Purification, Crystallization, and Preliminary X-ray Diffraction Analysis of an M.*Hha*I–AdoMet Complex†

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Received May 29, 1992; Revised Manuscript Received June 30, 1992

ABSTRACT: The type-II DNA-(cytosine-5)-methyltransferase M.*Hha*I was overexpressed in *Escherichia coli* and purified to apparent homogeneity. The purification scheme exploits a unique high salt back-extraction step to solubilize M.*Hha*I selectively, followed by FPLC chromatography. The yield of purified protein was 0.75–1.0 mg per gram of bacterial paste. M.*Hha*I could be isolated in two forms: bound with its cofactor *S*-adenosylmethionine (AdoMet) or devoid of the cofactor. The AdoMet-bound form was capable of methylating DNA in vitro in the absence of exogenous AdoMet. From kinetic studies of the purified enzyme, values for K_m^{AdoMet} (60 nM), K_i^{AdoHyc} (0.4 nM), and K_{cat} (0.22 s^{-1}) were determined. The purified enzyme bound with its cofactor was crystallized by the hanging drop vapor diffusion technique. Crystals were of monoclinic space group $P2_1$ and had unit-cell dimensions of a = 55.3 Å, b = 72.7 Å, c = 91.0 Å, and β = 102.5°, with two molecules of M.*Hha*I in each of the two asymmetric units. The crystals diffract beyond 2.5 Å and are suitable for structure determination.

DNA-(cytosine-5)-methyltransferases (m5C-MTases)[1] catalyze the *S*-adenosylmethionine (AdoMet) dependent methylation of cytosine to 5-methylcytosine in specific DNA sequences. Several m5C-MTases have been purified and studied extensively: M.*Bam*HI (Nardone et al., 1986), M.*Bsu*RI (Gunthert et al., 1981a,b), M.*Eco*RII (Friedman, 1985), M.*Hae*III (Chen et al., 1991), M.*Msp*I (Dubey et al., 1992), *Ngo*AI (Piekarowicz et al., 1988), and M.*Hha*I (Wu & Santi, 1985, 1987, 1988). Comparative sequence analysis has shown that the prokaryotic m5C-MTases share five highly conserved and five weakly similar motifs (Posfai et al., 1989; Lauster et al., 1989). Many of these motifs are also present in the C-terminal domain of the mouse m5C-MTase (Bestor et al., 1988). These sequence similarities suggest a common architecture for the m5C-MTases; however, no molecular structures have been determined for any member of this group of proteins. The absence of known DNA binding motifs, as well as a lack of homology with their counterpart restriction endonucleases, suggests that a novel method may be used by m5C-MTases for interaction with their target DNA sequences.

M.*Hha*I from the bacterium *Haemophilus haemolyticus* (Roberts et al., 1976) is one of the smallest (37 kDa) members of the m5C-MTase family and methylates the internal C in the target sequence 5′-GCGC-3′ (Mann & Smith, 1979). The gene encoding M.*Hha*I has been cloned and sequenced (Barsomian et al., 1988; Caserta et al., 1987) and the protein product purified (Wu & Santi, 1988). M.*Hha*I is a type-II DNA methyltransferase. The general properties of these proteins have been reviewed previously (Razin et al., 1984; Adams & Burdon, 1985). As with other type-II DNA methyltransferases, M.*Hha*I is encoded as a single polypeptide and requires no cofactors other than the methyl donor AdoMet for enzymatic activity. Although their target DNA sequences usually display 2-fold symmetry, type-II methylases appear to interact with DNA as monomers, transferring only one methyl group per independent methylation event [reviewed in Adams and Burdon (1985)].

The transfer of methyl groups from AdoMet to C5 of cytosine by m5C-MTases is thought to be mechanistically similar to the reaction catalyzed by thymidylate synthase, involving a transient covalent protein–DNA intermediate between a Cys residue in the enzyme and the C6-position of cytosine (Santi et al., 1983; Wu & Santi, 1987). In agreement with this proposed mechanism, DNA containing 5-azacytosine or 5-fluorocytosine (both potent inhibitors of m5C-MTases) can form a covalent complex with m5C-MTases in the presence of AdoMet (Friedman, 1985; Osterman et al., 1988). Recently, the active site catalytic nucleophile in the m5C-MTase M.*Hae*III was identified as Cys_{71} (Chen et al., 1991). This Cys is part of a Pro-Cys (PC) motif that is absolutely conserved in m5C-MTases (Posfai et al., 1989; Lauster et al., 1989) and also found in the catalytic site of thymidylate synthase (Mathews et al., 1990a,b). Mutagenesis of the conserved Cys residue in M.*Eco*RII leads to a loss of methylating activity (Wyszynski et al., 1992). AdoMet can be photo-cross-linked in the vicinity of the PC motif in M.*Eco*RII (Som & Friedman, 1991).

In this paper, we describe a new method for high-yield purification of M.*Hha*I from an *E. coli* overexpression strain and report conditions for crystallization of an AdoMet–M.*Hha*I complex. We also present preliminary data from X-ray diffraction studies of the native crystals.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. M.*Hha*I overexpression was carried out in *E. coli* strain ER1727 [Δ(*mcr*BC-*hsd*RMS-*mrr*)2::Tn10, *mcr*A1272::Tn10,F′ *lac pro*AB *lac*I^q Δ(*lac*Z)-M15] (kindly provided by E. Raleigh). The IPTG-inducible expression vector pHSH0-1 has been described previously as construct H0 (Klimasauskas et al., 1991) and carries an I2L

† This work was supported by grants from the NIH (GM 46127) and NSF (DMB-8917650). Centralized facility support for oligonucleotide synthesis was from a Cancer Center Support grant from the NCI (CA 45508).

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[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; BSA, bovine serum albumin; DE81, diethylaminoethyl ion-exchange filters; EDTA, ethylenediaminetetraacetate; IPTG, isopropyl β-D-thiogalactopyranoside; m5C-MTase, DNA (cytosine-5)-methyltransferase; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; SDS–PAGE, SDS–polyacrylamide gel electrophoresis.

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mutant of M.*Hha*I which differs from wild type M.*Hha*I by the substitution of Leu for Ile at position 2. The original clone containing the gene for M.*Hha*I was obtained from Dr. G. Wilson (Caserta et al., 1987). Cultures of ER1727 containing pHSH0-1 were grown in LB medium supplemented with ampicillin (150 μg/mL) at 37 °C until the absorbance at 600 nm was approximately 1.0. At this point, the cells were induced with 0.15 mM IPTG. After 2 h, the cells were harvested by centrifugation and either used immediately or frozen as a cell paste at −80 °C.

Enzymes. All restriction enzymes and methylases were obtained from New England Biolabs and used under the conditions suggested by the manufacturer.

Purification. Unless noted, all procedures were carried out at 4 °C. Ten grams of cell paste was resuspended in 10 mM HEPES (pH 7.0), 5 mM Na_2EDTA, 10% glycerol, and 0.1% β-mercaptoethanol to a final volume of 40 mL. Cells were disrupted by two passes through a French press at 1200 psi. This suspension was centrifuged at 20000*g* for 20 min. The supernatant (S1) was discarded. The pellet was vigorously back-extracted twice with 20 mL of high-salt buffer [10 mM $KHPO_4$ (pH 7.4), 5 mM Na_2EDTA, 10% (v/v) glycerol, 0.1% (v/v) β-mercaptoethanol, 400 mM NaCl]. The high-salt suspensions were centrifuged at 20000*g* and the supernatants (S2) pooled. Nucleic acids were precipitated from the S2 supernatant by the slow addition of one-half volume of protamine sulfate (10 mg/mL) in high-salt buffer. After incubation at room temperature for 5 min, the precipitate was removed by centrifugation at 20000*g* for 40 min. The cleared S2 supernatant was concentrated approximately 8-fold using Amicon Centriprep-10 ultrafiltration units and then slowly diluted with low-salt buffer [10 mM $KHPO_4$ (pH 7.4), 5 mM Na_2EDTA, 50 mM NaCl, 10% (v/v) glycerol, 0.1% (v/v) β-mercaptoethanol] until its conductivity was equivalent to that of low-salt buffer containing 100 mM NaCl. Precipitated material was removed by passage through a 0.22-μm Millex-GVS filter (Millipore). Just prior to separation by FPLC (Pharmacia), the diluted sample was adjusted to 100 μM AdoMet.

FPLC Chromatography. Diluted sample was applied to a Pharmacia MONO-S FPLC column (cation exchanger) equilibrated with 100 mM NaCl in buffer A [10 mM $KHPO_4$ (pH 7.4), 5 mM Na_2EDTA, 10% (v/v) glycerol, 0.1% (v/v) β-mercaptoethanol]. For preparative chromatography, an 8 mL bed volume HR10/10 column was used with flow rates between 1 and 2 mL/min. For small-scale analytical fractionation, a 1 mL bed volume HR5/5 column was used with a flow rate of 0.5 mL/min. The volumes listed below are for preparative fractionation. After washing with 40 mL of equilibration buffer, proteins were eluted with 120 mL of a linear gradient of NaCl (100–350 mM) in buffer A. Column fractions were assayed for protein content and methylase activity. M.*Hha*I eluted at approximately 200 mM NaCl. If the preparation was not sufficiently pure, an additional FPLC purification step was carried out. Fractions containing M.*Hha*I were pooled, diluted with buffer A until their conductivity was equivalent to that of 100 mM NaCl, and applied to a Pharmacia MONO-Q (anion exchange) column (HR5/5). The column flow-through containing M.*Hha*I was collected. Final fractions containing M.*Hha*I were pooled, concentrated to 10 mg/mL or greater using Amicon Centricon-30 ultrafiltration units, and stored at 4 °C. Protease inhibitor PMSF (phenylmethanesulfonyl fluoride, Sigma) was added to 100 μM prior to storage.

Methylation Assays. M.*Hha*I methylase activity was assayed in two ways. For qualitative analysis, the sensitivity of substrate DNA to cleavage by the cognate restriction enzyme (R.*Hha*I) was monitored after in vitro methylation. M.*Hha*I samples were incubated at 37 °C in 20 μL of TM buffer [10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 100 μg/mL BSA] (NEBuffer 4) with 0.4 μg of *Bst*EII–cleaved λ DNA (New England Biolabs) and 50 μM AdoMet (Sigma). After 15 min, the reaction was halted by heat inactivation at 70 °C for 5 min. R.*Hha*I (5 units) was then added to the reaction mixture, and incubation continued at 37 °C for 1 h. Samples were electrophoresed in 1% agarose gels in TBE buffer [90 mM Tris-borate (pH 8.3), 1 mM Na_2EDTA], and the DNA was visualized by ethidium bromide staining and UV illumination.

Quantitative analysis of methyltransferase activity was carried out by measuring the incorporation of ^3H-labeled methyl groups into substrate DNA. The reactions were performed in 600 μL of TM buffer containing 20 μg/mL *Bst*EII–cleaved λ DNA and 0.9–8.0 μM [^3H]AdoMet (New England Nuclear, 15 Ci/mmol). The enzyme concentration was approximately 100 pM for most reactions. Ninety-microliter aliquots were removed at timed intervals and pipetted onto Whatman DE81 filters in a vacuum manifold. The filters were washed sequentially with 0.2 M NH_4HCO_3 (5 × 1 mL, 4 °C), H_2O (4 × 1 mL), and 100% ethanol (3 × 1 mL). After drying, the filters were placed in 10 mL of scintillation fluid (Cytoscint ES, ICN) and incorporation of ^3H into the trapped DNA was measured by scintillation counting.

Measurement of Kinetic Constants. Data from the methylation assays was plotted and analyzed as described in Wu and Santi (1987) for determination of K_i^{AdoHcy}. K_m and K_{cat} were derived graphically by direct linear plotting (Eisenthal & Cornish-Bowden, 1974) of initial velocity measurements under reaction conditions where the concentration of AdoHcy was less than 1 nM. Median estimates of K_m and V_{max} were used as suggested by Cornish-Bowden and Eisenthal (1974).

Protein Analysis. Protein concentration was determined using the Biorad Coomassie-plus assay kit. Standard curves were established using BSA. Electrophoretic analysis of protein samples was carried out in 12% SDS–polyacrylamide gels using Laemmli buffer. Proteins were visualized by staining with Coomassie brilliant blue. N-Terminal sequencing was performed by automated Edman degradation on an Applied Biosystems 475A protein sequencer at the Cold Spring Harbor Protein Sequencing Facility.

Crystallization. Crystallization trials were performed with concentrated purified M.*Hha*I (10 mg/mL or greater) in the FPLC column elution buffer (see above) supplemented with 50 μM AdoMet. A sparse matrix screening method (Jancarik & Kim, 1991) was used to search for initial conditions for crystallization by the hanging drop vapor diffusion technique (McPherson, 1982) using silanized microscope cover slips inverted over 1 mL of precipitant solution in 24-well tissue culture plates (ICN). After needle-like crystals were first observed, the concentrations of precipitant, salt, pH, and temperature were carefully refined to optimize growth of large single crystals. For data collection, single crystals were mounted in 0.7- or 1.0-mm glass capillary tubes (Charles Supper Company) between plugs of mother liquor and sealed with dental wax and Duco cement at both ends.

X-ray Diffraction. X-ray diffraction data were collected on a FAST television area detector (Enraf-Nonius, Delft, The Netherlands) at beam line X12-C at the National Synchrotron

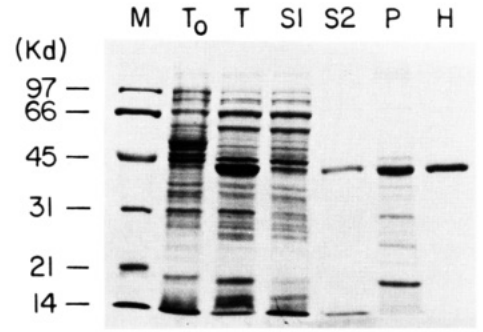

FIGURE 1: SDS–PAGE analysis of aliquots from successive stages of M.*Hha*I purification. Proteins were visualized by staining with Coomassie brilliant blue. Sample volumes were adjusted to allow direct comparison of protein concentration at each purification step, with the exception of the pellet lane, which is twice as concentrated. M: molecular weight markers. T_0: preinduction lysate. T: total lysate after induction. S1: soluble protein of first supernatant. S2: high-salt-extracted protein (second supernatant). P: insoluble pellet after high-salt extraction. H: FPLC-purified M.*Hha*I.

Light Source, Brookhaven National Laboratory, and evaluated by the program MADNES (Messerschmidt & Pflugrath, 1987) followed by profile fitting (Kabsch, 1988). The following parameters were used for data collection: the wavelength was 1.10 Å; the collimator aperture was 0.2 mm; the rotational increments were 0.1°; the exposure times were 5 or 10 s; the specimen-to-detector distance was 120 mm; the detector swing angle was 12–19°; the detector was run at a gain setting of SETD 7 1. The unit cell parameters were refined by the program MADNES during data reduction. The data sets were divided into sectors containing roughly 1000–2000 reflections each for scaling. The programs FS and PROTEIN were used to scale the data (Weissman, 1982; Steigemann, 1974).

RESULTS AND DISCUSSION

Purification of M.HhaI. M.*Hha*I (M_r 37 kDa) was purified from *E. coli* ER1727 cells carrying an IPTG-inducible overexpression vector (pHSH0-1) constructed in our laboratory (Klimasauskas et al., 1991). The recombinant M.*Hha*I encoded by this plasmid differs from the wild type enzyme only at position 2, where an Ile residue has been substituted by Leu. After induction, the recombinant M.*Hha*I product accounts for an estimated 10% of the total protein in the cell (Figure 1, lane T). An unusual property of the enzyme was exploited to simplify the purification and enhance the yield of M.*Hha*I. After induction and cell lysis, the majority of the enzyme (>70%) remained associated with the disrupted cellular debris. Back-extraction of this pelleted material with high-salt (0.4 M NaCl) buffers selectively solubilized M.*Hha*I. The resulting soluble fraction (termed S2) was greatly enriched for M.*Hha*I, which made up approximately 80% of the fraction (Figure 1, lane S2). Nucleic acids were removed by protamine sulfate precipitation, and the cleared S2 supernatant was fractionated by ion-exchange chromatography on a MONO-S (cation exchange) FPLC column (see Materials and Methods). This single-column procedure yielded 0.75–1.0 mg of M.*Hha*I per gram of bacterial cell paste, with 98% purity or better as judged from Coomassie stained gels (Figure 1, lane H). Remaining contaminants could be removed by an additional ion-exchange-chromatography step using a MONO-Q (anion exchange) FPLC column. Under the conditions used, M.*Hha*I could be isolated from the MONO-Q column flow-through.

SDS–PAGE analysis of the purified recombinant M.*Hha*I showed that it migrated as a single band of apparent molecular weight 37 kDa (Figure 1, lane H). N-Terminal sequencing by automated Edman degradation indicated that the 25 residues at the N-terminus matched the sequence predicted from the DNA sequence of the cloned gene, including the Ile to Leu substitution. The sequence specificity of the purified M.*Hha*I was identical to that expected for the wild type protein: substrate DNA methylated in vitro with M.*Hha*I was resistant to cleavage by the cognate restriction enzyme R.*Hha*I, but could be digested with restriction enzymes R.*Hpa*II, R.*Eco*RI, and R.*Bam*HI (data not shown).

The majority of the overexpressed M.*Hha*I is insoluble in low-salt buffer immediately after cell lysis (cf. Figure 1, lanes T and S1) and pellets with the cellular debris. However, at least 50% of this insoluble M.*Hha*I could be recovered in an enzymatically active form by high-salt extraction of the pelleted cellular debris. The high-salt-extracted enzyme (fraction S2) and the low-salt soluble form (fraction S1) have identical electrophoretic mobilities in SDS–PAGE and similar FPLC elution profiles. In kinetic studies (see below), M.*Hha*I purified from the S2 fraction had K_m^{AdoMet}, K_i^{AdoHcy}, and K_{cat} values comparable to those reported previously for M. *Hha*I isolated by an alternative method which does not utilize high-salt extraction (Wu & Santi, 1987, 1988). From these comparisons, the enzyme isolated from the S2 fraction appears to be biochemically similar to the low-salt soluble form. However, we did not purify the small S1 fraction of M.*Hha*I to homogeneity, so a direct comparison was not possible. The unrecoverable methylase that remains insoluble after the high-salt extraction (Figure 1, lane P) may be trapped in inclusion bodies. In previous work with overexpression of another m5C-mTase, M.*Msp*I, large inclusion bodies were formed under the growth conditions used in this study (Dubey et al., 1992).

M.HhaI Can Be Isolated in Two Forms. A significant finding made during the development of the purification scheme was that M.*Hha*I exists as a mixture of two forms. On MONO-S columns, M.*Hha*I was observed to elute as two peaks (peaks A and B, Figure 2A): peak B apparently contains the enzyme bound with its cofactor AdoMet, while peak A is devoid of AdoMet. Support for this interpretation came from the observation that M.*Hha*I from peak B was capable of methylating DNA in vitro in the absence of exogenous AdoMet (Figure 2B). Tight AdoMet binding was previously reported during the purification of the type-III DNA methyltransferase *Hinf*III (Piekarowicz & Brzezinski, 1980). In that study also, in vitro methylation could occur in the absence of exogenous AdoMet. Addition of AdoMet (100 μM) to the cleared S2 fraction prior to FPLC chromatography yielded a single elution peak coincident with peak B, presumably consisting of a homogeneous preparation of AdoMet-bound M.*Hha*I (Figure 2A). This peak was disrupted if the competitive inhibitor AdoHcy was added to the sample prior to MONO-S chromatography (data not shown).

The association between AdoMet and M.*Hha*I was quite stable, being capable of surviving multiple rounds of FPLC purification over a period of a week. Part of this stability may be due to the high glycerol content (10%) and low temperatures (4 °C) maintained during purification and storage. The binding of AdoMet is more labile when glycerol is removed and the temperature raised (S. Klimasauskas, personal communication). Substrate DNA does not appear to be required for AdoMet binding, as M.*Hha*I in the nucleic acid-free S2 supernatant readily binds AdoMet (Figure 2A). This observation was surprising because the mechanism of

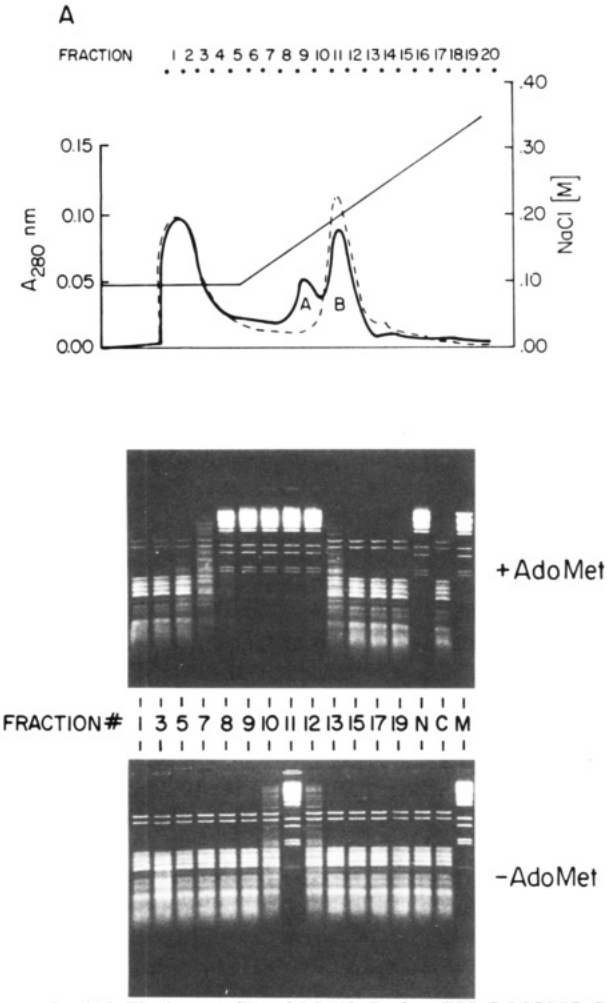

FIGURE 2: (A) Elution profile of M.*Hha*I after FPLC MONO-S ion-exchange chromatography: dashed line, 100 μM AdoMet added prior to chromatography; solid line, no additional AdoMet added. (B) In vitro methylation assay of MONO-S column fractions in the presence and absence of exogenous AdoMet. Selected column fractions from the solid line trace in panel A were assayed for methylase activity in vitro in 20-μL reactions containing 0.4 μg of *Bst*EII-cleaved λ DNA. After methylation at 37 °C for 15 min, the samples were heat inactivated, and the DNA was challenged with restriction enzyme R.*Hha*I. The samples in the top panel contained 0.2 μL of 1:10 dilutions of each column fraction and 100 μM exogenous AdoMet in the methylation reaction. The lower panel samples contained 0.2 μL of each column fraction but no exogenous AdoMet. Lane N: 0.2 μL (1 unit) of M.*Hha*I obtained from New England BioLabs. Lane C: no methylase. Lane M: marker DNA (*Bst*EII-cleaved λ DNA).

M.*Hha*I methylase activity outlined by Wu and Santi (1987) proposes that DNA binding precedes the binding of AdoMet. Our results do not address the catalytic mechanism of M.*Hha*I, but do show that DNA need not be present for AdoMet to bind to M.*Hha*I.

Titration experiments were carried out to estimate the fraction of M.*Hha*I molecules from peak B containing bound AdoMet. Increasing amounts of purified M.*Hha*I were incubated with a fixed amount of substrate DNA in in vitro methylation reactions in the absence of exogenous AdoMet. After methylation, the DNA was challenged with restriction enzyme R.*Hha*I. Nearly complete resistance to cleavage by R.*Hha*I was observed only after the molar ratio of M.*Hha*I to double-stranded DNA target sites exceeded 0.87 (Figure 3). This suggests that nearly every M.*Hha*I molecule is associated with AdoMet, assuming that R.*Hha*I is unable to cleave a hemimethylated recognition site efficiently. R.*Hha*I sensitivity to hemimethylated substrates is not known, but

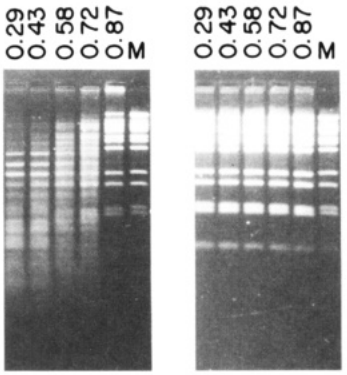

FIGURE 3: Titration of methylase activity in the absence of exogenous AdoMet. In vitro methylation reactions were carried out using the indicated molar ratios of enzyme:double-stranded target sites in the absence of exogenous AdoMet at 37 °C for 1 h. The substrate DNA (*Bst*EII-cleaved λ DNA) was subsequently digested with restriction enzyme R.*Hha*I and the sensitivity to cleavage measured by agarose gel electrophoresis as shown in the left panel. The right panel shows a parallel set of reactions supplemented with 50 μM AdoMet. M: marker lane (input DNA).

such inhibition was reported for several other methyltransferases [reviewed by Kessler and Manta (1990)]. The results do not rigorously exclude the possibility that two molecules of AdoMet are bound to each M.*Hha*I molecule, although this seems unlikely. Homogeneous preparations of cofactor-bound M.*Hha*I were used for crystallization studies.

Kinetic Analysis of M.HhaI. Kinetic parameters for M.*Hha*I were established using in vitro methylation reactions. Transfer of ^{3}H-labeled methyl groups from AdoMet to *Bst*EII-cleaved λ DNA containing 215 *Hha*I target sites was measured as a function of time for various concentrations of AdoMet as described in Materials and Methods. Progress curves were plotted for the reaction at increasing initial concentrations of AdoMet (data not shown). A rapid departure from linearity was observed at low concentrations of AdoMet, consistent with strong inhibition from a product of the reaction, AdoHcy (K_i = 1 nM), as previously reported by Wu and Santi (1987). K_i^{AdoHcy} for our preparation was calculated as 0.4 nM by applying the graphical analyses described by Wu and Santi (1987). Initial velocity measurements were used to calculate K_{cat} for the reaction and K_m for AdoMet under conditions where the maximum concentration of AdoHcy generated was less than 1 nM. Experimental values for K_{cat} and K_m were 0.22 s^{-1} and approximately 60 nM, respectively. These values are similar to those previously reported (0.08 s^{-1} and 14 nM, respectively) (Wu and Santi, 1987, 1988). Differences in K_{cat} may be linked to the DNA substrate as well as differences in the reaction buffer. Limited experiments with poly[d(G-C)], the substrate used by Wu and Santi, for unknown reasons yielded 10-fold-lower incorporation of [^{3}H]methyl groups relative to λ DNA in our hands (data not shown).

Crystallization of M.HhaI. Single well-formed crystals were grown at either 16 °C or room temperature (22 °C) in 10-μL hanging drops containing equal volumes of protein solution (10 mg/mL in FPLC buffer A, 0.2 M NaCl) and mother liquor [25% (w/v) PEG-4000, 50 mM ammonium sulfate, and 100 mM citrate buffer at pH 6.6]. After protein and precipitant were mixed, oil-like droplets first formed and then gradually disappeared, accompanied by the appearance of crystals. Eventually, the crystals reached maximum size when the oil droplets vanished. The crystals grown at higher temperature were larger in size and reached a maximum size of approximately 0.9 × 0.3 × 0.3 mm after a week (Figure 4). These conditions have consistently yielded crystals from

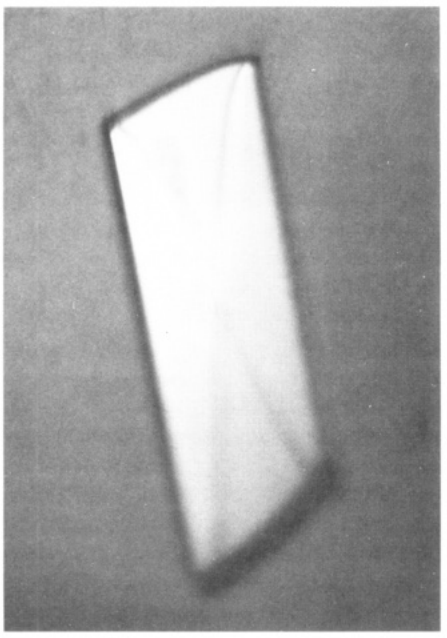

FIGURE 4: Photomicrograph of crystallized M.*Hha*I–AdoMet complex grown by the hanging drop vapor diffusion method at 22 °C under the optimal conditions described in Materials and Methods. The crystal shown has dimensions 0.9 mm × 0.3 mm × 0.3 mm.

separate preparations of the protein. Since the M.*Hha*I protein was purified and crystallized in the presence of AdoMet, we believe that the crystal obtained is likely to be a more informative cocrystal between M.*Hha*I and AdoMet. Initial attempts at crystallizing M.*Hha*I in the absence of AdoMet yielded only extremely small crystals or amorphous aggregates.

Previous attempts to crystallize purified M.*Hpa*II and M.*Msp*I were unsuccessful in our hands. The success of our present effort may be attributable to several factors. First, M.*Hha*I is one of the smallest of the m5C-MTases. The size differences between members of this family of enzymes are primarily localized to the N- and C-termini flanking conserved motifs I and X, respectively (Posfai et al., 1989). The shorter termini of M.*Hha*I may favor crystal packing. Also, the molecule may have an ordered structure in the absence of N- and C-terminal regions that may adopt irregular conformations. Second, the presence of AdoMet may serve to induce or stabilize a conformation of the enzyme that is more amenable to crystallization. Studies with adenine methyltransferases, M.*Eco*RI and *dam*, suggest that AdoMet binding is associated with a conformational change in the methylase (Bergerat et al., 1991; Reich & Mashhoon, 1991; Kriebardis & Guschlbauer, 1987). It was recently reported that the binding of AdoMet and an inhibitor appears to be required for the growth of large crystals of rat catechol *O*-methyltransferase (Vidgren et al., 1991). Our own experience with the AdoMet-free form of M.*Hha*I was similar. Finally, the recognition that two forms of the protein existed allowed us to take steps to ensure that only a homogeneous preparation was used for crystallization.

X-ray Diffraction. The crystals were of monoclinic space group $P2_1$ and had unit-cell dimensions of $a = 55.3$ Å, $b = 72.7$ Å, $c = 91.0$ Å, and $\beta = 102.5°$. The space group is based on systematic absences of reflections along the $0k0$ axis. The $0k0$ reflections with $k = 2n$ had $F/\sigma(F) > 14$, while $R = 2n$ had $F/\sigma(F) \leq 1$. Assuming that the crystals have an average packing density of approximately 2.4 Å^3/Da (Matthews, 1968), the molecular weight of the asymmetric unit is approximately 74 000 (=2 × 37 000). This is twice the predicted molecular weight (37 000) of one polypeptide of M.*Hha*I and suggests that there are two molecules in each of the two asymmetric units.

Strong diffraction was observed to 2.5-Å resolution. The crystals were stable in the beam on the synchrotron source for approximately 100 min (60° rotations, 0.1° increment, and 10-s exposure at 2.5 GeV, ≈200–110 mA). Each crystal was aligned with the ORIENT command of MADNES so that the b axis was parallel to the rotational ω axis. A native data set from four crystals was collected with 2-fold redundancy. The data set was 80.3% complete to 4.25 Å, 90.5% between 4.26 and 3.39 Å, 79.4% between 3.39 and 2.96 Å, 55.4% between 2.96 and 2.69 Å, and 31.8% between 2.69 and 2.50 Å. We merged 37 160 measurements of 16 666 unique reflections to 2.5-Å resolution with an overall error (R-merge) of 6.98% with $F/\sigma(F) > 2$, where F is the structure factor.

Currently, work is continuing toward solving the structure of the M.*Hha*I–AdoMet complex. Toward this end, two useful heavy-atom derivatives have been obtained. Data sets that are more complete at higher resolution are being collected for both the native and derivative crystals. In addition, we are attempting cocrystallization of the enzyme with DNA substrates and competitive inhibitors (*S*-adenosyl-L-homocysteine and sinefungin). In parallel, biochemical analysis of cofactor binding by site-specific mutagenesis is also planned. The latter studies are using comparative sequence analysis to predict AdoMet-binding sites within the protein to be targeted for mutagenesis.

ACKNOWLEDGMENT

We thank Peter Lee for valuable assistance with the data collection and Robert M. Sweet for generous access to beamline X-12C at Brookhaven National Laboratory in the Biology Department single-crystal diffraction facility of the National Synchrotron Light Source. This facility is supported by the Office of Health and Environmental Research of the U.S. Department of Energy. It is a pleasure to acknowledge Dr. G. Wilson, who supplied the original clone containing the *Hha*I methylase gene that enabled these studies to be carried out. The *E. coli* strain ER1727 was kindly provided by E. Raleigh. We also thank S. Klimasauskas, A. Dubey, and D. Roberts for helpful discussions of the work and M. Ockler, J. Duffy, and P. Renna for art and photography. And finally, we thank C. Carreras and D. Santi for their generosity and insight in examining our kinetic data prior to publication and providing assistance in the calculation and interpretation of kinetic constants.

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