The Deoxyribonucleic Acid-Modification Enzyme of Bacteriophage P1

SUBUNIT STRUCTURE

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The bacteriophage P1 modification enzyme was purified 1400-fold from induced lysogens of a thermoinducible mutant of bacteriophage P1. The most purified fraction, when analysed by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate, showed two principal stained bands. The two bands co-sedimented in a glycerol gradient with the modification activity, at a rate which, when compared with the rate of sedimentation of marker proteins, corresponds to a sedimentation coefficient in water of 6S. The mobilities of the bands on sodium dodecyl sulphate–polyacrylamide-gel electrophoresis corresponded to polypeptides of molecular weight 70000 and 45000 and they were present in equimolar amounts. It was concluded that the 6S species of the enzyme is a dimer of unlike subunits.

In a cell that is lysogenic for bacteriophage P1, the P1 genome codes for the synthesis of endonuclease R.P, the DNA restriction enzyme of bacteriophage P1. This enzyme cleaves bacteriophage DNA into discrete fragments of high molecular weight, unless such DNA has been protected by bacteriophage P1 'modification' (Meselson & Yuan, 1968). This modification involves methylation of specific adenine residues in the nucleotide sequence recognized by the restriction enzyme. We have described the partial purification and properties of DNA methylase M.P, the DNA modification enzyme of bacteriophage P1 (Brockes *et al.*, 1972). Endonuclease R.P and DNA methylase M.P are examples of several restriction and modification enzymes whose properties have recently been reviewed (Meselson *et al.*, 1972).

The present paper describes the purification of DNA methylase M.P to near-homogeneity, and an analysis of its subunit structure. The principal conclusion is that the enzyme is a dimer of unlike subunits.

The nomenclature of bacteriophages and bacteria has been given previously (Arber & Linn, 1969; Brockes *et al.*, 1972); thus λ K is bacteriophage λ grown on *Escherichia coli* strain K.

Materials and Methods

Bacterial and bacteriophage strains

Escherichia coli K140 (PIC₁ 162) (Scott, 1970) was obtained from Dr. J. R. Scott of Emory University, Atlanta, Ga., U.S.A. *E. coli* W1485 (Δ C₁ 857 S7) was

obtained from Dr. N. E. Murray of this department and was lysogenized with bacteriophage Plk_c (Lennox, 1955). All other strains have been described previously (Brockes *et al.*, 1972).

Enzymes and chemicals

Bacterial alkaline phosphatase was purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Aristar-grade (NH₄)₂SO₄, acrylamide, methylenebisacrylamide, *NNNN'*-tetramethylethylenediamine and sodium dodecyl sulphate (especially pure) were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. Coomassie Brilliant Blue, phosphorylase *a*, horse liver alcohol dehydrogenase, yeast alcohol dehydrogenase and myoglobin were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. L-Amino acid oxidase (*Crotalus terrificus*) and pig heart fumarase were obtained from Boehringer (London) Ltd., London W5 2TZ, U.K. CM-Sephadex C25 was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Whatman DE23 cellulose was obtained from H. Reeve Angel and Co. Ltd., London E.C.4, U.K. All other enzymes and chemicals have been described previously (Brockes *et al.*, 1972).

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis

This was performed essentially as described by Weber & Osborn (1969). The samples (volume 0.05–0.1 ml) were mixed with tracking dye [5 μ l of 0.05% (w/v) Bromophenol Blue], sodium dodecyl sulphate

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(10%, w/v; 5 μ l), glycerol (1 drop) and 2-mercaptoethanol (5 μ l), and heated (100°C, 2min) before electrophoresis. The gels [8% (w/v) acrylamide, 0.27% (w/v) methylenebisacrylamide] were polymerized as described by Weber & Osborn (1969) and run at a constant current of 8mA/gel until the tracking dye had moved at least 8cm. The gels were stained in Coomassie Brilliant Blue (Weber & Osborn, 1969) and de-stained by soaking for 7–10 days in 7.5% (v/v) acetic acid–5% (v/v) methanol. The gels were scanned at 550nm by using the 2410-S linear transport attachment to the Gilford model 2000 spectrophotometer.

DNA preparations

Bacteriophage 82c.C600 (P1_{rP1}–m_{P1}–) DNA and 82c.C600 (P1_{rP1}–m_{P1}+) DNA were prepared as described previously (Brockes *et al.*, 1972).

To prepare bacteriophage λ C₁ 857 S₇.K DNA and λ C₁ 857 S₇.K(P1) DNA, *E. coli* W1485 (λ C₁ 857 S7) or *E. coli* W1485 (λ C₁ 857 S7) (P1) were grown to 5×10^8 cells/ml at 30°C in 1 litre of L broth. The temperature was raised to 42°C for 10min and then lowered to 37°C for 3–4h. The cells were collected by centrifugation (5000g, 20min, 2°C), resuspended in 20ml of λ dil medium (Kaiser & Hogness, 1960) and lysed with 5ml of chloroform. The suspension was clarified by centrifugation (10000g, 30min, 2°C), and the bacteriophage were recovered from the supernatant by centrifugation, purified by forming bands in 41.5% (w/w) CsCl, and extracted with freshly distilled phenol, all as described previously for bacteriophage 82c DNA (Brockes *et al.*, 1972).

Purification of the modification enzyme

This procedure differs in several respects from that previously used (Brockes *et al.*, 1972) and is thus described in detail. All buffers contained 5% (v/v) glycerol. The molarities quoted for the potassium phosphate buffers refer to the concentration of phosphate ion. The enzyme was assayed as described previously (Brockes *et al.*, 1972).

Growth of cells. Strain K140 (P1C₁ 162) was grown in L broth (50 litres) at 30°C to approx. 1×10^9 cells/ml. The temperature was raised to 42°C for 15–20min and lowered to 0–5°C by adding frozen 0.05M-NaCl. The cells were harvested in an Alfa-Lavall centrifuge and stored at –20°C. All subsequent operations were performed at 4°C.

Step 1: preparation of crude extract. Cells (850g) were resuspended in 900ml of breaking buffer (0.05M-Tris-HCl, pH8.0, 0.1mM-EDTA, 1mM-2-mercaptoethanol, 0.01M-MgCl₂), then 1.5kg of acid-washed glass beads was added and the mixture was blended (with intermittent cooling) in a Waring Blendor (5min at low speed, 7.5min at high speed). The supernatant was decanted from the beads, which

were washed with breaking buffer (200ml). Pancreatic deoxyribonuclease (5mg) was added to the pooled supernatant and washings, which were incubated at 5–8°C for 40min and then centrifuged at low speed (10000g, 20min, 0°C), and at high speed (MSE Super-speed 65 centrifuge, 6 $\times$ 250ml rotor, 18000rev./min, 2°C, 4h). The pellets from these centrifugations were discarded.

Step 2: (NH₄)₂SO₄ fractionation. (NH₄)₂SO₄ (233g) was added slowly to the supernatant from Step 1 (volume 1.2 litres) at 0°C. The precipitate was collected by centrifugation (12000g, 20min, 2°C) and discarded. The supernatant was precipitated with (NH₄)₂SO₄ (183g). This precipitate was collected by centrifugation (12000g, 20min, 2°C), dissolved in 300ml of 0.02M-PM buffer (0.02M-potassium phosphate, pH6.7, 5mM-2-mercaptoethanol) and dialysed extensively against 0.02M-PM buffer.

Step 3: fractionation on DE23 cellulose. The dialysed (NH₄)₂SO₄ fraction was diluted to 2 litres with 0.02M-PM buffer and applied to a column (17cm $\times$ 9.5cm diam.) of Whatman DE23 cellulose that had been equilibrated with 0.02M-PM buffer. The column was washed with 800ml of 0.02M-PM buffer, 800ml of 0.05M-PM buffer (0.05M-potassium phosphate, 5mM-2-mercaptoethanol) and then eluted with 0.3M-PM buffer (0.3M-potassium phosphate, 5mM-2-mercaptoethanol). The conductivity of the effluent was equal to that of 0.3M-PM after the passage of 940ml of the buffer. All of the fraction that was eluted after commencing to apply the 0.05M buffer was taken and concentrated by adding (NH₄)₂SO₄ (950g), the precipitate was collected by centrifugation (12000g, 20min, 2°C), dissolved in 90ml of 0.02M-PM buffer and dialysed extensively against 0.05M-PM buffer.

Step 4: chromatography on DE52 cellulose. The fraction from DE23 cellulose was applied to a column (14.5cm $\times$ 6.25cm diam.) of DE52 cellulose that had been equilibrated with 0.05M-PM buffer. The column was washed with 600ml of 0.05M-PM buffer and eluted with a linear 2-litre gradient of 0.05–0.3M-PM buffer. Fractions of volume 30ml were collected and the activity was found in six neighbouring fractions (with a mean phosphate concentration of 0.12M), which were concentrated by adding (NH₄)₂SO₄ (155g). The precipitate was collected by centrifugation, dissolved in 30ml of 0.05M-PEM buffer (PM buffer containing 0.5mM-EDTA) and dialysed extensively against 0.05M-PEM buffer.

Step 5: chromatography on P11 phosphocellulose. The fraction from DE52 was diluted to 80ml with 0.05M-PEM buffer and applied at approx. 40ml/h to a column (7.2cm $\times$ 3.75cm diam.) of Whatman P11 phosphocellulose that had been equilibrated with 0.05M-PEM buffer. The column was washed with 75ml of 0.05M-PEM buffer and eluted with a linear 500ml gradient of 0.05–0.3M-PEM buffer. Fractions (10ml) were collected and the activity was found in

three neighbouring fractions (with a mean phosphate concentration of 0.13M), which were pooled, concentrated by ultrafiltration in a Diaflo Amicon pressure cell (Amicon Corp., Lexington, Mass. 02173, U.S.A.) with a PM10 membrane at 140kPa (20lb/in²) of N₂, and the solvent was changed by concentration on four occasions after addition of a 10-fold excess of 0.02M-PED buffer (0.02M-potassium phosphate, pH6.5, 0.5mM-EDTA, 1mM-dithiothreitol). The volume of the final concentrate was 5ml (1.3mg of protein/ml).

Step 6: chromatography on CM-Sephadex. Of the P11 fraction, 4ml was applied to a column (5cm × 1.25cm diam.) of CM-Sephadex C25 that had been equilibrated with 0.02M-PED buffer. The column was washed with 20ml of 0.02M-PED buffer and eluted with increasing concentrations of potassium phosphate: 0.1M-PED buffer (30ml), 0.2M-PED buffer (20ml) and 0.4M-PED buffer (30ml). Fractions (5ml) were collected and the activity was eluted sharply in the first two fractions of the 0.4M-PED buffer step. These were pooled and concentrated in the Amicon cell, and the solvent was changed to 0.01M-potassium morpholinoethanesulphonate, pH6.5, 1mM-dithiothreitol, 0.1mM-EDTA, by successive concentration as employed for the fraction from P11. The concentrate was stored at 0°C and lost approx. 80% of its activity after 7 months.

Results

Purification of the modification enzyme

To purify the modification enzyme it is helpful to increase its specific activity in extracts. Scott (1970) has described a thermoinducible mutant of bacteriophage P1. This bacteriophage seemed an attractive source of the enzyme, since the induction of the pro-

phage should lead to a considerable increase in the number of gene copies (see, e.g., Kondo *et al.*, 1970). The specific activity of the modification enzyme in fractionated extracts of the induced lysogen is approximately 4-fold higher than that in the lysogenic strain 1100r_K-(P1), which was previously used as a source of enzyme (J. P. Brockes, unpublished work). The enzyme was therefore purified from the induced lysogen (see the Materials and Methods section), and the purification is summarized in Table 1. The CM-Sephadex fraction is thus purified approximately 1400-fold from the (NH₄)₂SO₄ fraction.

The purity of the CM-Sephadex fraction was investigated by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate. The anionic detergent dissociates oligomeric proteins into their component subunits, which run on the gel with a mobility that is inversely proportional to the logarithm of their molecular weight (Shapiro *et al.*, 1967; Weber & Osborn, 1969). The CM-Sephadex fraction showed two prominent bands and five trace bands when 24 µg of protein was analysed in this way.

Do these two bands represent the component subunits of the modification enzyme? A portion of the CM-Sephadex fraction was sedimented through a long glycerol gradient that was calibrated by reference to marker proteins run in a sister bucket of the same rotor (see legend to Fig. 1). A single peak of modification activity was found at approximately the position occupied by a molecule with a sedimentation coefficient in water of 6S. The active fractions were concentrated by acid precipitation and analysed by sodium dodecyl sulphate-polyacrylamide-gel electrophoresis. As shown in Plate 1 the intensities of the two bands increased and decreased co-ordinately across the peak of activity. Although close inspection of the gels revealed trace bands in some of them, none of these bands exhibited such a change in intensity across the peak of activity. On the basis of the

Table 1. *Summary of enzyme purification*

A unit of activity makes 0.16pmol of methyl groups acid-insoluble/5 µg of unmodified bacteriophage 82 DNA per 60min incubation at 30°C. The blank value for incorporation into modified DNA is subtracted (Brockes *et al.*, 1972). The recovery value for the CM-Sephadex fraction is corrected for purification of total pooled P11 fraction. Protein in subcellular fractions was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Purification stage	Protein (mg)	Activity (units)	Specific activity (units/mg)	Recovery (%)
High-speed supernatant of crude extract	26400	—	—	—
(NH ₄) ₂ SO ₄ fraction	17800	106800	6.0	100
DE23 fraction	5640	98352	17.4	92
Pooled DE52 fraction	400	48481	121.2	45
Pooled P11 fraction	6.5	17563	2702	16
Pooled CM-Sephadex fraction	1.12	9814	7869	9

Fig. 1. Analysis of the CM-Sephadex fraction by glycerol-gradient sedimentation

A 0.5ml portion (0.3mg of protein) of the CM-Sephadex fraction was layered on a 12–25% (v/v) glycerol gradient (volume 13.5ml) in 0.01M-potassium morpholinoethanesulphonate, pH6.5, containing 1mM-dithiothreitol and 0.5mM-EDTA. After centrifugation (MSE Superspeed 75 centrifuge, 6×15 ml SW rotor, 70h, 24000rev./min, approx. 2°C), the gradient was collected in 15-drop fractions. Samples (0.02ml) were assayed for methylation of DNA from bacteriophages λC_1 857 S7.K ($\circ$) and λC_1 857 S7.K(P1) ($\bullet$) in a reaction mixture (volume 0.05ml) containing 0.05M-potassium morpholinoethanesulphonate, pH6.0, 5mM-2-mercaptoethanol, 0.5mM-EDTA, $5\mu\text{M}$ -*S*-adenosyl-L-[methyl- ^3H]methionine (8.9Ci/mmol) and $5\mu\text{g}$ of bacteriophage DNA. The mixture was incubated and assayed for acid-precipitable ^3H radioactivity as described previously (Brockes *et al.*, 1972). A parallel gradient was layered with a solution (volume 0.5ml) containing 5mg of bovine haemoglobin (Hb) and 0.4mg of bacterial alkaline phosphatase (BAP). After sedimentation and fractionation, the haemoglobin was located by means of its E_{410} and the phosphatase by following (at 410nm) the hydrolysis of 1mM-*p*-nitrophenyl phosphate in 1M-Tris-HCl, pH8.0.

association of these two bands through the purification procedure and sedimentation analysis it is concluded that they are the components of the 6S modification enzyme. It is possible, but unlikely, that undetectable components are responsible for the activity.

Molecular weights and molar proportions of the two components

The molecular weights of the two components were estimated from their mobilities (with respect to

Fig. 2. Plot of the mobility of various proteins on sodium dodecyl sulphate-polyacrylamide gels versus the logarithm of their molecular weight

The mobilities of the two components (arrows), and of various marker proteins ($\bullet$), with respect to Bromophenol Blue, were determined as described by Weber & Osborn (1969). The two components (applied as $12\mu\text{g}$ of CM-Sephadex fraction) were run on one gel and the markers (approx. $10\mu\text{g}$ of each) were run in two groups of four on parallel gels. The molecular-weight markers were: 1, phosphorylase *a* (94000); 2, bovine serum albumin (68000); 3, L-amino acid oxidase (61000); 4, fumarase (49000); 5, ovalbumin (43000); 6, liver alcohol dehydrogenase (41000); 7, yeast alcohol dehydrogenase (37000); 8, myoglobin (17200).

Bromophenol Blue) in sodium dodecyl sulphate-polyacrylamide-gel electrophoresis relative to eight reference samples (Shapiro *et al.*, 1967; Weber & Osborn, 1969). In the experiment described in Fig. 2, the mobilities of the two components of the P1 modification enzyme corresponded to molecular weights of 70000 ± 5000 and 45000 ± 5000 . This result is the average of four determinations, each of which was carried out in parallel with the eight reference proteins.

The molar proportion of each component was estimated in the following way. The stained gels of the CM-Sephadex and glycerol gradient fractions of

EXPLANATION OF PLATE I

Analysis of fractions from the glycerol gradient by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis

Fractions 10–15 from the glycerol gradient of Fig. 1 were precipitated by adding 10% (w/v) trichloroacetic acid (0.4ml). After 15min at 0°C, the precipitate was collected by centrifugation (Quickfit microcentrifuge, 20000g, 5min) and dissolved in 0.01M-sodium phosphate, pH7.0, containing 1% (w/v) sodium dodecyl sulphate and 1% (w/v) 2-mercaptoethanol (0.05ml), before sodium dodecyl sulphate–polyacrylamide-gel electrophoresis (see the Materials and Methods section).

the enzyme were scanned in a recording densitometer. The areas under the two peaks were determined from the weight of each peak on a Xerox copy of the densitometer traces. The amounts of Coomassie Brilliant Blue bound to various proteins differ by less than 10% on a weight-for-weight basis (Fazekas de St. Groth *et al.*, 1963). Thus if the two components are present in equimolar amounts, the areas under the densitometer peaks should be in the ratio 7:4.5, that is 1.55. The average ratio found from the experiments shown in Fig. 1 was 1.52. The association of these two components in a molecule that sediments at 6S (Fig. 2) precludes the possibility of anything other than a 1:1 dimer, assuming that the conformation is not grossly abnormal.

Discussion

Genetic analysis of the restriction and modification systems of *E. coli* strain K, *E. coli* strain B and bacteriophage P1 has led to the formulation of a three-gene model for the components of these activities (Arber & Linn, 1969). According to this model, the restriction enzyme is composed of three polypeptide chains, two of which are common to the modification enzyme (Hubacek & Glover, 1970). The results given here on the subunit structure of DNA methylase M.P and DNA methylase M.B are consistent with this model. For DNA methylase M.B it has been shown that the two subunits are also components of endonuclease R.B, which has one extra subunit (Lautenberger *et al.*, 1972).

The detailed role of the two subunits in the modification mechanism is unknown. The study of

the nucleotide sequences around the methylated base should provide some insight into the mechanism of sequence recognition by these enzymes.

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