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Self-methylation of *Bsp*RI DNA-methyltransferase

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ABSTRACT

The DNA (cytosine-5)-methyltransferase (m5C-MTase) M.*Bsp*RI is able to accept the methyl group from the methyl donor S-adenosyl-L-methionine (AdoMet) in the absence of DNA. Transfer of the methyl group to the enzyme is a slow reaction relative to DNA methylation. Self-methylation is dependent on the native conformation of the enzyme and is inhibited by S-adenosyl-L-homocysteine, DNA and sulfhydryl reagents. Amino acid sequencing of proteolytic peptides obtained from M.*Bsp*RI, which had been methylated with [methyl-^{3}H]AdoMet, and thin layer chromatography of the modified amino acid identified two cysteines, Cys156 and Cys181 that bind the methyl group in form of S-methylcysteine. One of the acceptor residues, Cys156 is the highly conserved cysteine which plays the role of the catalytic nucleophile of m5C-MTases.

INTRODUCTION

Prokaryotic DNA (cytosine-5)-methyltransferases (m5C-MTases) catalyze transfer of methyl group from S-adenosyl-L-methionine (AdoMet) to the C5 carbon of cytosine in specific sequences. This group of enzymes includes modification methyltransferases of several restriction modification systems as well as enzymes like Dcm of *E.coli* or some phage encoded methyltransferases which do not have an endonuclease counterpart (1). Prokaryotic m5C-MTases share a common architecture. There are 10 blocks of conserved amino acids and a variable region with limited or no homology between m5C-MTases of different recognition specificity (1). Some of the conserved sequence elements were also found in a eukaryotic m5C-MTase (2). It has been shown that sequence specificity is determined by the variable region (3,4).

m5C-MTases have been proposed to operate by the same catalytic mechanism as thymidylate synthase (5). In this model, a transient covalent bond is formed between an enzyme nucleophile and the C6 carbon of the pyrimidine ring, converting the cytosine C5 into a powerful nucleophile which then attacks the methyl group of AdoMet, resulting in the methylation of the cytosine C5 and the release of S-adenosyl-L-homocysteine (AdoHcy). The reaction ends with the release of the protein by β-elimination. This model is supported by several lines of experimental evidence, such as the analysis of the steric course of the methyl transfer reaction (6), isolation and characterization of the protein–DNA intermediate formed between the enzyme and DNA containing the mechanism based inhibitor 5-fluoro-2′-deoxycytidine (7–11) and mutagenesis of the highly conserved cysteine that initiates the nucleophilic attack (11–15). The active site cysteine is part of the invariant Pro–Cys doublet present in all m5C-MTases (1).

Much of our understanding of the enzyme–DNA and enzyme–AdoMet interaction comes from the recently solved X-ray structure of the M.*Hha*I–AdoMet complex (16) and of a trapped intermediate formed by M.*Hha*I methyltransferase, AdoHcy and the fluorinated target DNA (17). The structure illustrated a novel mode of sequence specific DNA recognition and suggested functional roles for several conserved residues. The most interesting aspect of the covalent enzyme–DNA complex was the flipping out of the target cytosine from the normal double helix structure (17). The localized strand separation is apparently needed to make the target base accessible for the chemical attack as had been hypothesized (18,19).

M.*Bsp*RI methyltransferase of the *Bsp*RI restriction–modification system recognizes GGCC and methylates the inner cytosine to yield 5-methylcytosine. The gene encoding M.*Bsp*RI has been cloned (20) and sequenced (21). The DNA sequence predicts a protein consisting of 424 amino acids. Here, we report an interesting phenomenon displayed by M.*Bsp*RI: the enzyme can accept a methyl group from the methyl donor AdoMet in the absence of DNA.

MATERIALS AND METHODS

Enzymes and chemicals

M.*Bsp*RI was purified from an *E.coli* strain carrying the *bspRIM* gene. Construction of the overproducer and enzyme purification will be described elsewhere. Enzyme preparations that were at least 95% pure as judged by one dimensional SDS polyacrylamide gel electrophoresis were used in this study. M.*Bsp*RI was stored in TDEG buffer (50 mM Tris–HCl pH 7.5, 1 mM ditiothreitol, 1 mM EDTA, 10% glycerol) at −20°C. In this buffer the enzyme can be repeatedly frozen and thawed without loss of activity.

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Trypsin (B grade) was purchased from Calbiochem, alpha chymotrypsin from L'Industrie Biologique Française, carboxypeptidase Y, S-methyl-L-cysteine and S-adenosyl-L-homocysteine from Sigma.

S-adenosyl-L-[methyl-^{3}H]methionine (3.1 TBq/mmol), S-adenosyl-L-[methyl-^{14}C]methionine (2.1 GBq/mmol), S-adenosyl-L-[carboxyl-^{14}C]methionine (1.85 GBq/mmol) were purchased from New England Nuclear. [^{14}C]-labeled AdoMet preparations were used directly, [^{3}H]-labeled AdoMet was diluted with unlabeled AdoMet (Sigma) to a specific activity required by the experiment. All other materials were analytical grade commercial products.

Methyltransferase assay

Reaction mixtures contained MTase assay buffer (50 mM Tris−HCl pH 8.0, 10 mM EDTA, 7 mM 2-mercaptoethanol), 4−15 μM [methyl-^{3}H]AdoMet, 2−5 μg lambda phage or calf thymus DNA and 0.5−5 pmol enzyme. After incubation at 37°C for 5−20 minutes, the reaction mixtures were pipetted onto 3 cm^2 DE81 (Whatman) paper disks. The disks were washed in 50 mM Na_2HPO_4 and in ethanol as described (22), then the filter-bound radioactivity was determined in a toluene-based scintillator liquid.

Self-methylation reactions contained the same buffer, 4−15 mM [methyl-^{14}C] or [methyl-^{3}H]AdoMet, M.*Bsp*RI but no DNA. Samples were incubated at 37°C for 20 minutes to several hours. AdoMet is known to be unstable at the pH of the assay buffer, therefore, if longer incubation times (more than four hours) were used, AdoMet was again added later during the incubation to compensate for the decomposition of the methyl donor. Radioactivity bound to the protein was determined by the DE81 paper disk method as described above. M.*Bsp*RI binds to DE81 paper and remains bound under the washing conditions of the methyltransferase assay. For testing the effect of sulfhydryl reagents, M.*Bsp*RI was dialyzed free of ditiothreitol and reactions were performed in 50 mM K-phosphate pH 6.8. Enzyme activity in this buffer was only slightly lower than in the standard Tris/EDTA/mercaptoethanol buffer.

To study the effect of self-methylation on the DNA methyltransferase activity, 50 μg (1000 pmol) M.*Bsp*RI was preincubated in 100 μl reaction volumes containing 1750 pmol [methyl-^{14}C]AdoMet in methyltransferase assay buffer. After 3 hours at 37°C, 5 μl (1750 pmol) of [methyl-^{14}C]AdoMet was added and the incubation was continued. Control reactions contained water instead of AdoMet. After the self-methylation reaction, the samples were dialyzed against TDEG buffer. To estimate the extent of self-methylation, the radioactivity of 15 μl aliquots of the dialyzed enzyme was measured in a dioxane-based scintillator. The DNA methyltransferase activity of the dialyzed enzyme samples was determined using [methyl-^{3}H] AdoMet.

Gel-electrophoresis and quantitation of proteins

Analytical gel electrophoresis of proteins was done in 10 or 15 % SDS−polyacrylamide gels (23). Proteins were stained with Coomassie Brilliant Blue R. For fluorographic detection of ^{3}H or ^{14}C labeled proteins, gels were soaked in Amplify solution (Amersham), then were dried and exposed to X-ray film for 3−7 days. Protein concentration of a homogenous M.*Bsp*RI preparation was determined spectrophotometrically from the absorption at 205 nm (24), then this preparation was applied to standardize the Biorad Protein Assay (25) which was used in routine measurements.

Isolation of radioactively labeled peptides

For the isolation of radioactively labeled peptides, 100 μg of M.*Bsp*RI was methylated in 100 mM Tris−HCl pH 8.0, 8 μM [methyl-^{3}H] AdoMet in 100 μl for 30 minutes at 37°C. After adding 8 μl 10 mM unlabeled AdoMet and incubation for 5 minutes at 37°C, the protein was separated from uninorporated AdoMet on a 4 ml Sephadex G50 column (in 10 mM Tris−HCl pH 8.0). The pooled excluded fractions (1.8 ml, 70 000 cpm) containing the radioactively labeled protein were concentrated by reducing the volume to 100 μl in SpeedVac, then 1 μg trypsin was added and the mixture was incubated at 37°C overnight.

For chymotrypsin digestion pCMB-treated M.*Bsp*RI was used. 100 μg M.*Bsp*RI that had been reacted with 100 μM pCMB in 50 mM potassium phosphate pH 6.8 in 100 μl for 10 minutes at 25°C, then separated from the unreacted pCMB on a Sephadex-G50 column, was incubated in 50 mM potassium phosphate pH 6.8, 8 μM [methyl-^{3}H]AdoMet at 37°C for 30 minutes. Unlabeled AdoMet was added and the protein was purified from unincorporated AdoMet as described above. The methylated protein (12 000 cpm) was digested overnight at 37°C in 100 μl containing 1 μg chymotrypsin, 100 mM potassium phosphate pH 6.8 and 10 mM $CaCl_2$.

The trypsin and chymotrypsin digests were fractionated on a 3.9×150 mm Delta-Pak (Waters) C18 reversed phase HPLC column packed with 5 μm particles (pore size: 300 Å). The column was equilibrated with 0.1 % trifluoroacetic acid. Peptides were eluted with a linear gradient of 0−70 % acetonitrile in 0.1 % trifluoroacetic acid (flow rate 1 ml/min). Absorbance was measured at 220 nm and fractions were collected manually. Radioactivity of the eluted fractions was determined by pipetting aliquots on DE81 disks and measuring radioactivity in liquid scintillation fluid without prior washing of the disks.

Thin layer chromatography

Thin layer chromatography was performed by following a procedure described earlier (26). Radioactive peptides (4000 cpm) isolated by HPLC were digested with 300 μg/ml carboxypeptidase Y in 100 mM triethanolammonium acetate pH 5.0, for 18 h at 25°C. The digest was lyophilized, then dissolved in a minimal amount of water and subjected to thin layer chromatography on silica gel plates (Merck) in butanol:acetic acid:water (4:1:1) using S-methyl-L-cysteine as standard. After staining with ninhydrin, 5×10 mm areas of the chromatographic plate were scraped off and their radioactivity was determined in liquid scintillation fluid. To detect the oxidized derivative, part of the digested sample was treated with 5 % H_2O_2 for 2 h at 25°C.

Protein sequencing

Peptide sequences were determined by Edman degradation using an Applied Biosystems 471A gas-phase protein sequencer.

RESULTS

M.*Bsp*RI can methylate itself

In the course of our studies with M.*Bsp*RI, using the filter binding assay described in Materials and Methods, we noticed that if longer incubation times and/or larger enzyme aliquots were used, an above-background radioactivity could be detected bound to

Figure 1. Methylation of M.*Bsp*RI. Fluorographic image of an SDS–polyacrylamide gel. **1.**10 μg of M.*Bsp*RI incubated with [methyl-^{3}H]AdoMet. **2.** The same sample but heated to 100°C for 5 minutes in loading buffer (23).

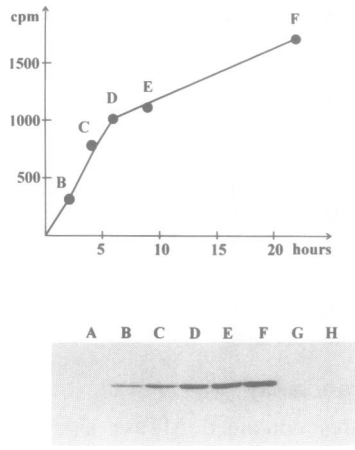

Figure 2. Time course of M.*Bsp*RI self-methylation. 10 μg (200 pmol) aliquots of M.*Bsp*RI were incubated with 700 pmol (14 μM) [methyl-^{14}C]AdoMet in 50 μl for 0 (**A**), 2 (B), 4 (**C**), 6 (**D**), 9 (**E**) and 22 (**F**) hours. 700 pmol [methyl-^{14}C]AdoMet aliquots were added at 4 h to samples **D**, **E** and **F**, then at 8 h to sample F as described in Materials and Methods. Reactions were stopped by adding 5 μl 20% SDS. Half of the samples was applied to DE81 disks to determine the incorporated radioactivity (*upper panel*), the other half was run in a 10% SDS–polyacrylamide gel and analyzed by fluorography (*lower panel*). Sample **G**: [carboxyl-^{14}C]AdoMet was substituted for [methyl-^{14}C]AdoMet. Sample **H**: 2% SDS was added at the beginning of the reaction. Samples G and H were incubated for 4 hours.

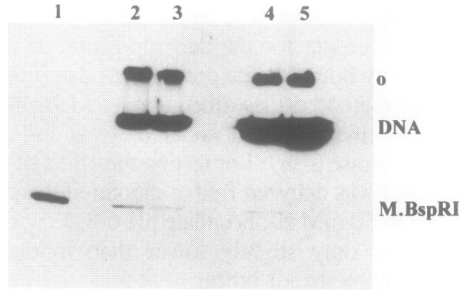

Figure 3. Effect of DNA on self-methylation of M.*Bsp*RI. Flurographic image of a 10% SDS gel. 2.5 μg (50 pmol) M.*Bsp*RI was incubated in 50 μl containing MTase assay buffer, 210 pmol [methyl-^{3}H]AdoMet and linearized pES2 or pBR322 plasmid DNA at 37°C for 1 hour. (pES2 and pBR322 cleaved with *Bam*HI and *Eco*RI, respectively was used in this experiment. The use of linearized plasmid DNA was necessary because the circular form did not enter the gel). **1.** M.*Bsp*RI, no DNA; **2.** M.*Bsp*RI and 0.4 μg pES2 DNA; **3.** M.*Bsp*RI and 4 μg pES2; **4.** M.*Bsp*RI and 1.3 μg pBR322 (20 pmol acceptor cytosines); **5.** M.*Bsp*RI and 6.5 μg pBR322 (100 pmol acceptor cytosines). 'o' marks the origin of the gel.

the DE81 paper disk (see Materials and Methods) even if DNA substrate was not present in the incubation mixture. It was known that at the pH of the assay buffer (pH 8.0) M.*Bsp*RI binds to DEAE cellulose, so it seemed likely that the radioactivity retained on the filter was bound to the methyltransferase even after the washing procedure. To test this, M.*Bsp*RI was incubated with [methyl-^{3}H or ^{14}C] labeled AdoMet and run in an SDS–polyacrylamide gel. After the run the gels were subjected to fluorography. Radioactivity was detected as a single band corresponding to the *Bsp*RI methyltransferase (Fig.1.) No loss of radioactivity in the protein band was observed if the sample was heated for 5 minutes at 100°C in loading buffer containing 2% SDS and 0.7M 2-mercaptoethanol, indicating that the radioactive label was bound by a covalent bond (Fig. 1.). If carboxyl-labeled AdoMet was used instead of methyl-labeled AdoMet in the incubation mixture, no radioactivity was detected in the methyltransferase (Fig. 2;G) showing that only the methyl group is transferred to the protein. 2% SDS abolished self-methylation (Fig. 2.; H).

The AdoMet analog AdoHcy which is a competitive inhibitor of M.*Bsp*RI-catalyzed DNA methylation, has also been found to inhibit self-methylation. At an AdoMet concentration of 14 μM, 2 μM AdoHcy inhibited DNA methylation as well as self-methylation by approximately 50 % (not shown).

To follow the time course of self-methylation, aliquots of M.*Bsp*RI were incubated with [methyl-^{14}C]AdoMet for different lengths of time and were analyzed by the filter binding assay and SDS–polyacrylamide gel electrophoresis followed by fluorography. Fig. 2 shows that methyl incorporation increased linearly for 4–6 hours, then it slowed down, but methyl incorporation proceeded even after 20 hours. We assume that slowing-down of self-methylation was mainly due to the accumulation of S-adenosylhomocysteine which competes with AdoMet for binding to the enzyme. Assuming a 50 % efficiency in scintillation counting, in 22 hours approximately 30 pmol methyl was incoporated in 100 pmol M.*Bsp*RI.

To test the effect of DNA on self-methylation, M.*Bsp*RI was incubated with [methyl-^{3}H]AdoMet in the presence of pES2 or pBR322 plasmid DNA. pES2 carries the *bsprIM* gene and its M.*Bsp*RI recognition sites are methylated. Both the modified and unmodified DNA suppressed self-methylation (Fig. 3.). In the samples containing DNA, a reduction of AdoMet concentration as a consequence of rapid DNA methylation may have contributed to weaker labeling of the protein, however, the AdoMet and DNA concentrations were such (see legend of Fig. 3.) that, with the exception of sample 5, this effect could only be negligible. Hence, the substantial reduction of self-methylation was caused by the direct inhibitory effect of DNA rather than by AdoMet depletion. Methyl incorporation in pES2 DNA (samples 2 and 3) may be due to incomplete *in vivo* modification of the plasmid DNA and/or to methylation of noncognate sites.

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M A I K I N E K G R G K F K P A P T Y E K E E V R Q L L M E K I N E E M E A V A T A T S D I S N D E  50
I Q Y K S D K F N V L S L F C G A G G L D L G F E L A G L E Q S L G T D K A L E A F K D R D V Y N A 100
I R H E S V F H T V Y A N D I F S E A L Q T Y E K N M P N H V F I H E K D I R K I K E F P S A N L↓V 150
I G G F P C P G F↓S E A G P R L V D D E R N F L Y I H F I R↓C L M Q V Q P E I F V A E N V K G M M T 200
L G G G E V F R Q I V E D F G A A G Y R V E A R L L N A R D Y G V P Q I R E R V I I V G V R N D I D 250
F N Y E Y P E I T H G N E E G L K P Y V T L E E A I G D L S L D P G P Y F T G S Y S T I F M S R N R 300
K K K W T D Q S F T I Q A S G R Q A P I H P G G L P M E K V D K N K W I F P D G E E N H R R L S V K 350
E I K R I Q T F P D W Y E F S D G G N M K V S V N N R L D K Q Y K Q I G N A V P V F L T R A V A K S 400
I A Q F A A D Y L K D N H P H E A P Q M K L F I
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Figure 4. Amino acid sequence of M.*Bsp*RI as derived from the DNA sequence of the *bspRIM* gene (21, accession number X15758). It is a revised version of the published sequence, it contains a Leu to Pro change at position 322. The three cysteines are printed in underlined italic and the most conserved residues (1) in bold. Double arrow marks the position of the trypsin cleavage site which created the amino terminus of peptides A and B. Single arrows indicate the positions of chymotrypsin cuts that produced peptide D.

M.*Bsp*RI contains three cysteines at positions 65, 156 and 181 (Figure 4). Cys156 is part of the conserved Pro-Cys doublet which is the catalytic center of m5C-MTases. Sulfhydryl reagents like p-chloromercuribenzoate (pCMB) and N-ethylmaleimide were known to inhibit DNA methylation by M.*Bsp*RI (unpublished). We tested how self-methylation was affected by pCMB. M.*Bsp*RI that had been treated with 10 μM pCMB at 25°C, retained approximately half of the self-methylating capacity. When the pCMB treatment was carried out at 37°C, self-methylation was almost completely abolished (not shown).

Identification of the radioactively labeled peptides

To localize the labeled amino acid, M.*Bsp*RI was methylated with [methyl-^{3}H]AdoMet, then the labeled protein was digested with trypsin and the digest was fractionated on a reversed phase HPLC column as described in Materials and Methods. Three radioactive peaks, each containing roughly the same amount of radioactivity, were detected. Peak A eluted at 15.6 ml (18.3 % acetonitrile), peak B at 38 ml (26.6 % acetonitrile), and peak C eluted in a broad front at the end of the gradient (Figure 5). Peaks A and B were rechromatographed on the same column (Figure 5) and then were subjected to amino acid sequencing. The peptide fragment in peak A (16 000 cpm) was sequenced for six cycles. The product appearing in the first cycle did not correspond to any of the PTH-amino acid standards (it eluted between Trp and Phe). The next five cycles yielded the sequence LMQVQ. This uniquely corresponds to the region between positions 182 and 186, thus the residue that could not be identified in the first cycle must be Cys181. After the sixth cycle, the filter containing the rest of the peptide was removed from the sequencer and its radioactivity was determined. 300 cpm were detected, thus most of the originally applied radioactivity was lost in the first six cycles.

Sequencing of peptide B gave an identical sequence, indicating that peptides A and B overlap and their amino termini result from the trypsin cleavage after Arg180 (Figure 4).

The third radioactive product (peak C) detected by HPLC of the tryptic digest could not be isolated as a sharp peak, therefore it was not analyzed by amino acid sequencing. Chymotrypsin digestion and rechromatography of the material recovered in peak C resulted in a single sharp radioactive peak (peak D) eluting at 19.4 ml (22.6 % acetonitrile). As mentioned before, preincubation with pCMB at 25°C was found to reduce self-

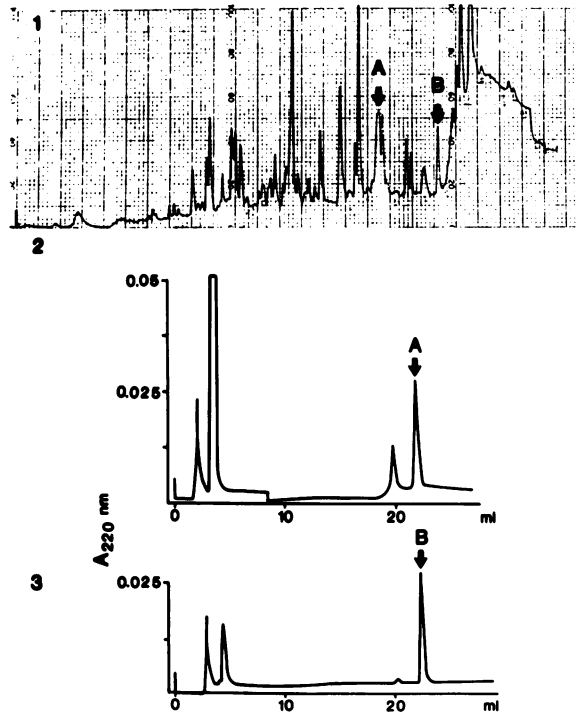

Figure 5. 1. Reversed phase HPLC chromatography profile of a tryptic digest obtained from M.*Bsp*RI self-methylated with [methyl-^{3}H]AdoMet. A and B mark the position of peptides A and B. **2.** Rechromatography profile of peptide A. **3.** Rechromatography profile of peptide B.

methylation by around 50 %. We tested how pCMB treatment changed the distribution of radioactivity in the peptide pattern. When M.*Bsp*RI that had been treated with pCMB at 25°C and subsequently labeled with [methyl-^{3}H]AdoMet, was digested with chymotrypsin, a single radioactive peak was detected by HPLC. This peak eluted at the same position as peak D, thus we concluded that the two fragments were identical. To analyze the structure of peptide D, 100 μg of pCMB treated M.*Bsp*RI was labeled with [methyl-^{3}H]AdoMet as described in Materials and Methods. Peptide D was purified by HPLC and an aliquot containing 6000 cpm was subjected to amino acid sequencing.

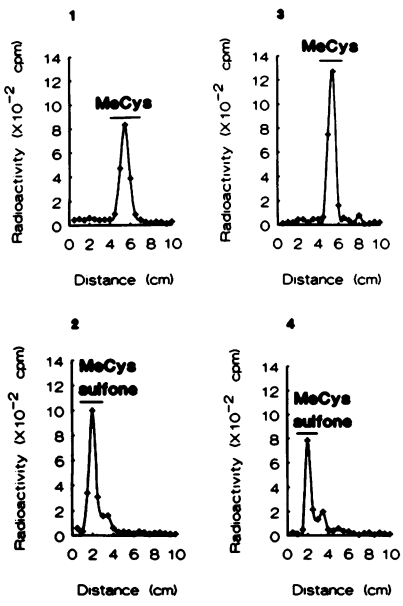

Figure 6. Thin layer chromatographic analysis of the modified amino acid in the proteolytic hydrolysate of M.*Bsp*RI labeled with [methyl-^{3}H]AdoMet. Aliquots (2000 cpm) of hydrolysates obtained from purified peptides were run on silica gel plates. **1** and **2**: peptide A, **3** and **4**: peptide D. *Upper panels*: before oxidation, *lower panels*: after oxidation with 5% H_2O_2. Horizontal bars indicate the position of the marker compounds. Similar results were obtained with peptide B (not shown).

Six cycles yielded the sequence VIGGXP (X, unidentified amino acid). The effluents of the next two cycles were collected manually and their radioactivity was determined. The effluents of cycle 7 and 8 contained 4200 cpm and 300 cpm, respectively. From cycle 9, sequencing of peptide D was continued in the standard arrangement. Cycle 9 yielded G and cycle 10 an amino acid that could not be identified. Cycles 11 and 12 did not give signal indicating that the peptide was sequenced in entirety. After the tenth cycle we determined the amount of radioactivity that remained attached to the membrane on which the sequenced peptide was loaded: only 300 cpm could be detected. Results with peptide C and D can be interpreted that peptide D was an internal peptide of peptide C and resulted from the chymotrypsin cleavage after Leu149 and Phe159. Elution in cycle 7 of the majority of radioactivity that was associated with peptide D identifies Cys156 as the modified amino acid.

Identification of the modified amino acid

Radioactively labeled peptides A, B and D purified by HPLC were digested by carboxypeptidase Y and the hydrolysate was analyzed by thin layer chromatography on silica gel plates. In all three experiments practically all of the recovered radioactivity comigrated with the S-methylcysteine standard. After oxidation with H_2O_2, the radioactivity comigrated with S-methylcysteine sulfone (Figure 6). We conclude that the labeled amino acid in all three peptides was S-methylcysteine.

Effect of self-methylation on the enzyme activity

As the cysteine of the conserved ProCys doublet is the active site nucleophile of m5C-MTases, methylation of the Cys156 of M.*Bsp*RI is expected to abolish the enzyme activity. It is not known whether the Cys181 thiol has any function in M.*Bsp*RI. To test the effect of self-methylation on the enzyme activity, we incubated M.*Bsp*RI with [methyl-^{14}C]AdoMet. After the reaction, the unreacted AdoMet was removed by dialysis and the DNA methylation activity of the dialyzed enzyme was determined. Table 1 shows that self-methylation was accompanied by a gradual loss of activity, suggesting that methylation leads to enzyme inactivation. A similar loss of activity was observed if unlabeled AdoMet was used for preincubation (not shown).

Table 1. Effect of self-methylation on the activity of M.*Bsp*RI. M.*Bsp*RI was preincubated with or without [methyl-^{14}C]AdoMet

Preincubation with	-			[methyl-^{14}C]AdoMet		
time (hours)	4	6	8	4	6	8
[^{14}C]cpm in 65 pmol M.*Bsp*RI				594	842	969
(pmol methyl)				(9.4)	(13.3)	(15.3)
[^{3}H]cpm in DNA	5736	3686	3838	3705	3156	2948

After dialysis, the DNA methyltransferase activity was determined using [methyl-^{3}H]AdoMet. The amount of incorporated [^{14}C]-methyl is shown in brackets. Enzyme and methyl picomoles are given on the assumption that no loss and dilution of the samples occured during dialysis.

DISCUSSION

We have found that the DNA methyltransferase M.*Bsp*RI can methylate itself utilizing its normal cofactor, AdoMet as methyl donor. AdoMet is a weak alkylating agent and known to methylate DNA in a nonenzymatic reaction (27). Our results suggest that methylation of M.*Bsp*RI is an enzymatic process, not a simple chemical alkylation. This is supported by data showing that both DNA methylation and self-methylation was inhibited by AdoHcy and that self-methylation required native enzyme conformation. *Bsp*RI self-methylation is a slow reaction.

We are aware of two papers reporting methylation of a DNA methyltransferase (10,28). Methyl transfer to Dcm, an m5C-MTase, was shown to be dependent on Cys177, which is the active site nucleophile of the enzyme (10). Enzyme methylation by AdoMet has also been demonstrated with M.*Eco*P1, a type III adenine methyltransferase (28). The modified amino acid has, however, in neither case been identified (10,28). The observation of self-methylation in two classes of DNA methyltransferases is interesting because m5C- and adenine methyltransferases share very little sequence homology (29) and have different catalytic mechanism (30).

In a broader context of self-methylation, O^6-methylguanine methyltransferases can be mentioned whose function is to transfer a methyl group from the mutagenic O^6-methylguanine or DNA methyl phosphotriesters to two of their own cysteines. In the reaction the acceptor cysteines are irreversibly modified to S-methylcysteine. O^6-methylguanine methyltransferases act stochiometrically as methylation renders them inactive. Both acceptor cysteines are located in a ProCys motif like the active site cysteine of m5C-MTases (31–33).

We have identified two cysteines of M.*Bsp*RI that played the role of the methyl acceptor. This assignment was based on amino acid sequence analysis of purified peptides and on thin layer chromatographic analysis of the modified amino acid. The modified amino acid was identified in both cases as S-methylcysteine. One of the two cysteines, Cys156, is part of the invariant ProCys doublet conserved in m5C-MTases, O^6-methylguanine methyltransferases and thymidylate synthase (1,5). The other modified cysteine was Cys181 which is not conserved. Even M.*Bsu*RI, which shares a very high degree of sequence homology with M.*Bsp*RI, has serine at the corresponding position (34). Cys181 is preceded by arginine in the M.*Bsp*RI sequence. The reason for the high conservation of proline on the immediate N-terminal side of the active cysteine is not fully understood (19). Methylation of Cys181 shows that a preceding proline is not always essential for a cysteine to act as a catalytic nucleophile. In this context it may deserve attention that, although it is unknown which amino acid in M.*Eco*P1 acts as methyl acceptor, none of the eight cysteines of the enzyme is preceded by a proline (35).

The ability of M.*Bsp*RI to transfer the methyl from AdoMet to two of its own cysteines suggest that in the enzyme–AdoMet complex the reactive cysteine thiols must be in close proximity of the donor methyl. Authors who investigated the UV-induced transmethylation of the active site Cys186 of M.*Eco*RII, reached a similar conclusion about the likely position of the Cys186 thiol (26). On the other hand, in the X-ray structure of the M.*Hha*I–AdoMet cocrystal, the donor methyl group of AdoMet is nearly 10 Å away from the sulphur atom of Cys81, the active site cysteine of M.*Hha*I, making a direct interaction between the two functional groups rather unlikely (17). It awaits further studies to determine whether self-methylation is a widespread property of DNA methyltransferases or it is just a peculiar side activity of some enzymes. In any case, the functional significance of this activity is unclear at the moment. It is possible that methyl transfer to the enzyme is an 'idle reaction', that takes place only if the enzyme is not bound to DNA. During DNA methylation, in the ternary complex, the target cytosine, the catalytic nucleophile and the donor methyl are held closely for methyl transfer to occur (17). In the absence of DNA, the binary complex may have greater flexibility which could occasionally result in a molecular accident: methyl transfer to the nearby reactive sulfhydryls.

We do not know whether self-methylation takes place *in vivo* and if it does, what functional significance, if any, it has in the cell. An important aspect of this reaction is that methylation of the active site cysteine inactivates the enzyme. Our *in vitro* data showed that if DNA was present, methyl transfer to the enzyme was very low, nevertheless it was detectable. We assume that in the cell, with DNA at a high concentration, most methyltransferase molecules are bound to DNA which prevents significant self-methylation.

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REFERENCES

1. Kumar, S., Cheng, X., Klimasauskas, S., Mi, S., Psfai, J., Roberts, R.J. and Wilson, G.G. (1994) Nucl. Acids Res. **22,** 1–10.
2. Bestor, T.H., Laudano, A., Mattaliano, R. and Ingram, V. (1988) J. Mol. Biol. **203,** 971–983.
3. Balganesh, T.S., Reiners, L., Lauster, R., Noyer-Weidner, M., Wilke, K. and Trautner, T.A. (1987) EMBO J. **6,** 3543–3549.
4. Klimasauskas, S., Nelson, J.L. and Roberts, R.J. (1991) Nucl. Acids Res. **19,** 6183–6190.
5. Wu, J.C. and Santi, D.V. (1987) J. Biol. Chem. **262,** 4778–4786.
6. Ho, D. K., Wu, J. C., Santi, D. V. and Floss, H. G. (1991) Arch. Biochem. Biophys. **284,** 264–269.
7. Chen, L., MacMillan, A.M., Chang, W., Ezaz-Nikpay, K., Lane, W.S. and Verdine, G.L. (1991) Biochemistry **30,** 11018–11025.
8. Smith, S.S., Kaplan, B.E., Sowers, L.C. and Newman, E.M. (1992) Proc. Natl. Acad. Sci. USA **89,** 4744–4748.
9. Friedman, S. and Ansari, N. (1992) Nucl. Acids Res. **20,** 3241–3248.
10. Hanck, T., Schmidt, S. and Fritz, H.-J. (1993) Nucl. Acids Res. **21,** 303–309.
11. Wyszynski, M.W., Gabbara, S. Kubareva, E.A., Romanova, E.A., Oretskaya, T.S., Gromova, E.S., Shabarova, Z.A. and Bhagwat, A.S. (1993) Nucl. Acids Res. **21,** 295–301.
12. Wilke, K., Rauhut, E., Noyer-Weidner, M., Lauster, R., Pawlek, B., Behrens, B. and Trautner, T.A. (1988) EMBO J. **7,** 2601–2609.
13. Wyszynski, M.W., Gabbara, S. and Bhagwat, A.S. (1992) Nucl. Acids Res. **20,** 319–326.
14. Mi, S. and Roberts, R.J. (1993) Nucl. Acids Res. **21,** 2459–2464.
15. Chen, L., MacMillan, A.M. and Verdine, G.L. (1993) J. Am. Chem. Soc. **115,** 5318–5319.
16. Cheng, X., Kumar, S., Pósfai, J., Pflugrath, J.W. and Roberts, R.J. (1993) Cell **74,** 299–307.
17. Klimasauskas, S., Kumar, S., Roberts, R.J. and Cheng, X. (1994) Cell **76,** 357–369.
18. Erlanson, D.A., Chen, L. and Verdine, G.L. (1993) J. Am. Chem. Soc. **115,** 12583–12584.
19. Verdine,G.L. (1994) Cell **76,** 197–200
20. Szomolányi, É., Kiss, A. and Venetianer, P. (1980) Gene **10,** 219–225.
21. Pósfai, G., Kiss, A., Erdei, S., Pósfai, J. and Venetianer, P. (1983) J. Mol. Biol. **170,** 597–610.
22. Greene, P.J., Poonian, M.S., Nussbaum, A.L., Tobias, L., Garfin, D.E., Boyer, H.W. and Goodman, H.M. (1975) J. Mol. Biol. **99,** 237–261.
23. Laemmli, U. K. (1970) Nature 227, 680–685.
24. Scopes, R.K. (1974) Anal. Biochem. **59, 277**–282.
25. Bradford, M. (1976) Anal. Biochem **72,** 248–254.
26. Som, S. and Friedman, S. (1991) J. Biol. Chem. **266,** 2937–2945.
27. Rydberg, B. and Lindahl, T. (1982) EMBO J. **1,** 211–216.
28. Hornby, D.P., Müller, M. and Bickle, T.A. (1987) Gene **54,** 239–245.
29. Klimasauskas, S., Timinskas, A., Menkevicius, S., Butkiene, D., Butkus, V. and Janulaitis, A.A. (1989) Nucl. Acids Res. **17,** 9823–9832.
30. Pogolotti, A.L., Ono, A., Subramaniam, R. and Santi, D.V. (1988) J. Biol. Chem. **263,** 7461–7464.
31. Demple, B., Sedgwick, B., Robins, P., Totty, N., Waterfield, M.D. and Lindahl, T. (1985) Proc. Natl. Acad. Sci. USA **82,** 2688–2692.
32. Myers, L.C., Verdine, G.L. and Wagner, G. (1993) Biochemistry, **32,** 14089–14094.
33. Moore, M.H., Gulbis, J.M., Dodson, E.J., Demple, B. and Moody, P.C. (1994) EMBO J. **13,** 1495–1501.
34. Kiss, A., Pósfai, G., Keller, C.C., Venetianer, P. and Roberts, R.J. (1985) Nucl. Acids Res. **13,** 6403–6421.
35. Hümbelin, M., Suri, B., Rao, D.N., Hornby, D.P., Eberle, H., Pripfl, T., Kenel, S. and Bickle, T.A. (1988) J. Mol. Biol. **200,** 23–29.