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Purification and properties of the Hpa I methylase

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ABSTRACT

The purification and catalytic properties of the homogeneous Hpa I methylase is described. The enzyme exists as a single polypeptide chain with a molecular weight of 37,000 ± 2,000 was shown by sedimentation equilibrium and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The Hpa I methylase transfers methyl groups of S-adenosylmethionine to adenine present in the recognition sequence d(G-T-T-A-A*-C), A* is the N^6 methyl adenosine. An average of 2.1 methyl groups per recognition site are transferred by the Hpa I methylase.

INTRODUCTION

Since their discovery by Smith in 1970 (1), Type II restriction and modification enzymes have been invaluable both as tools for the study of gene structure and as models for sequence-specific DNA-protein interactions (2). In particular, comparison of a Type II endonuclease with its cognate methylase may reveal valuable information on two distinct proteins that interact with the same nucleotide sequence.

Previously, this laboratory has purified to homogeneity and characterized the Hpa I endonuclease, which cleaves DNA at the base sequence d(G-T-T-A-A-C) (3). We now report the purification to homogeneity and catalytic properties of the Hpa I methylase from Haemophilus parainfluenzae. Our studies show that Hpa I methylase is active as a monomer, methylates an adenosine in the Hpa I recognition sequence, and exhibits Michaelis-Menten kinetics with respect to DNA and AdoMet.

MATERIALS AND METHODS

Materials

S-Adenosyl-L-(methyl-^{3}H)methionine (15 Ci/mmol) was purchased from Amersham. Dr. K. Subramanian generously provided SV40 DNA. f_1RF DNA and f_1(+) strand DNA were prepared according to published procedures (4,5). The

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octanucleotide d(G-G-T-T-A-A-C-C) was chemically synthesized by a modified triester method (6). The Hpa I endonuclease and Hpa II endonuclease were purified as previously described (7).

Methods

Analytical centrifugation employed a Beckman Model E centrifuge with a photoelectric scanner. Prior to sedimentation, the protein was dialyzed exhaustively against 20 mM sodium phosphate, pH 7.6, 0.25 M NaCl, 0.1 mM EDTA, 0.1 mM dithiothreitol. Sedimentation equilibrium centrifugation was carried out at 21,740 rpm at 13.7° for 60 hr at a protein concentration of 100 μg/ml of Hpa I methylase. The sample was scanned at 280 nm.

SDS-polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborn (9) on 10% polyacrylamide slab gels (13 x 14 x 0.15 cm). The protein bands were visualized by staining with 0.1% Coomassie brilliant blue. Electrophoresis of DNA was performed on 1% agarose slab gels (15 x 15 x 0.3 cm) as previously described (10). DNA was visualized by staining with 1 μg/ml of ethidium bromide.

The assay for methylase measured transfer of ^{3}H-methyl groups from S-adenosyl-L-(methyl-^{3}H)methionine to either DNA or octanucleotide and was performed as described by Rubin and Modrich (8). One unit of Hpa I methylase catalyzes the transfer of one pmole of ^{3}H-methyl groups to 2 μg SV40 DNA when incubated with 0.55 μM S-adenosyl-L-(methyl-^{3}H)methionine at 37° for one hour in 24 μl buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 5mM NaCl, 10 mM 2-mercaptoethanol, 40 μg/ml bovine serum albumin).

The octanucleotide was phosphorylated with T_4-polynucleotide kinase and [γ-^{32}P]ATP as previously described (11).

The position of methylation in the recognition sequence was identified by using an octanucleotide substrate. 5.4 μM (5'-^{32}P)d(G-G-T-T-A-A-C-C), 14 μM [^{3}H]AdoMet, and 150 units Hpa I methylase were incubated in 14 μl buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 7 mM 2-mercaptoethanol, 40 μg/ml bovine serum albumin) for 3.5 hr at room temperature. After the addition of 300 units Hpa I methylase, incubation was continued for 18.5 hr. The methylated octanucleotide was partially hydrolyzed with snake venom phosphodiesterase as previously described (12). The oligonucleotides were separated on DEAE-cellulose thin layer plates by one-dimensional homochromatography (13). The ^{32}P-labeled spots were detected by autoradiography, eluted, and analyzed for ^{3}H by scintillation counting.

The methylated adenosine in f_1RF DNA was identified by the procedure of Rozim *et al.* (14). 150 units Hpa I methylase, 11 μg f_1RF DNA, and 2.6 pmoles

[^{3}H]AdoMet were incubated for one hour and 45 min at 37° in 41 µl buffer (50 mM Tris-HCl, pH 7.8, 10 mM 2-mercaptoethanol, 5 mM EDTA, 13 µg/ml bovine serum albumin). 150 units Hpa I methylase and 2.5 µg f_1RF DNA were added, and the incubation was continued for one additional hour at 37°. The reaction was mixed with the unlabeled methylated base standards, lyophilized, dissolved in 0.3 ml trifluoroacetic acid, sealed under vacuum, and heated at 175° for 15 min. After evaporation and suspension in 10 µl of water, 5 µl of the mixture was spotted on a plastic-backed cellulose thin layer sheet with UV fluorescent indicator and developed in butanol: water (86:14 v/v) with NH_3 saturating the vapor. The thin layer chromatography sheet was cut in 1 cm strips, and the strips were analyzed for tritium by scintillation counting. This chromatographic system separates 1-methyladenine, N6-methyladenine, and N6-dimethyladenine increasing Rf values (14).

The melting curve of the octanucleotide was determined by measuring absorbances at 260 nm at temperatures from 6° to 70°. The solution contained 11.4 µM (total strand concentration) of d(G-G-T-T-A-A-C-C) in 0.25 ml buffer (25 mM Tris-HCl, pH 7.5, 2.5 mM NaCl).

RESULTS AND DISCUSSION

The purification of the Hpa I methylase from 1 kg of Haemophilus parainfluenzae cells is summarized in Table 1. Unless indicated otherwise, all the steps were carried out at 4°, and centrifugation was at 12,000 g for 30 min in Sorval GSA or GS3 rotors. Buffers used were as follows: buffer A, 50 mM Tris-HCl, pH 8.0, 10 mM 2-mercaptoethanol, 5% glycerol, and 0.1 mM EDTA, and buffer B, 20 mM sodium phosphate, pH 7.6, 10 mM 2-mercaptoethanol, 10% glycerol, and 0.1 mM EDTA.

Step 1: Preparation of Extract

Haemophilus parainfluenzae cells (1 kg) were suspended in buffer A (3 l) with gentle stirring overnight at 4°. The suspended paste was passed twice through a Manton-Gaulen homogenizer at 7500 p.s.i. The suspension was clarified by centrifugation at 35,000 rpm in a 45 Ti rotor for 60 min, and the supernatant (3.66 l) was diluted with buffer A to an A_{260} of 60 (fraction I, 8.74 l).

Step 2: Streptomycin Sulfate Fractionation

A freshly prepared streptomycin sulfate solution (5%, 2.76 l) was added dropwise to fraction I over a period of 45 min with gentle stirring. After an additional 30 min of stirring, the supernatant was collected by centrifugation (fraction II, 10.88 l).

TABLE 1. Purification of Hpa I methylase from 1 kg of Haemophilus parainfluenzae.

FRACTION	STEP	TOTAL PROTEIN (mg)	TOTAL UNITS[c] ($\times 10^{-3}$)	SPECIFIC ACTIVITY ($\frac{\text{units}}{\text{mg}} \times 10^{-3}$)	PERCENT RECOVERY
I	Crude Extract	54,900[a]	---	---	---
II	Streptomycin Sulfate	49,300[a]	---	---	---
III	Ammonium Sulfate	30,700[a]	---	---	---
IV	Heparin Agarose	86.6[a]	1,000	12.7	100
V	DEAE-Sephadex	12.6[a]	941	74.7	85.5
VI	Phosphocellulose	1.8[a]	743	412.8	67.5
VII	SP-Sephadex	0.4[b]	594	1,485	54

[a]Protein concentration determined by the method of Schaffner and Weissman (16); [b]protein concentration determined by the method of Lowry et al. (17); [c]units not determined until IV because of other methylases present in I, II, and III.

Step 3: Ammonium Sulfate Fractionation

Powdered ammonium sulfate (4,406 g) was added to fraction II to 65% saturation with stirring over a 60 min period. After gentle stirring overnight, the precipitate was collected by centrifugation, resuspended in 3 l of buffer B, and dialyzed against 10 volumes of buffer B for 6 hr (fraction III, 4.78 l).

Step 4: Heparin-agarose Chromatography

Fraction III was applied at 140 ml/hr to a heparin-agarose column (75 x 14.6 cm^2) equilibrated with buffer B. The column was washed with 1.5 column volumes of buffer B, and the protein was eluted with an 8 l NaCl gradient (0.05 M - 0.80 M) in buffer B. Fractions containing Hpa I methylase were pooled (fraction IV, 480 ml). Hpa I methylase eluted between 0.33 M and 0.38 M NaCl.

Step 5: DEAE-Sephadex (A-50) Chromatography

Fraction IV was dialyzed three times against 10 volumes of buffer B for a total of 12 hr and applied to a DEAE-Sephadex (A-50) column (27 x 3 cm^2) equilibrated with buffer B. The column was washed with 0.5 column volume of buffer B, and the protein was eluted with a 1.4 l NaCl gradient (0.05 M - 0.60 M) in buffer B at a flow rate of 10 ml/hr. Fractions containing Hpa I methylase were pooled (fraction V, 90 ml). Hpa I methylase eluted between

0.18 M and 0.25 M NaCl.

Step 6: Phosphocellulose Chromatography

Fraction V was dialyzed against 10 volumes of buffer B for 6 hr and applied to a phosphocellulose column (30 ml syringe) equilibrated with buffer B. The column was washed with 30 ml buffer B, and the protein was eluted with 240 ml NaCl gradient (0.05 M - 1.0 M) in buffer B at a flow rate of 13 ml/hr. Fractions containing Hpa I methylase were pooled (fraction VI, 25.5 ml). Hpa I methylase eluted between 0.52 M and 0.60 M NaCl.

Step 7: SP-Sephadex C-25 Chromatography

Fraction VI was dialyzed three times against 10 volumes of buffer B for a total of 12 hr and applied to a SP-Sephadex C-25 column (10 ml syringe) equlibrated with buffer B. The protein was eluted with a 50 ml NaCl gradient (0.05 M - 0.60 M) in buffer B at a flow rate of 5 ml/hr. Fractions containing Hpa I methylase were pooled and dialyzed against buffer B supplemented with 50% glycerol (fraction VII, 2.0 ml). Hpa I methylase eluted between 0.20 M and 0.30 M NaCl.

400 μg of Hpa I methylase was isolated from 1 kg of Haemophilus parainfluenzae cells. 67% of the Hpa I methylase activity present after heparin-agarose chromatography was recovered after phosphocellulose chromatography (step 5). Although the Hpa I methylase at this stage of purification was free of nuclease activities, it contained a trace of high molecular weight contaminating protein. Chromatography on SP-Sephadex removed the contaminant and yielded homogeneous enzyme. The specific activity of the homogeneous Hpa I methylase was 1.5×10^6 units/mg.

After electrophoresis through a 10% polyacrylamide gel containing 0.1% SDS, the denatured and reduced Hpa I methylase appeared as a single band on the gel with a molecular weight of 37,500 $\pm$ 1,000 (Fig. 1). The molecular weight of the native Hpa I methylase, as determined by sedimentation equilibrium analysis at an initial protein concentration of 100 μg/ml, was 37,000 $\pm$ 2,000. Plots of the logarithm of protein concentration versus r^2 were linear indicating physical homogeneity. Thus, the Hpa I methylase exists as a 37,000 dalton monomer. The Hpa I methylase is also active as a monomer, since the initial velocity of methylation of DNA by the Hpa I methylase was first order with respect to Hpa I concentration up to 800 pM.

The absence of phosphatase, exo-, and nonspecific endonuclease activities was demonstrated by incubation of [5'-^{32}P]d(G-G-T-T-A-A-C-C) and f_1RF DNA with Fraction VII. As shown in Fig. 2B, neither labeled phosphate was released from the 5'-labeled octanucleotide nor the octanucleotide was

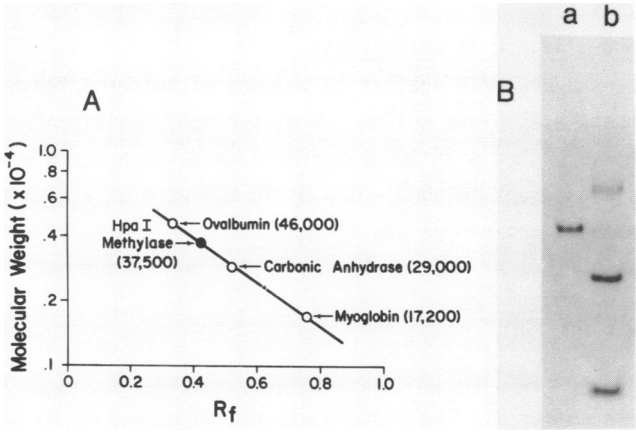

FIGURE 1. Molecular weight and homogeneity determination for the Hpa I methylase. A, subunit molecular weight of the Hpa I methylase with respect to standard proteins. B, SDS-polyacrylamide gel electrophoresis of Fraction VII and standard proteins. Lane a, Hpa I methylase; lane b, standard proteins.

cleaved to homologs. Similary, f_1RF DNA was resistant to cleavage on treatment with Fraction VII as shown in Fig. 2A, lane c. In addition, Fraction VII was also free of Hpa II methylase and other methylases, since it did not methylate f_1RF DNA that had been cleaved by the Hpa I endonuclease (Fig. 2A, lane b).

The Hpa I methylase and Hpa I endonuclease act on the same base sequence in DNA was demonstrated by employing f_1RF and SV40 DNAs as substrates. f_1RF DNA methylated by Hpa I methylase is not cleaved by Hpa I endonuclease but is cleaved by Hpa II endonuclease (Fig. 2A, lanes d, e, and f). Likewise, f_1RFI DNA cleaved by Hpa I endonuclease is not methylated by Hpa I methylase (Fig. 2A, lanes b and c). The small amount of linear DNA in lane d, Fig. 2A, is due to incomplete methylation of f_1RFI DNA. These results clearly show that Hpa I endonuclease and Hpa I methylase sites on f_1RFI DNA are identical.

For determining the nature and position of the nucleotide methylated in the recognition sequence by the Hpa I methylase, we employed the labeled octanucleotide [5'-^{32}P]d(G-G-T-T-A-A-C-C). The 5'-labeled octanucleotide treated with Hpa I methylase and [^{3}H]AdoMet was resistant to Hpa I endonuclease as shown in Fig. 2B, lane d. The [^{32}P] and [^{3}H] labeled octanucleotide thus generated was partially cleaved by snake venom phosphodiesterase to

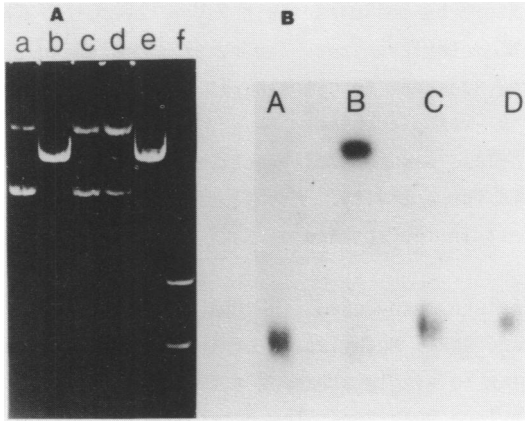

FIGURE 2. Determination of specificity of Hpa I methylase using f_1RFI DNA and an oligonucleotide. A, f_1RFI DNA (1.1 µg) and 132 pmol [^{3}H]AdoMet in 40 µl reaction buffer were incubated with appropriate amount of the Hpa I methylase at 37° for 30 min. The DNA bands were analyzed by 1% agarose gel electrophoresis. A, no enzyme treatment; b, digestion with 5 units of Hpa I endonuclease followed by treatment with 150 units of Hpa I methylase; c, treatment with 150 units of Hpa I methylase; d, treatment with 150 units of Hpa I methylase followed by 5 units of Hpa I endonuclease; e, digestion of Hpa I methylase followed by digestion with 20 units of Hpa II endonuclease. B, each reaction contained 0.71 µM [5'-^{32}P]d(G-G-T-T-A-A-C-C) (sp. activity 1.0 x 10^6 cpm/pmol) and 14 µM AdoMet in 14 µl of buffer. Reactions in lanes C and D were treated with 300 units of Hpa I methylase for 24 hr at room temp. Then $MgCl_2$ and NaCl were added to a conc. of 7.5 mM and 150 mM respectively. The final concentration of octanucleotide and AdoMet in 20 µl buffer was 0.5 µM and 9.8 µM respectively. Reactions in lanes B and D were digested with 3000 units of Hpa I endonuclease for 5 hr at room temp. followed by addition of additional 2000 units of Hpa I endonuclease and the digestion was continued for a total of 20 hr. Reaction in lane A was incubated under the same conditions without the addition of any enzyme. 2 µl of each enzyme was spotted in DEAE-cellulose thin layer plate which was developed in 2% RNA homomix and autoradiographed.

generate all possible homologs. These homologs were separated by one-dimensional homochromatography and their positions were localized by autoradiography. Each of these oligonucleotides were isolated and analyzed for the presence of tritium. The shortest oligonucleotide which contained 88% of the [^{3}H] radioactivity was identified as the hexanucleotide, ^{32}Pd(G-G-T-T-A-A). These data clearly indicate that adenosine adjacent to cytosine in the recognition sequence (G-T-T-A-A*-C, A* is methylated adenosine) is the nucleotide methylated by the Hpa I methylase. To identify the methylated base, f_1RF DNA was methylated by the Hpa I methylase and [^{3}H]AdoMet. The resulting methylated DNA was hydrolyzed to bases by trifluoroacetic acid (14).

Analysis of the mixture by cellulose thin layer chromatography which identified [^{3}H] base as N^6-methyladenine. The results presented above strongly suggest that adenine adjacent to cytosine is methylated by the Hpa I methylase to yield N^6-methyladenine.

The Hpa I methylase was maximally active at pH 6.8 to 7.0 and does not require Mg^{2+} or EDTA for activity. Methylation of f_1RF DNA by the Hpa I methylase was fastest in the absence of NaCl and completely inhibited by 150 mM NaCl.

The Hpa I methylase methylated f_1RF DNA 300 times faster than single stranded f_1(+) strand DNA. Methylation of the octanucleotide by the Hpa I methylase was similar to single stranded f_1(+) strand DNA. Under the conditions of Hpa I methylase reactions, the octanucleotide is mostly single stranded, since the Tm of the octanucleotide was only 9°. These studies show that Hpa I methylase clearly prefers double-stranded DNA as a substrate. An average of 2.1 methyl groups per recognition site are transferred by the Hpa I methylase when SV40 DNA was used as a substrate.

The steady state kinetic studies shown in Fig. 3 were performed in the presence of a saturating concentration of the invariant substrate. The turnover number determined from Fig. 3A is 0.91 methyl transfers/min/monomer at 37° C, while that obtained from the experiment shown in Fig. 3B is similar, being 0.96 min^{-1}. The corresponding Km values are 90 nM for SV40 DNA (in

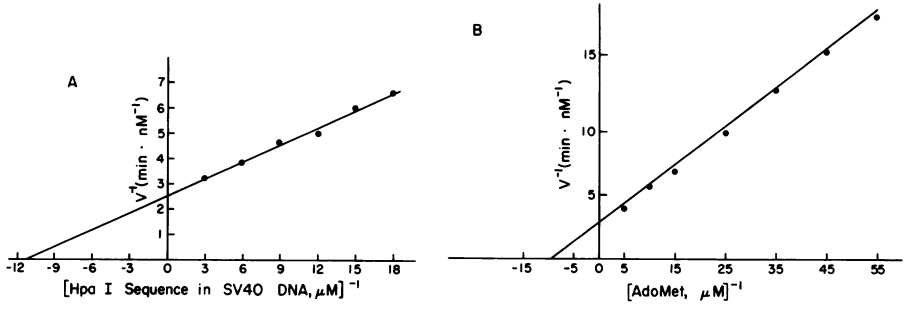

FIGURE 3. Steady state kinetics of Hpa I methylase. A, determination of Km for SV40 DNA. Reactions (25 µl) contained 50 mM Tris-HCl, pH 7.0, 5.0 mM EDTA, 40 µg/ml BSA, 10 mM 2-mercaptoethanol, 1.06 µM [^{3}H]AdoMet, 432 pM of Hpa I methylase and indicated concentrations of SV40 DNA. Incubation was at 37° for 30 min. DNA concentrations are in terms of Hpa I sequence. B, determination of Km for AdoMet. Reactions (26.5 µl) contained 50 mM Tris-HCl, pH 7.0, 5 mM EDTA, 40 µg/ml BSA, 10 mM 2-mercaptoethanol, 0.48 µM Hpa I sequence in SV40 DNA, 367 pM Hpa I methylase and indicated concentrations of [^{3}H]AdoMet. Incubation was at 37° for 30 min.

terms of Hpa I sequence) and 0.10 μM for AdoMet. The Km of octanucleotide was 17.3 μM, 190 times greater than Km of SV40 DNA (90 nM) and 600 times greater than the Km of f_1RFI DNA (27 nM). The turnover number for the Hpa I methylation of octanucleotide (0.015 min^{-1} at 20°) is sixty fold less than the turnover number of 1 min^{-1} for Hpa I methylation of SV40 DNA. These kinetic parameters for Hpa I methylation of DNA are similar to those determined for EcoRI methylase of ColEI DNA (8).

Recently, we have determined the contacts of the Hpa I endonuclease and the recognition sequence (15) by employing oligonucleotide containing base analogs as substrates. Similar studies with the Hpa I methylase reveals that the two enzymes do not share common contacts. Further studies aimed at determining the remaining contacts between the Hpa I endonuclease and the cogmethylase will reveal the details of DNA-protein interactions.

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REFERENCES

1. Smith, H.O. and Wilcox, K.W. (1970) J. Mol. Biol. 51, 379-391.
2. Modrich, P. (1980) CRC Crit. Rev. Biochem. In press.
3. Hines, J.L. and Agarwal, K.L. (1979) Fed. Proc. 38, 294.
4. Mazur, B.J. and Model, P. (1973) J. Mol. Biol. 78, 285-300.
5. Model, P. and Zinder, N.D. (1974) J. Mol. Biol. 83, 231-251.
6. Agarwal, K.L. and Riftina, F. (1978) Nucleic Acids Res. 8, 2809-2823.
7. Hines, J.L. (1979) "The Purification and Characterization of the Sequence Specific Endonucleases Hpa I and Hpa II" Ph.D. dissertation, University of Chicago.
8. Rubin, R.A. and Modrich, P. (1977) J. Biol. Chem. 252, 7265-7272.
9. Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.
10. Sharp, P.A., Sugden, B., and Sambrook, J. (1973) Biochemistry 12, 3055-3066.
11. Sgaramella, V. and Khorana, H. (1972) J. Mol. Biol. 72, 427-444.
12. Agarwal, K.L. and Riftina, F. (1978) Nucleic Acids Res. 5, 2809-2824.
13. Jay, E., Bambara, R., Padmanabhan, R. and Wu, R. (1971) Nucleic Acids Res. 1, 331-353.
14. Razim, A., Sedat, J.W. and Sinsheimer, R.L. (1970) J. Mol. Biol. 53, 251-259.
15. Dwyer-Hallquist, P., Kézdy, F.J. and Agarwal, K.L. (1982) Biochemistry, in press.
16. Schaffner, W. and Weismann, C. (1973) Anal. Biochem. 56, 502-514.
17. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.