by asterisks. Note that each mole of duplex DNA contains 2 mol of methylatable substrate.

Kinetic Burst Experiment. For the AdoMet-preincubation burst experiment, 2.2 μM M·*Pvu*II was preincubated for 3 min at 37 °C with 4−32 μM AdoMet in 30 μL of methyltransferase buffer (described above). Control reactions were incubated in buffer only, and both DNA and AdoMet were added at time zero. The reactions were started by adding 25 μL of preincubation mixture to prewarmed tubes containing the DNA substrate (1.3 μM duplex, or 2.6 μM methylatable sites), methyltransferase buffer, and enough AdoMet to keep the concentration and specific radioactivity unchanged from preincubation conditions. The concentration of enzyme fell to 0.27 μM. The final volume was 200 μL, and 25 μL samples were taken every 5 s for 30 s. Samples were spotted onto Whatman DE 81 paper disks. The filters were washed 3 times for 10 min with gentle shaking, in buffer containing 50 mM NaH_2PO_3, 10 mM EDTA, and 5% SDS at pH 7.2, and washed once in 95% ethanol. After being dried for 10 min at 65 °C, filters were placed in 2 mL of Scinti-safe scintillation fluid, and C^{3}H$_3$ incorporation was determined

RESULTS

M·PvuII Is Initially Cleaved into Large Fragments by Proteases. If the two internally homologous regions of M·*Pvu*II define similar domains in the tertiary structure of the methyltransferase, then a protease might preferentially cleave between them. M·*Pvu*II was in fact preferentially cleaved in a central region (after Arg^{183} and Lys^{186}), but these cleavage points are within the carboxyl-proximal homologous region (see Figure 1). The SDS−polyacrylamide gel shown in Figure 3 reveals that limited trypsin digestion of M·*Pvu*II rapidly released a 19.6 kDa fragment (fragment sizes were determined by electrospray mass spectroscopy); the N-terminal sequence of this fragment was found to match that of the intact protein (in both cases with the initiator

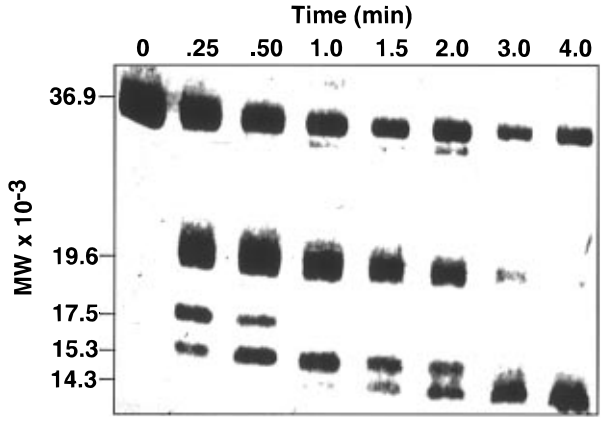

FIGURE 3: M·*Pvu*II is initially cleaved by trypsin into large fragments. Purified *Pvu*II methyltransferase was digested with 1.0% w/w of trypsin/methyltransferase for the times shown at the top of the figure. The image shown is from a CCD photograph of a Coomassie-stained 15% SDS−polyacrylamide gel. The MWs of the intact enzyme and of its cleavage products were determined by electrospray mass spectroscopy (19.6, 15.3, 14.3) or relative mobility (17.5, 13.0), and are indicated to the left of the figure.

methionine removed). The 17.5 kDa fragment is probably the primary carboxyl fragment resulting from the cleavage that generates the 19.6 kDa band (expected size = 17 173 Da; and the 19.6 and 17.5 kDa fragments would sum to 37.1 kDa); if so, it is rapidly cleaved further at both ends (Figure 1). The smaller 15.3 kDa fragment is from the carboxyl portion of the protein, as revealed by N-terminal sequence analysis, and is further shortened at its amino end to yield fragments of 14.3 and 13.0 kDa (the 13.0 kDa band is not shown in Figure 3). In parallel experiments, neither 440 μM AdoMet nor 4 mM sinefungin had any effect when added before the trypsin (not shown). Limited digestion of M·*Pvu*II with two other proteases, LysC endoprotease and chymotrypsin, gave patterns of cleavage comparable to that of trypsin (not shown). The protease digestion results are consistent with a two-lobed structure for M·*Pvu*II.

M·PvuII Activity Is Abolished by Primary Trypsin Cleavage. Because the initial cleavage might yield a nicked complex rather than two separate fragments, and because each half of M·*Pvu*II has candidate catalytic motifs such as DPPY and FXGXG (Figure 1), we tested the catalytic activity of the cleaved methyltransferase. Trypsin digestions were carried out as described under Experimental Procedures, and at various times, samples were removed and the reaction was stopped with SBTI. Part of each sample was used to assay its capacity to incorporate C^3H_3 from AdoMet into λ DNA, and the rest was resolved on a 15% polyacrylamide−SDS gel to determine the amount of uncleaved M·*Pvu*II. Residual M·*Pvu*II activity was directly proportional to the amount of uncut protein throughout the trypsin time course (Figure 4), indicating that cleavage abolished activity: theoretically, the slope of residual activity *vs* residual uncut protein would be 1.0, and it is 0.96 (Figure 4, inset). The two large fragments of M·*Pvu*II do not remain together in an active conformation (at least at the protein concentration we used) and lack independent activity.

Photochemically Labeling M·PvuII with AdoMet Labels Both Large Fragments. Many methyltransferases can be photochemically labeled by [^{3}H-CH_3]AdoMet using short-wavelength UV (Chen et al., 1995; Finta et al., 1995; Krishnamurthy & Rao, 1994; Reich & Everett, 1990; Som & Friedman, 1990; Wenzel et al., 1991). This labeling

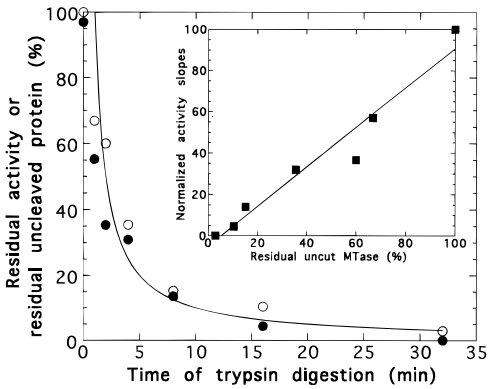

FIGURE 4: M·*Pvu*II activity is abolished by the primary tryptic cleavage. M·*Pvu*II was digested with 4% w/w trypsin, and at 0−32 min thereafter, samples were removed to tubes containing excess trypsin inhibitor. These samples were added to methylation reactions or saved for later loading onto a 10−20% gradient SDS−polyacrylamide gel. Densitometry of the stained gel indicated the amount of remaining uncut methyltransferase (●). At 0, 5, 10, and 15 min, 25 μL from each methylation reaction was added to 20% TCA, spotted onto Whatman S40 filters, and counted to determine C^3H_3 incorporation into bacteriophage λ DNA. Activity was determined from the slope of incorporated cpm *vs* time. These activity slopes are plotted *vs* time in trypsin (○). Inset: Methylation activity *vs* residual uncut M·*Pvu*II. The slope from linear regression is 0.96, with a correlation coefficient of 0.97.

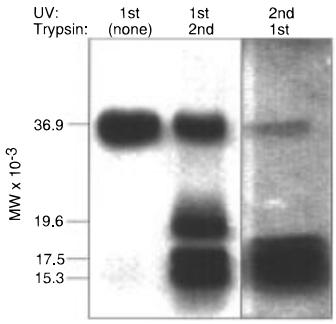

FIGURE 5: UV photochemical labeling of M·*Pvu*II by [^{3}H-CH_3]-AdoMet. Intact or trypsin-cleaved M·*Pvu*II was UV-irradiated in the presence of 1.6 μM [^{3}H-CH_3]AdoMet. The fluorogram shows M·*Pvu*II irradiated either before ("1st") or after ("2nd") cleavage by trypsin, and resolved on a 15% acrylamide−SDS gel. The leftmost lane shows photolabeled intact protein. Molecular weights were determined by electrospray mass spectroscopy as described in Figure 1.

reflects specific AdoMet binding based on the following control results, specifically confirmed for M·*Pvu*II (data not shown): labeling is absolutely dependent on UV irradiation, it is saturable, and it is inhibited by unlabeled AdoMet or by the product of methylation reactions (AdoHcy). Furthermore, molecular weight standards (consisting of proteins that do not specifically bind AdoMet) do not become labeled in parallel experiments (Drummond et al., 1993). At least in the case of M·*Eco*RII labeling is due to UV-dependent transfer of the tritiated methyl group by itself, presumably via a radical mechanism, to a nearby nucleophilic amino acid side chain (a cysteine in this case; Som & Friedman, 1990).

We sought to determine the ability of each trypsin-defined portion of M·*Pvu*II to bind AdoMet. Figure 5 shows the results of UV-irradiating M·*Pvu*II in the presence of [^{3}H-CH_3]AdoMet, and then cleaving with trypsin. Both the amino (19.6 kDa fragment) and carboxyl (17.5 and 15.3 kDa fragments) portions of the methyltransferase were labeled by [^{3}H-CH_3]AdoMet. No conclusion can be drawn from the fact that the carboxyl portion is labeled more efficiently than the amino portion, since the labeling efficiency appears to

Table 1: Molecules of AdoMet Bound by M·*Pvu*II or Lrp[a]

protein	protein recovered[b] (nmol)	AdoMet recovered[c] (nmol)	AdoMet/protein
M·*Pvu*II	$(3.4 \pm 0.3) \times 10^{-2}$	$(6.4 \pm 0.4) \times 10^{-2}$	1.9 ± 0.12
	$(5.8 \pm 0.2) \times 10^{-2}$	$(1.0 \pm 0.09) \times 10^{-1}$	1.7 ± 0.04
Lrp	$(3.3 \pm 0.4) \times 10^{-2}$	$(3.3 \pm 0.3) \times 10^{-4}$	0.01
	$(3.5 \pm 0.07) \times 10^{-2}$	$(3.3 \pm 0.3) \times 10^{-4}$	0.01

[a] Proteins were incubated with 16 μM [^{3}H-CH$_3$]AdoMet for 20 min at room temperature in a 150 μL volume, and then separated from unbound AdoMet by spun column gel filtration as described under Experimental Procedures. All procedures were carried out in triplicate, and standard error values are shown. [b] Protein recovery was determined, for half of each eluate, with the Bio-Rad protein assay kit. The input amount of protein was 10 μg per assay, which corresponds to ~0.27 nmol in each case. [c] Bound [^{3}H-CH$_3$]AdoMet was determined, for half of each eluate, by liquid scintillation.

depend on the proximity of a nucleophilic amino acid side chain (see above) and this could vary between binding sites.

If M·*Pvu*II was cleaved by trypsin before UV illumination, photolabeling of the carboxyl portion did not appear to be less efficient than it was in the intact protein. In contrast, while a labeled band corresponding to the 19.6 kDa amino portion of the methyltransferase was visible, it could only be seen after several weeks of exposure to film (not shown in Figure 5); the reduction in labeling is not due to proteolytic degradation of the 19.6 kDa fragment, which is clearly visible in the Coomassie-stained gel corresponding to the rightmost lane in Figure 5 (not shown) and is essentially as stable to trypsin as the 15.3 kDa fragment (Figure 3). This result raised two possibilities. First, there might be two AdoMet-binding sites, with the amino-proximal site being conformationally unstable after cleavage or cooperatively dependent upon the carboxy-proximal site. Alternatively, there might be a single AdoMet-binding site, with the one AdoMet molecule able to photolabel either portion of M·*Pvu*II. We were unable to distinguish between these two possibilities by carrying out photochemical labeling of intact M·*Pvu*II at varying concentrations of AdoMet and then measuring the relative labeling of the two tryptic fragments (not shown); however, this approach would only distinguish between two binding sites that had substantially different K_d^{AdoMet} values.

Determining the Number of Molecules of AdoMet Bound to M·PvuII. To measure the number of molecules of AdoMet bound by M·*Pvu*II, we used centrifugal gel filtration (spun columns). Lrp (Matthews & Calvo, 1994), a 37.6 kDa DNA-binding protein with a charge similar to that of M·*Pvu*II, was used as a control for nonspecific binding. In each assay, 10 μg of protein was incubated for 20 min at room temperature with [^{3}H-CH$_3$]AdoMet concentrations either fixed at 16 μM (Table 1) or ranging from 2.5 to 100 μM (Figure 6). Protein-bound and free AdoMet were separated by centrifugation for 2 min over beaded dextran columns. The recovered eluate was divided; part was used to determine protein recovery, and an equal volume was counted to determine the amount of [^{3}H-CH$_3$]AdoMet that coeluted with the protein. Protein recovery was determined by a dye-binding method, the accuracy of which had been confirmed by A_{280} measurements using a molar extinction coefficient of 56 540 M^{-1} cm^{-1} (Experimental Procedures). As shown in Table 1, M·*Pvu*II bound more than one molecule of AdoMet, while the control protein Lrp, as expected, did not bind AdoMet. Figure 6 shows the number of molecules of AdoMet bound *vs* AdoMet concentration. The inset is a Scatchard plot of these data; the line

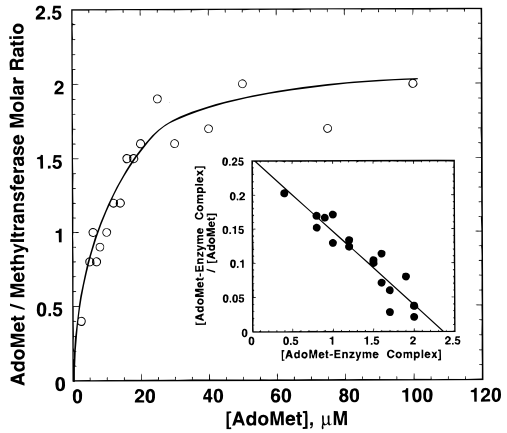

FIGURE 6: Number of molecules of AdoMet bound by M·*Pvu*II. The number of molecules of AdoMet bound was determined by incubation of M·*Pvu*II with a range of concentrations of [^{3}H-CH$_3$]-AdoMet (2−100 μM) and subsequent separation of bound from free AdoMet using spun column chromatography over beaded dextran. The number of molecules bound by the methyltransferase is plotted *vs* the total concentration of AdoMet. Inset: Scatchard plot of binding data. The points were fitted using linear regression. The correlation coefficient was 0.95 with a slope of −0.11. From this slope, the net K_d was determined to be 9.3 μM.

extrapolates to 2.3 for the number of AdoMet molecules that each molecule of M·*Pvu*II can bind, and the net K_d^{AdoMet} was calculated from the slope to be 9.3 μM. From these data alone, we cannot tell where on M·*Pvu*II the two AdoMet molecules bind, but the photolabeling and gel filtration results together suggest that the two trypsin-defined portions of M·*Pvu*II can each bind one molecule of AdoMet.

Because both binding sites appeared to have indistinguishable K_d^{AdoMet} values, we tried to distinguish between the two sites by an isotope exchange experiment. The similar K_d^{AdoMet} values mean that the ratio of k_{on}/k_{off} is similar for each site, but the values of k_{on} and k_{off} themselves could be quite different for the two sites. Accordingly, 2 μM M·*Pvu*II was preincubated with 50 μM [^{14}C-CH$_3$]AdoMet; at time zero, an equal volume of 50 μM [^{3}H-CH$_3$]AdoMet was added, and at various times thereafter, the ^{3}H/^{14}C ratio in enzyme-bound AdoMet was determined by passing portions of the mixture over a spun column as above. A difference between the two sites should have resulted in a transient plateau at half the final ^{3}H/^{14}C ratio when this ratio was plotted *vs* time. Controls included separately loading the ^{14}C·enzyme and ^{3}H mixes onto a column to show that no exchange takes place on the column, and premixing the [^{14}C]- and [^{3}H]AdoMet before adding enzyme. The results (not shown) failed to distinguish between the two binding sites within the time range of this manual experiment: M·*Pvu*II reached its equilibrium isotope ratio within 10 s of adding the [^{3}H]AdoMet.

Burst Kinetics of the Methyltransferase−AdoMet Complex. We next sought to determine whether or not M·*Pvu*II with either one or two molecules of prebound AdoMet is catalytically competent. Our interest in this question stems from the surprising structural similarity seen between two binding pockets on the structurally-characterized DNA methyltransferases; overlaying the pocket for the adenosine moiety of AdoMet and the pocket for the methylatable base yields an rms deviation of <1 Å for the C$^\alpha$ atoms (Malone et al., 1995); this is now known to be true for M·*Pvu*II as well (Gong et al., 1997). If the second AdoMet molecule binds in the target base pocket, it might be inhibitory. We

sought to determine the presence and size of a kinetic burst when M·*Pvu*II was preincubated with AdoMet. We used an AdoMet concentration range of 4−32 μM which, from the data in Figure 6, should result in averages of ~0.7 up to ~1.8 bound AdoMet molecules per molecule of M·*Pvu*II with the AdoMet distributed equally between the two binding sites.

Figure 7A shows the results of an experiment in which M·*Pvu*II was preincubated for 3 min at 37 °C with 4−32 μM [^{3}H-CH$_3$]AdoMet, followed by dilution into a prewarmed reaction mix containing the DNA substrate and enough AdoMet to maintain a constant AdoMet concentration and specific radioactivity. A burst of product formation was seen in all cases. Figure 7B shows data from a parallel control experiment, in which the M·*Pvu*II was incubated for 3 min in reaction buffer (no AdoMet) prior to the addition of a prewarmed mix containing both substrates. A burst was not seen, but as expected the resulting incorporation slopes closely matched the corresponding post-burst slopes from Figure 7A (Table 2). In both cases, the maximal reaction rates seen, in 32 μM AdoMet, correspond to about two turnovers per minute, which is in the range reported for several other DNA methyltransferases. These results confirm that M·*Pvu*II can bind AdoMet in the absence of DNA, and indicate that the resulting binary complex is catalytically competent.

These results also suggest that a ternary complex consisting of M·*Pvu*II plus two molecules of AdoMet is also catalytically competent. If the second bound AdoMet were inhibitory, then the size of the burst should have peaked at and declined above a concentration giving an average of one bound AdoMet per molecule of M·*Pvu*II, since above that point a decreasing fraction of the enzyme would have prebound AdoMet only in the AdoMet pocket and not at the second binding site (see Figure 7C).

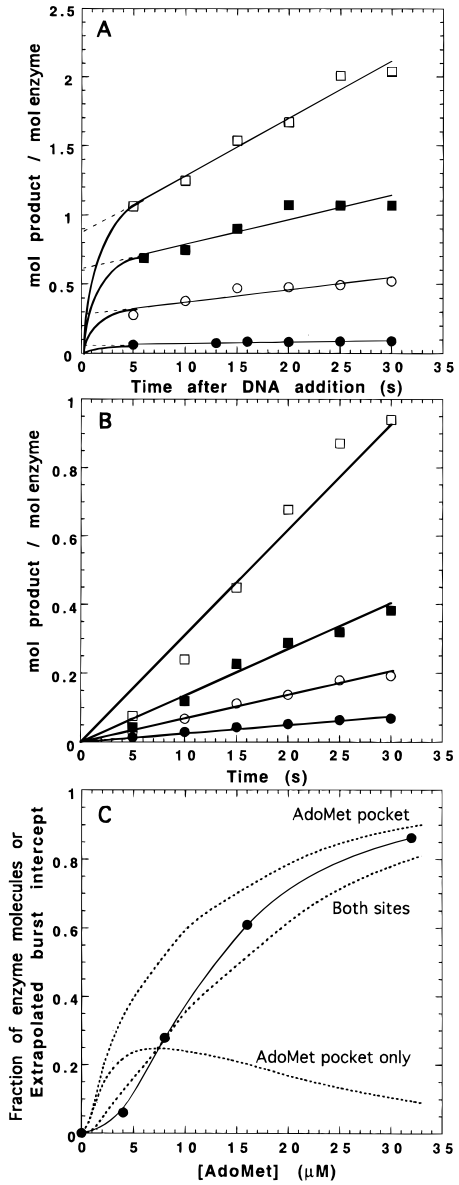

FIGURE 7: Kinetic burst assay of M·*Pvu*II preincubated with AdoMet. (A) M·*Pvu*II (2.2 μM) was preincubated for 3 min at 37 °C with 4 (●), 8 (○), 16 (■), or 32 (□) μM labeled AdoMet in reaction buffer. Reactions were initiated with a prewarmed reaction mixture containing DNA (1.3 μM duplex, or 2.6 μM methylatable sites); the concentration and specific radioactivity of AdoMet were maintained during DNA addition, but the concentration of enzyme fell to 0.27 μM. The final volume was 200 μL, and 25 μL samples were taken every 5 s for 30 s. (B) Parallel experiment, but the preincubations contained only M·*Pvu*II and buffer. Both substrates, AdoMet and duplex DNA, were added at time zero. (C) The *y*-axis intercepts from the experiment shown in panel A were plotted *vs* [AdoMet] (●). The three dotted lines are estimates of the fractions of M·*Pvu*II expected to have AdoMet bound to the "AdoMet pocket" (irrespective of binding to the second site), "Both sites" simultaneously, or the "AdoMet pocket only" (and not the second site). These estimates were made by assuming that the two binding sites have equal K_d^{AdoMet} values (see Figure 6 inset) and extrapolating from the fractional saturation data shown in Figure 6. Thus, for example, at 20 μM AdoMet, at which concentration there is ~1.6 mol of AdoMet bound per mole of M·*Pvu*II, the AdoMet will be evenly distributed between the two sites (0.8 mol of AdoMet per mole of either site); the fraction of M·*Pvu*II molecules having both sites occupied is $(0.8)^2$, the fraction having the AdoMet pocket occupied irrespective of the other site is $[(0.8 \times 0.2) + (0.8)^2]$, and the fraction having only the AdoMet pocket occupied is (0.8×0.2).

DISCUSSION

*Symmetry in the Structure of M·Pvu*II. The structurally characterized DNA methyltransferases share a highly conserved architecture (Schluckebier et al., 1995). Within these enzymes (M·*Hha*I, M·*Hae*III, and M·*Taq*I), the C$^\alpha$ atoms of the AdoMet adenine-binding pocket can be superimposed on those for the methylatable base-binding pocket with less than 1 Å rms deviation (Malone et al., 1995); this has recently been confirmed to be true of M·*Pvu*II as well (Gong et al., 1997). The portions of the M·*Pvu*II amino acid sequence associated with each of these remarkably similar α/β-binding pockets have been predicted from structure-guided sequence alignment (Malone et al., 1995), and confirmed by X-ray crystallography (Gong et al., 1997), to be essentially as indicated in Figure 1. It was appealing to think that the predicted structural homology of the two binding pockets corresponded to the sequence-level homology of the two parts of M·*Pvu*II (Tao et al., 1989), but they do not quite coincide.

The cleavage pattern resulting from limited trypsin digestion of M·*Pvu*II is consistent with a bilobal structure. The primary cleavage occurs between the predicted substrate-binding pockets and just within the carboxyl homologous region (Figures 1, 3). The implication of a bilobal structure is consistent with the results of limited digestion of another methyltransferase, M·*Eco*RI (Reich et al., 1991), and with the three-dimensional structures determined for M·*Hha*I,

Table 2: Reaction Rates (s^{-1}) of M·*Pvu*II following an AdoMet-Dependent Kinetic Burst or without Preincubation with AdoMet[a]

[AdoMet] (μM)	following burst[b]	without preincubation[c]
4	0.0009	0.0018
8	0.0074	0.0056
16	0.0148	0.0112
32	0.0346	0.0306

[a] Rates are expressed as moles of product per mole of enzyme per second, and are derived from linear regression of the data in Figure 7. [b] From the data in Figure 7A, following the kinetic burst. [c] From the data in Figure 7B.

M·*Taq*I, and M·*Hae*III (Cheng et al., 1993; Labahn et al., 1994; Reinisch et al., 1995).

While the DNA methyltransferases appear to share a common tertiary structure, they fall into three groups based on the linear order in which the AdoMet pocket, target nucleotide pocket, and DNA-binding region occur (Malone et al., 1995). M·*Pvu*II belongs to the β group of DNA methyltransferases, in that the predicted DNA-binding region is between the two binding pockets, and is the first member of this class to be structurally characterized (Gong et al., 1997). From the known structures, the DNA-binding region is expected to be a preferential target for proteases, and 4 of 5 identified cleavages (after Arg^{183}, Lys^{186}, Lys^{198}, and Lys^{208}) occur in that predicted region of M·*Pvu*II (Figure 1; the fifth cleavage, after Arg^{323}, occurs 13 amino acids from the carboxyl end). This is consistent with the fact that in the structure of the M·*Pvu*II−AdoMet complex (DNA was not present), the region from Pro^{179} to Gly^{216} was too flexible to be modeled (Gong et al., 1997).

M·PvuII Binds Two Molecules of AdoMet. When we photochemically labeled M·*Pvu*II with [^{3}H-CH_3]AdoMet and then digested with trypsin, we found that the large amino and carboxyl fragments had all been labeled (Figure 5). Direct measurement, by rapid (spun column) gel filtration, of the number of AdoMet molecules bound to intact M·*Pvu*II confirmed that two are bound (Figure 6). If M·*Pvu*II was cleaved before UV irradiation, only the carboxyl portion still labeled well; it is the carboxyl portion of M·*Pvu*II that includes the AdoMet-binding pocket (Malone et al., 1995; Gong et al., 1997). Nevertheless, some photolabeling of the amino portion of the methyltransferase was also seen.

These results are consistent with the possibility that the second molecule of AdoMet can occupy the binding pocket which, during catalysis, is occupied by the target nucleotide. It is interesting in this regard that M·*Hha*I binds tightly to an oligonucleotide containing a base mismatch which flips an adenine into the target-cytosine-binding pocket (Klimasauskas & Roberts, 1995b).

Structural analysis of one crystal form of M·*Pvu*II, grown in the presence of 80 μM AdoMet, did confirm our results by revealing two molecules of AdoMet associated with each molecule of protein (Gong et al., 1997); however, in this structure, the second AdoMet molecule was stacked onto the first in the AdoMet-binding pocket and was not located in the putative cytosine-binding pocket. It is possible that some methyltransferase crystals will be found with AdoMet in the target-base-binding pocket, but the existing M·*Pvu*II−$(AdoMet)_2$ structure suggests another possible interpretation of our results. In this structure, the first AdoMet (in the position occupied in single-AdoMet complexes) forms part of the binding site for the second AdoMet. If the K_d for the second AdoMet is substantially lower than that for the first one, then M·*Pvu*II would bind AdoMet in a strongly cooperative manner. This could explain why we were unable to distinguish between the two binding sites by isotope exchange or photochemical labeling, and why they appear to have the same K_d. One potential problem with this interpretation is that our burst kinetics indicate that the two-AdoMet complex is catalytically competent, while it is not obvious from the structure whether or not this complex could bind DNA.

AdoMet concentrations in *E. coli* are reported to be in the 300−500 μM range (Javor, 1983), which is 32−54 times the apparent K_d of 9.3 μM. We would accordingly expect the great majority of M·*Pvu*II molecules to be associated with two molecules of AdoMet *in vivo*, unless even nonspecific association with DNA affects binding of the second AdoMet molecule.

Two other DNA methyltransferases have also been shown to bind two molecules of AdoMet: M·EcoDam via kinetic and NMR studies (Bergerat & Guschlbauer, 1990; Bergerat et al., 1991), and M·Dam from bacteriophage T4 via equilibrium dialysis (Kossykh et al., 1993). Both M·EcoDam and M·T4Dam, like M·*Pvu*II, are amino methyltransferases; binding two molecules of AdoMet may be a general property of these enzymes or even of DNA methyltransferases in general.

Possible Roles for the Second Bound AdoMet Molecule. A major question raised by this work is what roles, if any, are played by the molecule of AdoMet that is not used as the methyl donor. One possibility is that this second AdoMet molecule increases the occupancy of the catalytically-relevant AdoMet-binding site. This might involve raising the local concentration of AdoMet through rapid binding and release or, based on the two-AdoMet structure described above, reducing k_{off} for the catalytically-relevant AdoMet molecule.

A second possible role for the second AdoMet molecule is suggested by the fact that M·EcoDam (Bergerat & Guschlbauer, 1990; Bergerat et al., 1991), M·*Hha*I (Klimasauskas & Roberts, 1995b), and M·*Pvu*II (preliminary data, not shown) show evidence for AdoMet affecting the degree of sequence specificity. In the case of M·EcoDam, the two AdoMet that bind were reported to play distinct roles: methyl donor and allosteric effector (Bergerat & Guschlbauer, 1990). AdoMet binds first to the higher-affinity allosteric site, causing a conformational change in M·EcoDam which increases the apparent affinity for its substrate sequence in competition with nonspecific DNA. A similar result has been reported for another methyltransferase, M·*Eca*I (Szilak et al., 1993); increasing amounts of AdoHcy progressively reduced inhibition of M·*Eca*I by a methylated DNA competitor. Some of these effects have been ascribed to a decreased k_{off} for specific sites when AdoMet or an analog is bound (Bergerat & Guschlbauer, 1990; Bergerat et al., 1991; Dubey & Roberts, 1992; Ford et al., 1993; Szczelkun & Connolly, 1995; Szilak et al., 1993).

An alternative interpretation is that AdoMet might act by increasing k_{off} at nonspecific DNA sites rather than decreasing it at substrate sites. DNA binding by methyltransferases appears to consist of at least three phases: first, nonspecific binding followed by hopping or sliding until a specific substrate sequence is found (Jeltsch et al., 1994); second, recognition of the specific substrate sequence; and third, rotation of the target nucleotide such that it projects into the catalytic pocket [base flipping (Klimasauskas et al., 1994)].

Site recognition and base flipping may or may not be concerted (Cheng & Blumenthal, 1996), but complexes between DNA methyltransferases and nonspecific DNA might be stabilized by base flipping as suggested by the greatly increased stability of M·*Hha*I−DNA complexes in which the flippable base is mispaired (Klimasauskas & Roberts, 1995a,b). We suggest that methyltransferases such as M·*Pvu*II can form dead-end complexes, having a base flipped into the target-base-binding pocket, with nonspecific DNA. If the binding pocket for the methylatable base were occupied by AdoMet (or if a second AdoMet molecule in the AdoMet-binding pocket interfered with base flipping in some way), base flipping would presumably require displacement of the AdoMet, and this could reduce the effective affinity for nonspecific sites. At a substrate DNA site, multiple recognition interactions hold the methyltransferase in position for a longer time than at a nonspecific site, so a larger number of base-flipping attempts could occur before the enzyme diffused away.

This model makes several testable predictions. First, nonspecific DNA containing a mispaired base should show increased ability to form stable complexes with M·*Pvu*II, and this ability should be reduced by high AdoMet concentrations. Second, the k_i for nonspecific DNA should rise with increasing AdoMet concentration up to the point at which AdoMet is saturating. Third, DNA (particularly if it contains a substrate sequence for M·*Pvu*II) should selectively displace one of the two AdoMet, though because of catalytic turnover this experiment would have to employ a nonreactive AdoMet analog or a noncatalytic mutant version of M·*Pvu*II.

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