The Deoxyribonucleic Acid Modification Enzyme of Bacteriophage P1

PURIFICATION AND PROPERTIES

By JEREMY P. BROCKES
Medical Research Council Molecular Genetics Unit, Department of Molecular Biology, University of Edinburgh, Edinburgh EH9 3JR, U.K.

and PAUL R. BROWN and K. MURRAY
Department of Molecular Biology, University of Edinburgh, Edinburgh EH9 3JR, U.K.

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The bacteriophage P1 modification enzyme, assayed by the specific methylation of unmodified bacteriophage 82 DNA, has been purified 500-fold from a bacteriophage P1 lysogen of Escherichia coli. The enzyme catalyses the incorporation of approximately 20–24 methyl groups per bacteriophage 82 DNA molecule. The sole product of methylation is 6-methylaminopurine. Methylation of unmodified bacteriophage DNA confers protection against a challenge by purified bacteriophage P1 restriction enzyme. The pH optimum is 6.0–6.25; the apparent $K_m$ for $S$-adenosyl-L-methionine is $5 \times 10^{-4} \text{M}$.

The mechanism by which certain proteins are able to bind to or recognize particular nucleotide sequences is a central problem in molecular biology (Yarus, 1969). The enzymes of DNA restriction and modification offer an excellent opportunity for studying this problem (Arber & Linn, 1969). Many strains of Escherichia coli are able to degrade or 'restrict' foreign DNA that enters the cell. The enzyme responsible is a product either of the bacterial genome or of plasmids such as bacteriophage P1 (Lederberg, 1957) that are harboured by the bacteria. Several restriction enzymes have been purified and shown to be specific endonucleases which cleave bacteriophage DNA into discrete fragments of high molecular weight (Meselson & Yuan, 1968; Roulland-Dussoix & Boyer, 1969; Linn & Arber, 1968; Smith & Wilcox, 1970). This highly specific cleavage is believed to be a consequence of recognition of particular nucleotide sequences (Kelly & Smith, 1970).

The bacterial DNA, and any bacteriophage DNA that successfully infects the cell, is protected from the restriction endonuclease by host-induced 'modification' of the DNA (Arber & Dussoix, 1962). Yuan & Meselson (1970) have shown that purified E. coli K restriction enzyme will bind to unmodified bacteriophage λ DNA, but not to bacteriophage λ DNA carrying the strain K modification. With E. coli B, the modification in vivo of bacteriophage fd DNA involves methylation of four adenine residues at the 6-amino position (Smith et al., 1972). Genetic analysis (reviewed by Arber & Linn, 1969) of the E. coli B, E. coli K and bacteriophage P1 restriction and modification systems indicates that the modification enzymes have at least one and probably two (Hubacek & Glover, 1970) polypeptide chains in common with the corresponding restriction enzymes. This presumably reflects the common specificities of the enzymes, since the adenine residue that is methylated on modification is believed to be located in the sequence recognized by the restriction enzyme (Kühnlein & Arber, 1972). The E. coli B modification enzyme has been partially purified (Kühnlein et al., 1969), this being based on an infectivity assay for bacteriophage fd DNA (Benzinger, 1968).

The present paper describes a 500-fold purification of the bacteriophage P1 modification activity and some of the properties of this activity. The activity has been assayed by measuring the transfer of tritium-labelled methyl groups from $S$-adenosyl-L-methionine to unmodified DNA.

In this paper the general nomenclature of bacteriophages and bacteria given by Arber & Linn (1969) is followed. Thus bacteriophage P1 that has lost its restriction and modification properties is written $P_{1\text{R}^K_{1}}$. Bacteriophage 'pedigree' is indicated afterno point by the strain of the bacterial host, e.g. 82.C600($P_{1\text{R}^K_{1}}$) is bacteriophage 82 grown on E. coli C600 lysogenized with the appropriate bacteriophage P1, and hence carrying bacteriophage P1 modification. Endonuclease R.P and endonuclease R.K are the bacteriophage P1 and E. coli K restriction enzymes respectively.

Materials and Methods

Bacterial and bacteriophage strains

The endonuclease I− strain E. coli 1100 of Dürwald & Hofman-Berling (1968) (obtained from Dr. I. R.
Lehman, Department of Biochemistry, Stanford University, California, U.S.A.) was made \( r_k^{-m_k} \) by Dr. N. E. Murray of this department by using ethylmethanesulphonate mutagenesis (Hubacek & Glover, 1970), and was lysogenized with bacteriophage Plkc (Lennox, 1955). \( E. \ coli \) C600 and its derivatives C600(P1\(r_p\)\(-m_p\)), C600(P1\(r_p\)\(-m_p^+\)) and C600 (P1) were obtained from Dr. S. W. Glover of this department. Bacteriophage 82c, a clear-plaque mutant of bacteriophage 82, was obtained from Dr. W. Arber, Biozentrum der Universität, Basel, Switzerland, and bacteriophage Plkc from Dr. W. J. Brammar of this department.

**Enzymes and chemicals**

Pancreatic deoxyribonuclease and snake-venom phosphodiesterase were purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Endonuclease R.P and endonuclease R.K, both purified to the glycerol-gradient stage as described by Meselson & Yuan (1968), were the generous gifts of Mr. R. W. Old and Dr. R. Yuan, both of this department. Dithiothreitol, bovine liver haemoglobin, bovine serum albumin, adenine, S-adenosyl-L-methionine, 6-methylaminopurine, 5-methylcytosine and N-7-methylguanine were obtained from Sigma (London) Chemical Co., London S.W.6, U.K., Whatman DE52 cellulose (microgranular, pre-swollen), Whatman P11 phosphocellulose and AE81 aminoethyl-cellulose paper were obtained from H. Reeve Angel and Co. Ltd., London E.C.4, U.K. Glass beads (ballotini no. 11) were obtained from Jencons (Scientific) Ltd., Hemel Hempstead, Herts., U.K. Streptomycin sulphate was a kind gift from Glaxo Laboratories Ltd., Ulverston, Lancs., U.K. S-Adenosyl-L-methionine was purified by elution from Zeo-Karb 226 with 4M-acetic acid (Meselson & Yuan, 1968), and stored in acetic acid at \(-10^\circ C\). Thin-layer cellulose sheets (MN300) were obtained from Macherey Nagel and Co., Duren, Germany. \([methyl-^3H]\)-S-Adenosyl-L-methionine (8.9Ci/mmol, 80\(\mu\)M) in \( H_2SO_4 \) (pH 2.5-3.5) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K., and used without further purification. L broth (pH 7.2) contained, per litre of water, Difco Tryptone (10g), yeast extract (5g) and NaCl (10g).

**DNA preparations**

\(^3H\)-labelled bacteriophage \( \lambda C_{1857}S_{17}, C \) DNA and unlabelled bacteriophage \( \lambda C_{1857}S_{17}, C \) DNA. These were kindly provided by Dr. N. E. Murray of this department. \(^32P\)-labelled bacteriophage \( \lambda C_{1857}S_{17}, K \) (P1) DNA was kindly provided by Mr. R. W. Old. Bacteriophage 82c DNA. \( E. \ coli \), either C600 (P1\(r_p\)\(-m_p^+\)) or C600 (P1\(r_p\)\(-m_p^+\)), was grown in 1 litre of L broth at 37°C to \( E_{650} = 0.5 \) (approx. 5\(\times\)10\(^6\) cells/ml). The culture was made 1mm in MgSO\(_4\) and bacteriophage 82c was added at a multiplicity of infection of 0.3-0.5 phage/bacterium. The culture was shaken vigorously at 37°C for 3-4h and lysed with 5ml of chloroform. The lysate was clarified by centrifugation (10000g for 15min at 4°C) and the titre determined on the appropriate indicator strains to check the presence or absence of bacteriophage P1 modification. The bacteriophages (generally 10\(^11\) plaque-formers/ml) were collected by centrifugation (45000g for 2½h), resuspended by gentle agitation overnight in 25ml of bacteriophage \( \lambda \) dil medium (Kaiser & Hogness, 1960) and twice banded by centrifugation (30h, 27000rev./min, MSE Superspeed 65 centrifuge, \( 3 \times 23 \)ml swing-out rotor) in 41.5% (w/w) CsCl. The final band was collected, dialysed against 10mm-tris–HCl–1mm-EDTA, pH7.4, and extracted three times by rolling with freshly distilled phenol that had been equilibrated with 0.5m-tris–HCl, pH8. The aqueous layer was dialysed exhaustively against 10mm-tris–HCl–1mm-EDTA–50mm-NaCl, pH7.4, and stored at 0°C.

**Methylation assay**

The reaction mixture contained, in a total volume of 200\(\mu\)l, 0.05m-potassium phosphate, pH6.5, 5mm-2-mercaptoethanol, 0.5mm-EDTA, 12\(\mu\)m-[methyl-\(^3H\)]S-adenosyl-L-methionine (3Ci/mmol), bacteriophage 82 DNA (5\(\mu\)g) and the sample to be assayed. After 60min incubation at 30°C, 0.5% bovine serum albumin (0.1ml), 0.2m-tetrasodium pyrophosphate (0.2ml) and 1m-HClO\(_4\)-2mm-sodium pyrophosphate (0.5ml) were added. After 5min at 0°C cold water (1ml) was added and the precipitate collected by centrifugation (5000g, 5min). The precipitate was dissolved in 0.2m-NaOH (0.3ml) and re-precipitated with 0.2m-sodium pyrophosphate (0.2ml) and 1m-HClO\(_4\)-2mm-sodium pyrophosphate (0.5ml). This washing procedure was repeated twice more and the precipitate was finally collected by filtration under reduced pressure through a 2.1cm Whatman GFC glass-fibre disc. The disc was washed with 1m-HClO\(_4\) (5ml), water (5ml) and ether (2ml). After drying under an i.r. lamp the discs were counted for radioactivity in 2.5ml of scintillant [4g of 5-(4-biphenyl)-2-(4-butylyphenyl)-1-oxa-3,4-diazole/l of AnalAR toluene] in a Nuclear–Chicago Unilux liquid-scintillation spectrometer. Assays were always done in parallel with P1 modified and unmodified bacteriophage 82 DNA. The blank value for incorporation into modified DNA was subtracted. This blank was always 100–150c.p.m., except for the dialysed (NH\(_4\))\(_2\)SO\(_4\) fraction, where it was 300–500c.p.m. owing to the presence of non-specific methylating activity.
Degradation of DNA

Acid hydrolysis. Bacteriophage 82 DNA from a methylation reaction was purified by acid precipitation as described in the methylation assay, except that calf thymus DNA (0.2ml, 1.2mg/ml) was used as carrier in place of serum albumin. The final precipitate was hydrolysed with 1M-HCl (0.2ml) at 100°C for 1h. The hydrolysate was evaporated to dryness (together with 20μl of 5mm solutions of various marker bases), the residue dissolved in 10% (v/v) acetic acid–10% (v/v) propan-2-ol (60μl) and applied to Whatman no. 1 paper. After descending chromatography in butan-1-ol–water (43:7, v/v) in an NH₃ atmosphere for 24h the paper was dried, markers were located under u.v. light and the paper was cut into strips (2cm×1cm); the radioactivity of each strip was determined by liquid-scintillation counting. The strips containing 6-methylaminopurine were swirled in toluene to remove scintillant, dried, eluted overnight with 0.1M-HCl and chromatographed on Whatman no. 1 paper in an ascending system of methanol–water–conc. HCl (7:1:2, by vol.) for 24h. The chromatogram was again examined under u.v. light, cut up and counted for radioactivity.

Methylation of bacteriophage λ DNA was investigated by hydrolysing 10μl of the product with 1M-HCl (50μl) for 45min at 100°C in the presence of 6-methylaminopurine and 5-methylcytosine. The hydrolysate was evaporated to dryness, the residue dissolved in 20μl of water and applied to a sheet (20cm×20cm) of MN300 thin-layer cellulose. The chromatogram was developed in two dimensions as described by Razin et al. (1970). Marker bases were located under u.v. light, eluted and counted for radioactivity, together with the origin region, as described by Razin et al. (1970).

Enzymic digestion. Bacteriophage 82 DNA was purified from a methylation reaction by extracting twice with an equal volume of phenol. The aqueous layer was extensively dialysed against 0.1M-sodium acetate (pH 5.0)–0.005M-MgCl₂ and a solution of pancreatic deoxyribonuclease (1mg/ml; 0.1ml) was added and incubated at 37°C for 2h. The reaction was adjusted to pH8.0 with 1m-tris–HCl, pH8.0 (10μl), and incubated with snake-venom phosphodiesterase (1mg/ml; 20μl; 37°C; 4h). The solution was evaporated to dryness, the residue dissolved in 50μl of water and applied, together with mononucleotide markers, to AE 81 cellulose paper. Electrophoresis was at 50 V/cm in pyridine acetate buffer, pH3.5, until the blue marker dye (Xylene Cyanol FF) had run 25cm. The paper was dried overnight at room temperature, examined under u.v. light, cut into strips (2cm×1cm) and the radioactivity determined in a liquid-scintillation spectrometer.

Results

P1 restriction of bacteriophage 82

The bacteriophage P1 modification enzyme was selected for study because the efficient restriction of certain bacteriophages by the bacteriophage P1 system presumably indicates a relatively large number of sites at which the restriction and modification enzymes act. This facilitates the use of a methylation assay for modification. The DNA of bacteriophage 82 was chosen as substrate rather than that of bacteriophage λ because of the relative efficiencies of plating of the two phages on a P1 lysogen (see Table 1). The assay for the P1 modification enzyme measures the extent of methylation of bacteriophage 82.C600(P1r₈₁−mp₁₊)/DNA compared with DNA from bacteriophage 82.C600(P1r₈₁−mp₁₊) modified in vivo. If modification in vivo is efficient, then the latter substrate should be inert to the activity in vitro.

Purification of the modification enzyme

All buffers contained 5% (v/v) glycerol, 5mm-2-mercaptoethanol and 0.5mm-EDTA unless otherwise stated. All potassium phosphate buffers were pH6.7. Protein in subcellular fractions was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard.

Step 1: preparation of dialysed (NH₄)₂SO₄ fraction. [Linn & Arber (1968), with minor modifications.] E. coli 1100r⁻ (P1) was grown in L broth at 37°C

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**Table 1. Efficiencies of plating of bacteriophages 82 and λ on P1 lysogenic hosts**

The efficiencies of plating (titre on bacteriophages P1 lysogenic host/titre on E. coli C600) of bacteriophage 82c and bacteriophage λ grown on various hosts were determined by standard bacteriophage and bacterial techniques.

<table>
<thead>
<tr>
<th>Bacteriophage</th>
<th>E. coli C600 (P1)</th>
<th>E. coli C600 (P1r₈₁−mp₁₊)</th>
</tr>
</thead>
<tbody>
<tr>
<td>82.C600</td>
<td>1×10⁻⁶–3×10⁻⁶</td>
<td>1</td>
</tr>
<tr>
<td>82.C600(P1r₈₁−mp₁₊)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>82.C600(P1r₈₁−mp₁−)</td>
<td>1×10⁻⁶–3×10⁻⁶</td>
<td>1</td>
</tr>
<tr>
<td>λ.C600(P1r₈₁−mp₁−)</td>
<td>1×10⁻⁵–3×10⁻⁵</td>
<td>1</td>
</tr>
</tbody>
</table>

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to $10^9$ cells/ml, sedimented in a continuous-flow Alfa-Lavall centrifuge and stored at $-20^\circ$C. All subsequent operations were performed at 4°C. Cells (150g) were resuspended in 225 ml of 0.05M-tris-HCl, pH 8.0, then 300 g of acid-washed glass beads was added and the mixture was blended for a total of 20 min with intermittent cooling in an ice–salt bath so that the temperature did not rise above 5°C. The supernatant was decanted from the beads, which were washed with 100 ml of 0.05M-tris-HCl, pH 8.0. The pooled supernatant and washings were centrifuged (10000g, 20 min). The supernatant (volume 354 ml) was made 0.035M in MgCl$_2$ and the resulting precipitate was removed by centrifugation (10000g, 15 min). Fresh 5% (w/w) streptomycin sulphate solution (125 ml) was added to the supernatant and, after 20 min at 0°C, the precipitate was removed by centrifugation (10000g, 15 min). The supernatant was precipitated by adding 180 g of solid (NH$_4$)$_2$SO$_4$, which was dissolved over 30 min at 0°C. The precipitate was collected by centrifugation in the MSE High-Speed 18 centrifuge (6 x 100 ml rotor, 17000 rev./min, 30 min, 2°C). The pellet was dissolved in 0.02M-potassium phosphate (30 ml) and dialysed against the same buffer.

**Step 2: chromatography on DEAE-cellulose.** The dialysed ammonium sulphate fraction was applied to a column (17 cm x 4.5 cm diam.) of Whatman DE52 cellulose that had been equilibrated with 0.02M-potassium phosphate. The column was washed successively with 500 ml of 0.02M-potassium phosphate, 500 ml of 0.05M-potassium phosphate, then eluted with a linear potassium phosphate gradient (1.5 litres) running from 0.05M to 0.3M. Fractions (approx. 45 ml) were collected and the enzyme activity was found in five neighbouring fractions with a mean phosphate concentration of 0.11 M (Fig. 1). The pooled fractions (volume 240 ml) were precipitated with (NH$_4$)$_2$SO$_4$ (150 g). The precipitate was collected by centrifugation, dissolved in 0.02M-potassium phosphate (30 ml) and dialysed extensively against 0.02M-potassium phosphate. The precipitate that formed during dialysis was removed by centrifugation; the total protein in the supernatant was 301 mg (volume 44 ml).

**Step 3: chromatography on phosphocellulose.** Of the concentrated DE fraction 34 ml was applied to a column (18 cm x 1.25 cm diam.) of Whatman P11 phosphocellulose that had been equilibrated with 0.02M-potassium phosphate. The column was eluted with 0.02M-potassium phosphate (75 ml), 0.1M-phosphate (100 ml), 0.2M-potassium phosphate (75 ml), 0.3M-potassium phosphate (100 ml) and 0.5M-potassium phosphate (100 ml). The methylation activity was found in the 0.2M step and was immediately concentrated by the addition of (NH$_4$)$_2$SO$_4$ (40 g). The precipitate was collected by centrifugation and dissolved in 0.02M-potassium phosphate (3 ml).

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**Fig. 1. Chromatography of dialysed ammonium sulphate fraction on DEAE-cellulose**

Adsorption and elution was as described in the Results section under ‘Purification of the modification enzyme’. Samples (0.15 ml) of the fractions were assayed in parallel for methylation of bacteriophage 82.C600 (P1 r$_{p1}^{-}\_$r$_{p1}^{-}$) DNA (●) and bacteriophage 82.C600 (P1 r$_{p1}^{-}\_$r$_{p2}^{-}$) DNA (○). The continuous line in (a) is the difference in radioactivity incorporated into the two substrates. Fractions enclosed by the bar were pooled and concentrated as described under ‘Purification of the modification enzyme’.   
(b) ———, $E_{280}$; ———, [potassium phosphate].

**Step 4: glycerol-gradient sedimentation.** After dialysis for 2 h against 0.02M-potassium phosphate (1 litre) the concentrated phosphocellulose fraction was layered in 1.2 ml portions on 20 ml of 10–25% (v/v) glycerol gradients made up in 0.02M-potassium phosphate – 0.1 M-EDTA – 5 mM-dithiothreitol, pH 6.5. After centrifugation for 36 h at 30000 rev./min in the MSE Superspeed 65 centrifuge, 3 x 23 ml swing-out rotor at 2°C, the gradients were collected in 1 ml fractions. The active fractions (see Fig. 2) were made 50% (v/v) in glycerol and stored at −20°C. No significant loss of activity (less than 10%) was observed over 4 months.

The purification of the enzyme is summarized in Table 2. The dilute column fractions were all unstable and the glycerol gradient fraction lost activity if stored at 0°C. It was found necessary to complete all steps before the glycerol gradient in 2–3 days. The glycerol-gradient fraction had no detectable bacteriophage P1 restriction activity, and no detectable endodeoxyribonuclease activity at pH 7.5 in 5mM-MgCl$_2$, as assayed by neutral sucrose-gradient sedimentation of bacteriophage λ DNA. Under the conditions of
methylation reactions [0.05 M-2-(N-morpholino)ethanesulphonic acid (pH 6.0)–0.25 mM-EDTA] it had no detectable effect on the sedimentation profile in neutral sucrose gradients of supercoiled bacteriophage M13 RFI DNA (kindly provided by Mr. G. G. Peters of this department).

The enzyme has also been purified from 27 g of bacteriophage P1-infected cells by an identical procedure, and is currently prepared from induced lysogens of the thermoinducible mutant P1.C600 (Scott, 1970). This increases the specific activity of enzyme five- to ten-fold.

![Glycerol-gradient sedimentation of concentrated phosphocellulose fraction](image)

**Fig. 2.** Glycerol-gradient sedimentation of concentrated phosphocellulose fraction

Sedimentation of the phosphocellulose fraction was as described in the Results section under ‘Purification of the modification enzyme’. Portions (25 µl) were assayed for methylation of bacteriophage 82.C600 (P1 r-p) DNA (●) or bacteriophage 82.C600 (P1 r-p) DNA (○). The 4.3S standard was bovine haemoglobin, which was located by its extinction at 410 nm. Fractions enclosed by the bar were stored as described under ‘Purification of the modification enzyme’. Sedimentation was from right to left.

### Table 2. Summary of enzyme purification

<table>
<thead>
<tr>
<th>Purification stage</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude supernatant</td>
<td>10700</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dialysed (NH₄)₂SO₄ fraction</td>
<td>4820</td>
<td>7520</td>
<td>1.56</td>
<td>100</td>
</tr>
<tr>
<td>Pooled DEAE-cellulose fraction</td>
<td>301</td>
<td>4420</td>
<td>14.7</td>
<td>59</td>
</tr>
<tr>
<td>Pooled phosphocellulose fraction</td>
<td>9.1</td>
<td>2161</td>
<td>230.8</td>
<td>29</td>
</tr>
<tr>
<td>Pooled glycerol-gradient fraction</td>
<td>1.5</td>
<td>1230</td>
<td>820</td>
<td>16</td>
</tr>
</tbody>
</table>

1 unit of activity makes 0.16 pmol of methyl groups acid-insoluble/5 µg of unmodified bacteriophage 82 DNA per 60 min incubation at 30°C. This is equivalent to 125 c.p.m. under the conditions of assay. The blank value for incorporation into modified DNA is subtracted, as noted in the Materials and Methods section. The recovery values for the phosphocellulose and glycerol-gradient fractions are corrected for purification of total pooled DEAE-cellulose fraction.

**Properties of the purified modification activity**

All these experiments were performed with the glycerol-gradient fraction.

**Requirements for the activity.** The requirements were investigated as shown in Table 3. The enzyme is specific for native, unmodified DNA and is stimulated 1.5-fold by 5 mM-Mg²⁺, a property also noted by Kühnlein et al. (1969) with the E. coli B modification activity. A threefold inhibition by 0.1 mM-ATP was also observed.

**pH-dependence.** The pH-dependence of the activity in 2-(N-morpholino)ethanesulphonic acid and piperazine-NN'-bis-2-ethanesulphonic acid buffers is shown in Fig. 3. In 2-(N-morpholino)ethanesulphonic acid buffer the pH optimum is between 6.0 and 6.25, although significant methylation was observed from pH 5.5 to pH 8.0. No significant methylation of bacteriophage P1-modified DNA was observed over this pH range.

**Time-course of methylation.** The time-course of methylation of unmodified bacteriophage 82 DNA was investigated by incubating a series of duplicate reaction mixtures for various times and then assaying for acid-precipitable radioactivity. As shown in Fig. 4, incorporation of methyl groups was complete after 3 h of incubation at 30°C. The extent of methylation was not limited by inactivation of the enzyme, since the addition of more enzyme after 5 h was without effect, whereas the addition of more DNA produced a detectable stimulation. The number of methyl groups incorporated at the plateau was about 20–24/DNA molecule (containing 10⁸ bases), although this number is difficult to estimate accurately because of differences in recovery and quenching during the acid-precipitation procedure.

**Dependence of methylation on S-adenosyl-L-methionine.** The S-adenosyl-L-methionine dependence was investigated as described in the legend to Fig. 5. Analysis of the results by a double-reciprocal plot...
Table 3. Requirements for methylation

The complete system contained, in a volume of 100μl: 0.05m-2-(N-morpholino)ethanesulphonic acid, pH 6.0, 0.25mm-EDTA, 5mm-2-mercaptoethanol, 4μM-[methyl-3H]-S-adenosyl-L-methionine (8.9Ci:mmol), 6μg of bacteriophage 82.C600 (P1rp1 mp1+) DNA and 25μl of glycerol-gradient fraction modification enzyme. After incubation for 45min at 30°C the mixtures were assayed for acid-precipitable radioactivity as described in the Materials and Methods section. Bacteriophage 82 DNA was denatured by heating at 100°C for 7min and cooling in ice. Enzyme was heat-inactivated at 100°C for 5min in a stoppered tube.

<table>
<thead>
<tr>
<th>Acid-insoluble 3H radioactivity (c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
</tr>
<tr>
<td>Minus DNA</td>
</tr>
<tr>
<td>Substitute bacteriophage</td>
</tr>
<tr>
<td>82.C600 (P1rp1 mp1+) DNA</td>
</tr>
<tr>
<td>Substitute heat-denatured DNA</td>
</tr>
<tr>
<td>Minus enzyme</td>
</tr>
<tr>
<td>Substitute heat-inactivated enzyme</td>
</tr>
<tr>
<td>Plus 5mm-MgCl2</td>
</tr>
<tr>
<td>Plus 0.1mm-ATP</td>
</tr>
<tr>
<td>Plus 5mm-MgCl2 and 0.1mm-ATP</td>
</tr>
</tbody>
</table>

Fig. 3. pH-dependence of methylation in 2-(N-morpholino)ethanesulphonic acid (pK<sub>a</sub> = 6.15) and piperazine-NN'-bis-2-ethanesulphonic acid (pK<sub>a</sub> = 6.8) buffers

The complete system was made up as for Table 1 in 0.05m-2-(N-morpholino)ethanesulphonic acid or 0.05m-piperazine-NN'-bis-2-ethanesulphonic acid buffers of the appropriate pH value, which was measured at 27°C on a Vibron model 39a pH-meter with manual temperature compensation and standardized against air-free phthalate buffer, pH 7.0. The reaction mixtures were incubated for 40min at 30°C and assayed as described in the text. Bacteriophage 82.C600 (P1 rp1 mp1) DNA: ●, 2-(N-morpholino)ethanesulphonic acid; △, piperazine-NN'-bis-2-ethanesulphonic acid. Bacteriophage 82.C600 (P1 rp1 mp1) DNA: ○, 2-(N-morpholino)ethanesulphonic acid; △, piperazine-NN'-bis-2-ethanesulphonic acid.

(Lineweaver & Burk, 1934) gave an apparent K<sub>m</sub> for S-adenosyl-L-methionine of 5 × 10<sup>-8</sup>m. The value obtained by Kühnlein & Arber (1972) for the E. coli B modification enzyme was 4 × 10<sup>-6</sup>m.

Product of methylation. A sample of methylated DNA was analysed by enzymic digestion to mononucleotides and high-voltage electrophoresis on amineoethylcellulose paper at pH 3.5 (see the Materials and Methods section). A single peak of radioactivity (Fig. 6a) migrated with dAMP, indicating that a single mononucleotide had been methylated. Another sample of methylated DNA was analysed by acid hydrolysis and paper chromatography (see the Materials and Methods section). The radioactivity migrated on two solvent systems with 6-methylaminopurine (see Figs. 6b and 6c). 6-Methylaminopurine is thus the sole detectable product of methylation.

Modification and restriction of bacteriophage λ DNA in vitro. The effect of this methylase can be demonstrated by incubating DNA that had been methylated in vitro with purified bacteriophage λ restriction enzyme. Unlabelled bacteriophage λ DNA was methylated with the enzyme and [methyl-<sup>3</sup>H]-S-adenosyl-L-methionine in the presence of 32P-labelled bacteriophage λ.K (P1) DNA. After the incubation the DNA species were purified by phenol extraction. After removal of the phenol and S-adenosyl-L-methionine by dialysis, a sample of DNA was analysed by acid hydrolysis, followed by t.l.c.; 85% of the <sup>3</sup>H was found in 6-methylaminopurine. The remainder of the <sup>3</sup>H-methylated DNA was
The reaction mixtures had the same composition as the complete system in Table 1, except that 4 μg of bacteriophage 82.C600 (P1 rP1 mP1) DNA (●) or bacteriophage 82.C600 (P1 rP1 mP1) DNA (○) was used. Reaction mixtures were incubated at 30°C for the times indicated and then assayed for acid-insoluble methyl groups as described in the Materials and Methods section. To one set of reaction mixtures (Δ) 3 μg of bacteriophage 82.C600 (P1 rP1 mP1) DNA was added after 5 h; to another set (□) a further 25 μl of enzyme was added. All tubes had a blank value of 129 c.p.m. subtracted for an incubation without enzyme assayed at 0 min. No attempt has been made to correct for any fluctuation of this blank with time of incubation.

The reaction mixtures contained, in a volume of 150 μl, 0.05 M 2-(N-morpholino)ethanesulphonic acid, pH 6.0, 0.2 mM EDTA, 5 mM 2-mercaptoethanol, 7.5 μg of bacteriophage 82.C600 (P1 rP1 mP1) DNA, 50 μl of enzyme and [methyl-3H]-S-adenosyl-L-methionine adjusted with the required amount of unlabelled S-adenosyl-L-methionine to give the concentrations shown in the figure. After 30 min incubation at 30°C the reaction mixtures were assayed for acid-insoluble methyl groups as described in the Materials and Methods section. Each result was corrected for a blank incorporation performed in the absence of enzyme.

Fig. 4. Time-course of methylation

Fig. 5. S-Adenosyl-L-methionine dependence of methylation

demonstrates the role of DNA methylation in protection against the P1 restriction enzyme.

Discussion

Three lines of evidence identify the methylase as the bacteriophage P1 modification enzyme: (a) bacteriophage 82 DNA that has been modified by bacteriophage PI in vivo is not methylated, whereas unmodified DNA is a substrate for methylation; (b) methylation of bacteriophage λ DNA protects against breakage by purified restriction enzyme; (c) the activity is found in the dialysed (NH₄)₂SO₄ fraction prepared from E. coli 1100 infected with bacteriophage P1, but is absent (<10%) on infection with bacteriophage P1 mP1 (P. R. Brown & J. P. Brookes, unpublished work).

The product of the activity, 6-methylaminopurine, is also the product of E. coli B modification both in vivo (Smith et al., 1972) and in vitro (Kühnlein & Arber, 1972), and of E. coli K modification in vitro (J. P. Brookes, unpublished work). 6-Methylaminopurine has been reported as the product of the bacteriophage T₄-induced methylase that protects

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(a) Mononucleotide analysis of methylated bacteriophage 82 DNA on aminoethylcellulose paper (see the Materials and Methods section). 'Blue' = Xylene Cyanol FF marker dye. (b) and (c) Base analysis of methylated bacteriophage 82 DNA by paper chromatography (see the Materials and Methods section) on butan-1-ol-water (NH₃ atmosphere) then methanol-water-HCl. The material remaining at the origin (indicated by an arrow) after the butan-1-ol-water system was eluted with 0.1 M-HCl and hydrolysed with 1 M-HCl at 100°C for 2 h. On re-chromatography more than 90% of the radioactivity remained at the origin. This material was also found in the hydrolysate of a reaction from which the DNA was omitted and hence is not a product of DNA methylation. Methylated DNA for both analyses was obtained by incubating 4 μg of bacteriophage 82 DNA for 4 h at 30°C in the complete system described in Table 2. 7MeG, N-7-methylguanine; Ad, adenine; 5MeC, 5-methylcytosine; 6MAP, 6-methylaminopurine.

Fig. 6. Analysis of the product of DNA methylation
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Methylation reactions. Reaction mixture 1 (350μl) contained 0.05M-2-(N-morpholino)ethanesulphonic acid, pH 6.0, 0.25 mM-EDTA, 5 mM-2-mercaptoethanol, 4μM-[methyl-3H]-S-adenosyl-L-methionine (8.9 Ci/mmol), 20μg of bacteriophage λ.C DNA, 2 × 10^4 c.p.m. of ^32P-labelled bacteriophage λ.K(P1) DNA and 225μl of enzyme. Reaction mixture 2 (115μl) contained 6.0μg of bacteriophage λ.C DNA, 6 × 10^3 c.p.m. of ^32P-labelled bacteriophage λ.K(P1) DNA and 5.5 × 10^3 c.p.m. of ^3H-labelled bacteriophage λ.C DNA (1μg), 75μl of enzyme in addition to 2-(N-morpholino)ethanesulphonic acid, EDTA and mercaptoethanol as for reaction mixture 1. After incubation for 3.0h at 30°C, both reactions were extracted three times with an equal volume of freshly distilled phenol. The aqueous layers were dialysed twice against 1 litre of 10 mM-tris-HCl-0.1 mM-EDTA-0.4 mM-NaCl, pH 7.4, then twice against 1 litre of the same buffer without NaCl. After both samples had been kept at 60°C for 10 min to dissociate concatenates, 10μl of reaction mixture 1 (volume after dialysis 510μl) was analysed by acid hydrolysis followed by t.l.c. (see the Materials and Methods section). The volume of reaction mixture 2 after dialysis was 154μl.

Restriction reactions. (a) (Challenge with endonuclease R.P.) The reaction mixture (360μl) contained 0.1M-N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid, pH 8.0, 1.2 mM-EDTA, 8 mM-MgCl₂, 60 mM-2-mercaptoethanol, 2.5 mM-ATP, 10 μM-S-adenosyl-L-methionine, 150μl of DNA from reaction mixture 1 (5500 c.p.m. of ^3H and 6300 c.p.m. of ^32P), 150μl of endonuclease R.P. (b) (Challenge with endonuclease R.K.) The reaction mixture (360μl) contained DNA, N-tris(hydroxymethyl)-methyl-2-aminoethanesulphonic acid, EDTA and mercaptoethanol and ATP as for (a), but 20μM-S-adenosyl-L-methionine and 100μl of endonuclease R.K instead of endonuclease R.P. (c) (No restriction enzyme.) As (a) except that water (150μl) replaced endonuclease R.P. (d) (Endonuclease R.P control.) As (a) except that 150μl of DNA from reaction mixture 2 (5200 c.p.m. of ^3H and 6000 c.p.m. of ^32P) replaced DNA from reaction mixture 1.

All reaction mixtures were incubated at 30°C for continued on page 10.
20 min; reactions were terminated with 10 \mu l of 0.5 M-EDTA, pH 8.0, and the mixture was layered on 6–20 % (w/v) sucrose gradients in 0.01 M-tris–HCl–1 mm-EDTA–0.04 % (w/v) sodium dodecyl sulphate, pH 7.4 (volume 4 ml). After centrifugation (MSE Superspeed 65) in a 6 × 5 ml swing-out rotor at 50 000 rev./min for 130 min at 20°C, 5 drop fractions were collected into vials and counted in a Beckman liquid scintillation spectrometer after the addition of 1.5 ml of scintillant (30 g of naphthalene, 2 g of 2,5-diphenyloxazole, 0.1 g of dimethyl 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene, 50 ml of methanol and 500 ml of \( p \)-dioxan). The arrows in (a)–(d) indicate the position of sedimentation of whole molecule \( ^{32} P \)-labelled bacteriophage \( \lambda \)K (P1) DNA and \( ^{3} H \)-labelled bacteriophage \( \lambda \)C DNA. The small peak of \( ^{3} H \) at the top of the gradient in (a), (b) and (c) is \( [\text{methyl-}\ ^{3} \text{H}] \)-S-adenosyl-L-methionine remaining after dialysis. Sedimentation was from right to left. \( \bullet \), \( ^{3} \text{H} \); \( \circ \), \( ^{32} \text{P} \).

The rather slow kinetics of methylation (Fig. 4) were also observed with \( E. \) coli B modification activity on bacteriophage fd DNA (Kühnlein & Arber, 1972). In collaboration with Dr. R. Yuan we have been studying the binding of the bacteriophage P1 modification enzyme to bacteriophage \( \lambda \) DNA by the filter-binding method (Riggs & Bourgeois, 1968; Yuan & Meselson, 1970). This technique indicates that in the presence of S-adenosyl-L-methionine the enzyme binds to both its substrate and its product (i.e. to both modified and unmodified DNA) and is thus released very slowly after methylation (R. Yuan, unpublished work). This effect may well provide a basis for the slow kinetics.

The investigation of the nucleotide sequences around the methylated base, and of the structure of the enzyme, may provide some insight into the recognition reaction.

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