Overproduction, purification and characterization of M.*Eco*HK31I, a bacterial methyltransferase with two polypeptides

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The two overlapping genes coding for *Eco*HK31I methyltransferase have previously been cloned, sequenced and expressed [Lee, Kam and Shaw (1995) *Nucleic Acids Res.* **23**, 103–108]. Here we describe protocols developed to purify polypeptides α and β together or separately, to apparent homogeneity by various chromatographic media. M.*Eco*HK31I is a heterodimer with a native molecular mass of 61 kDa. Its specific activity towards non-methylated lambda DNA was 3.0×10^5 units per mg of protein. The respective denatured molecular masses of polypeptides

α and β were 38 and 23 kDa, and their pI values were 8.7 and 6.8. Initial rate kinetic parameters of the native enzyme were 2.0 nM, 0.58 μ M and 3 min⁻¹ for K_m^{DNA} , K_m^{AdoMet} and k_{cat} , respectively, where AdoMet stands for S-adenosyl-L-methionine. Fully active enzyme was reconstituted by co-purifying the two separately synthesized polypeptides, and activity assays confirmed our previous finding that two polypeptides were needed to methylate substrate DNA.

INTRODUCTION

DNA methyltransferases (MTases) exist in most phyla. In higher eukaryotic organisms mC5-MTases are the only type of DNA MTase and they play an important role in gene regulation and development [2]. In bacteria there are three types of MTases, mC4-, mC5- and mA6-, classified according to the methylation site of the DNA. MTase and its cognate restriction endonuclease (ENase) form the restriction–modification system. The role of the MTase is to protect the host from its own ENase digestion while the ENase acts to degrade the invasion of foreign DNA [3]. Many restriction–modification systems reside on naturally occurring plasmids and these systems may ensure that the plasmid is not lost during replication [4].

mC5-MTases are homogeneous in organization. A typical bacterial mC5-MTase contains ten conserved motifs and a variable region lying between motifs VIII and IX [5–7]. Eukaryotic mC5-MTase is thought to be a fusion protein between a long N-terminal domain, containing a potential metal-binding region, and a bacterial mC5-MTase, because the motif organization at its C-terminus is similar to a bacterial mC5-MTase but its N-terminal sequence is unrelated and has a role in regulating the activity of the C-terminal MTase domain [8–10]. Functions of the conserved motifs in mC5-MTases are for the binding of S-adenosyl-L-methionine (AdoMet) cofactor and the transfer of the methyl group from AdoMet to cytosine 5 of the target sequence. The function of the variable region is for specific DNA interaction [11–13].

We have previously cloned and sequenced the locus of *Eco*HK31I MTase from a clinically isolated *Escherichia coli* HK31 strain. M.*Eco*HK31I methylates the inner cytosine of the sequence 5'-YGGCCR-3' and consists of polypeptides α and β , with the latter gene encoded in an alternative reading frame of the former [13a]. M.*AquI* is another naturally occurring mC5-

MTase with two polypeptides. Polypeptide α in M.*AquI* contains motifs I–VIII and polypeptide β contains motifs IX and X. Their open reading frames overlap by a few residues in the variable region [14]. Detached parts of closely related mC5-MTases have also been shown to complement each other [12,13]. Nevertheless it is not clear how these separate polypeptides are organized in space.

To compare the properties of M.*Eco*HK31I with other mC5-MTases and as a first step to understanding the structure–function relationship of the two polypeptides in M.*Eco*HK31I, we have developed protocols to purify the native recombinant enzyme and the two polypeptides to homogeneity and investigated their biophysical and biochemical properties. The present paper presents data on these studies.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

E. coli strain K802 [pEcoHK31G] was used to synthesize M.*Eco*HK31I. *E. coli* BL21 (DE3) [plysE, pET3a-M38] and BL21 (DE3) [plysS, pET3a-C23] were used to produce polypeptides α and β respectively [13a]. Plasmid pWM528 [15] was kindly provided by Dr. W. Mandelki. Bacterial strains were grown at 37 °C in M9ZB media [16] supplemented with ampicillin (50 μ g/ml) and chloramphenicol (34 μ g/ml). Cultures were induced with 0.4 mM isopropyl β -D-galactopyranoside (IPTG) at an A_{600} of about 0.6 and growth was continued for 3 h before harvesting the cells.

Purification of M.*Eco*HK31I

M.*Eco*HK31I was purified from *E. coli* K802 [pEcoHK31G]. pEcoHK31G was constructed by ligating a 1.4 kb *DraI*–*AsuII*

Abbreviations used: AdoMet, S-adenosyl-L-methionine; DTT, dithiothreitol; ENase, restriction endonuclease; Gu-HCl, guanidine hydrochloride; IPTG, isopropyl- β -D-thiogalactopyranoside; MTase, methyltransferase.

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fragment containing the methylase gene to pUC19 DNA. All purification steps were carried out at 4 °C unless otherwise stated.

10 g of cell paste was resuspended in 30 ml of buffer A [20 mM sodium phosphate, pH 7.0, containing 150 mM NaCl, 0.1 mM EDTA, 5 mM β -mercaptoethanol and 5% (v/v) glycerol] and PMSF was added to 1 mM final concentration. Cells were disrupted by sonication and the cell debris was removed by ultracentrifugation at 100 000 *g* for 1 h. Supernatant was applied to an Affi-gel heparin agarose column (Bio-Rad, 18 cm $\times$ 10 cm) equilibrated with buffer A at a flow rate of 0.3 ml/min. Proteins were eluted with 300 ml linear gradient of 0.15–1.0 M NaCl in buffer A. Active fractions containing MTase activity were dialysed against buffer A and applied to an FPLC-MonoS HR5/5 column (Pharmacia) at a flow rate of 1 ml/min. After being washed with 10 ml of buffer A, the proteins were eluted at 1 ml per fraction with a 20 ml linear gradient of 0.15–1.0 M NaCl in buffer A. Active fractions that were eluted at 0.37–0.42 M NaCl were pooled and dialysed against storage buffer [20 mM sodium phosphate, pH 7.0, containing 50 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 200 μ g/ml BSA and 50% glycerol]. The purified protein was stored at –20 °C.

Purification of polypeptide α

To purify polypeptide α , 6 g of *E. coli* BL21 (DE3) [plysE, pET3a-M38] cell paste was resuspended in 24 ml of buffer A and PMSF was added to a final concentration of 1 mM. The cells were disrupted by sonication and the cell debris was removed by centrifugation at 12 000 *g* for 30 min. The supernatant was collected and passed through a 0.2 μ m filter to remove fine particles. The filtrate (23 ml) was loaded onto a Fast Flow S cation-exchange column (Bio-Rad, 2.5 cm $\times$ 10 cm) that had been pre-equilibrated with buffer A at a flow rate of 2 ml/min. The column was washed with buffer A until the A_{280} of the eluate reached almost zero. Proteins were eluted at 4 ml per fraction with a 160 ml linear gradient of 0.15–1.0 M NaCl in buffer A. Active fractions (assayed by complementation with purified polypeptide β) were eluted from 0.35–0.45 M NaCl and were pooled, diluted with 2 vol. of buffer A and applied to an Affi-gel heparin agarose column at a flow rate of 0.3 ml/min. The column was washed with 50 ml of buffer A and the proteins were eluted at 3 ml per fraction with a 300 ml linear gradient of 0.15–1.0 M NaCl in buffer A. Active fractions eluted from 0.28–0.34 M NaCl were pooled, diluted with 2 vol. of buffer A and applied to an FPLC-MonoS HR5/5 column at a flow rate of 1 ml/min. After washing with 10 ml of buffer A, the proteins were eluted at 1 ml per fraction with a 20 ml linear gradient of 0.15–1.0 M NaCl in buffer A. Active fractions that were eluted at 0.48–0.52 M NaCl were pooled and dialysed against storage buffer (20 mM sodium phosphate, pH 7.0, containing 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 200 μ g/ml BSA and 50% glycerol). The purified protein was stored at –20 °C.

Purification of polypeptide β

To purify polypeptide β , 3 g of *E. coli* BL21 (DE3) [plysS, pET3a-C23] cell paste was resuspended in 20 ml of buffer B (20 mM Tris/HCl, pH 7.5, containing 0.1 mM EDTA, 5 mM β -mercaptoethanol and 5% glycerol) and PMSF was added to a final concentration of 1 mM. The cells were disrupted by sonication and centrifuged at 12 000 *g* for 30 min. The supernatant was discarded and the pellet washed with 20 ml of buffer B supplemented with 2% (v/v) Triton X-100. The inclusion

bodies were collected by centrifugation at 12 000 *g* for 30 min and dissolved in 6 M guanidine hydrochloride (Gu-HCl). The solubilized protein was applied to an FPLC-Superose 12 column (Pharmacia) pre-equilibrated with 6 M Gu-HCl in buffer B at a flow rate of 0.4 ml/min. Proteins were eluted with 6 M Gu-HCl in buffer B and collected in fractions. Fractions containing polypeptide β (detected by SDS/PAGE) were pooled and dialysed exhaustively with buffer B. Precipitated particles were removed by centrifugation at 12 000 *g* for 30 min and the supernatant was dialysed against storage buffer (10 mM Tris/HCl, pH 7.5, containing 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 200 μ g/ml BSA and 50% glycerol). The purified protein was stored at –20 °C.

Complementation of polypeptides α and β *in vitro*

Determination of methylation activity *in vitro* involves the addition of polypeptide α or β , or both, into 10 μ l of MTase reaction buffer (10 mM Tris/HCl, pH 7.5, containing 50 mM NaCl, 10 mM EDTA, 80 μ M AdoMet, 1 μ g of non-methylated lambda DNA and 5 mM β -mercaptoethanol) and incubation for 1 h at 37 °C. DNA samples after phenol/chloroform extraction were digested with 5 units of *R.Eco*HK31I in reaction buffer (10 mM Tris/HCl, pH 7.9, containing 10 mM $MgCl_2$, 50 mM NaCl and 1 mM DTT) for 1 h at 37 °C and analysed by gel electrophoresis.

Biophysical properties of polypeptides α and β

The molecular masses of the reduced and denatured proteins were determined by comparing the retention times with appropriate molecular mass markers on an FPLC-Superose 12 column (Pharmacia) with elution buffer C (6 M Gu-HCl in buffer B) or by SDS/PAGE (12% gel). The native molecular mass and subunit composition of *M.Eco*HK31I were determined by FPLC-Superose 12 (Pharmacia) chromatography in buffer A. Isoelectric points of polypeptides α and β were determined by the method of O'Farrell [17].

Initial rate kinetics of *M.Eco*HK31I

The method used to study *M.Eco*HK31I kinetics was based on that of Rubin and Modrich [18]. The standard assay for MTase activity measures the transfer of [3 H]methyl groups to pWM2372 DNA, which contains a unique *Eco*HK31I site. pWM2372 was constructed by ligating a 322 bp *Pvu*II fragment, which contains a unique *Eco*HK31I site (5'-TGGCCG-3') from pUC19 to the *Sma*I site of pWM528. Compositions of the reaction mixture are stated in Figure 4. After incubation at 37 °C for 10 min, 80 μ l samples were spotted onto Whatman DE81 filter papers, which were washed three times with 50 mM KH_2PO_4 , and treated for 10 min each with 80% ethanol, 95% ethanol and anhydrous diethyl ether. The filters were dried under an infrared lamp and labelled DNA that was bound to the filter papers was measured with a Beckman LS6000SC liquid-scintillation counter.

RESULTS

Overexpression and purification of *M.Eco*HK31I, polypeptides α and β

*M.Eco*HK31I was purified from a K802 strain transformed by a pUC19 plasmid carrying the MTase gene of *Eco*HK31I. A scheme was developed to purify this protein in large quantity. Figure 1(A) and Table 1 summarize the activities and purities

Figure 1 Purification profile of *M.EcoHK31I* (A), polypeptide α (B) and polypeptide β (C)

(A) Lane 1, molecular mass markers; lane 2, crude cell extract; lane 3, Affi-gel heparin pool; lane 4, FPLC-MonoS pool. (B) Lane 1, molecular mass markers; lane 2, crude cell extract; Lane 3, FFS-Sepharose pool; lane 4, Affi-gel heparin pool; lane 5, FPLC-MonoS pool. (C) Lane 1, molecular mass markers; lane 2, crude cell extract; lane 3, insoluble protein fraction; lane 4, washed insoluble protein fraction; lane 5, FPLC-Superose12 pool. Abbreviation: kDa.

achieved during the course of purification. The final yield and specific activity obtained were 9.6×10^5 units per g wet cells and 3.0×10^5 units per mg protein respectively.

The construction of expression vectors pET3a-M38 and pET3a-C23, which synthesized polypeptides α and β respectively under the control of a T7 RNA polymerase-driven promoter, has been described previously [13a]. Attempts to transform *E. coli* BL21 (DE3) [plysS] with pET3a-M38 were unsuccessful. It is likely that the basal expression of polypeptide α in this strain was

detrimental to the host, as BL21 (DE3) is proficient in methylation cytosine restriction (Mcr^+) as described in New England Biolabs Catalogue, 1995. However, we succeeded in transforming BL21 (DE3) [plysE] with pET3a-M38. The high level expression of lysozyme in this strain further suppressed the activity of T7 RNA polymerase [16]. On IPTG induction, polypeptide α accounted for about 5 % of total cellular protein. Polypeptide α was purified to near homogeneity after three chromatographic steps. A routine purification procedure starting with 6 g of IPTG-induced cells yielded 3.6 mg of protein (Figure 1B and Table 2).

Polypeptide β was synthesized in *E. coli* BL21 (DE3) [plysS, pET3a-C23] and accounted for up to about 20 % of total cellular protein on IPTG induction. Most of the induced proteins were found as inclusion bodies. The inclusion bodies, after being washed with 2 % Triton X-100 in buffer B, were dissolved in 6 M Gu-HCl and purified by FPLC-Superose 12 column chromatography. Gu-HCl was removed by dialysis and polypeptide β was purified to near homogeneity with a yield of about 7 mg/g wet cells (Figure 1C and Table 2).

As a means of reconstituting polypeptides α and β , IPTG-induced *E. coli* cells containing these polypeptides were mixed, lysed by sonication and purified on Affi-gel heparin and FPLC-MonoS columns by a scheme similar to that for recombinant *M.EcoHK31I*. The yield of active enzyme was about 5 mg/g wet cells.

Biophysical characterization of polypeptides α and β

The molecular masses of the denatured polypeptides α and β as judged by SDS/PAGE (12 % gel) were 38 and 23 kDa respectively (Figures 1B and 1C). This matched very well with the molecular masses determined with an FPLC-Superose 12 column under denaturing and reducing conditions. The native molecular mass of *M.EcoHK31I* determined by gel filtration was 61 kDa (Figure 2). The isoelectric points of polypeptides α and β were 8.7 and 6.8, respectively.

Methylation activity *in vitro*

Methylation experiments *in vitro* with purified proteins showed that neither polypeptide α nor β alone protected non-methylated lambda DNA from *R.EcoHK31I* digestion efficiently. However, the substrate DNA was completely modified when polypeptides α and β coexist (Figure 3). Similarly, a study of radiolabelled methyl group incorporation *in vitro* showed that there was an approx. 67-fold increase in methyl group transfer to non-methylated lambda DNA when both polypeptides were used (Table 3).

Initial rate kinetics of *M.EcoHK31I*

The reaction rate of *M.EcoHK31I* was examined with *A* *h*NI linearized pWM2372 DNA, which contains a unique *EcoHK31I* site. The initial rate of methylation was linear with respect to enzyme concentration up to 5 nM in the presence of saturating concentrations of substrates (20 nM pWM2372 DNA and 3 μ M AdoMet). The velocity of methylation was linear for 80 min in reactions with a DNA/enzyme concentration ratio of 5 or above. Furthermore the enzyme obeyed Michaelis-Menten kinetics with respect to both AdoMet and DNA (Figures 4A and 4B). The k_{cat} , determined from Figure 4(A) was 3.2 methyl transfers per

Table 1 Purification of *M.EcoHK311*

	$10^{-6} \times$ Total activity* (units)	Total protein† (mg)	$10^{-3} \times$ Specific activity (units/mg)	Yield (%)	Purification factor
Crude lysate	24	960	25	100	1
Affi-gel heparin	20	75	260	83	10.4
FPLC-MonoS	9.6	32.4	300	40	12

* One unit is the amount of enzyme required to protect 1 μ g of non-methylated lambda DNA (in 1 h at 37 °C in a 10 μ l reaction mixture) against cleavage by *R.EcoHK311*.

† Protein was determined by Bradford assay.

Table 2 Purification of polypeptides α and β

Purification step	Volume (ml)	Protein concn.* (mg/ml)	Total protein (mg)	Yield of protein (%)
Polypeptide α:				
Crude lysate	23	34.5	793.5	100
Econo-FFS	22.8	16.5	376.2	47
Affi-gel Heparin	14.8	1.17	17.3	2.2
FPLC-MonoS	6.0	0.6	3.6	0.5
Polypeptide β:				
Crude inclusion bodies	20	5	100	100
Washed inclusion bodies (in 6 M Gu-HCl)	20	2	40	40
FPLC-Superose 12	20	1	20	20

* Protein was determined by Bradford assay.

Figure 2 Determination of *M.EcoHK311* molecular mass in native conditions by FPLC-Superose 12 column chromatography

The elution volume is represented in terms of $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume, V_0 the void volume and V_t the bed volume. The elution position of *M.EcoHK311* is marked with an open circle. Abbreviation: kD, kDa.

min, whereas from Figure 4(B) it was 3.6. The corresponding K_m values were 0.58 μ M for AdoMet and 2.0 nM for pWM2372 DNA.

Figure 3 Methylase activity of the purified polypeptides α and β *in vitro* in protecting non-methylated lambda DNA

Details are described in the Materials and methods section. Lanes 1–3, DNA treated with 1, 0.5 and 0.25 μ g of polypeptide β respectively; lanes 4–6, DNA treated with 1, 0.5 and 0.25 μ g of polypeptide α respectively; lanes 7–9, DNA treated with 1, 0.5 and 0.25 μ g of both polypeptides respectively; lane 10, DNA treated with *M.EcoHK311*.

Table 3 Incorporation of [3 H]AdoMet to non-methylated lambda DNA using purified polypeptides

A fixed amount of purified protein was mixed with 1 μ g of non-methylated lambda DNA in the presence of 1 μ M [3 H]AdoMet in 50 μ l MTase reaction buffer and incubated for 1 h at 37 °C. DNA samples, after extraction with phenol/chloroform and precipitation with 10% (w/v) trichloroacetic acid, were spotted onto a glass-fibre filter (Whatman). The filter was washed with 10% trichloroacetic acid and ethanol and dried before radioactive counting.

Sample	Radioactivity (c.p.m.)
Water	42
Polypeptide α	951
Polypeptide β	448
Polypeptides α and β	30 031

DISCUSSION

M.EcoHK311 is a naturally occurring mC5-MTase with two subunits. The small subunit contains the conserved motif IX and is encoded in an alternative reading frame to that of the large subunit [13a]. To characterize the biochemical properties of *M.EcoHK311* and to purify it for crystallization and structure-

Figure 4 Steady-state kinetics of *M.EcoHK31I* Determination of K_m and K_{cat} for pWM2372 DNA (A) and AdoMet (B)

(A) Reactions (0.1 ml) contained 10 mM Tris/HCl, pH 7.5, 1 mM EDTA, 200 μ g/ml BSA, 5 mM β -mercaptoethanol, 3 μ M [3 H]AdoMet, 0.2 nM *M.EcoHK31I* and indicated concentrations of pWM2372 DNA. Incubation was at 37 °C for 10 min. (B) Reactions were as in (A) except that they contained 20 nM pWM2372 DNA, 1 nM *M.EcoHK31I* and the indicated concentrations of AdoMet.

function studies, the two polypeptides were synthesized together or separately in *E. coli* and protocols were developed to purify them to apparent homogeneity.

Much polypeptide β existed as inclusion bodies in *E. coli* on induction with 0.4 mM IPTG. It has been reported that the formation of *M.MspI* inclusion bodies can be prevented by lowering the induction temperature [19]. However, induction of BL21 (DE3) [plysS, pET3a-C23] at 30 °C and with 1 mM IPTG did not increase the amount of soluble polypeptide β in the cells. Nevertheless the inclusion bodies were easily purified from contaminating proteins and the soluble form after renaturation was able to complement polypeptide α .

As the native molecular mass of *M.EcoHK31I* measured by gel filtration was 61 kDa, it is believed that this methylase consists of a heterodimer of polypeptide α (38 kDa) and polypeptide β (23 kDa). Ionic interactions may contribute to their association, as polypeptide α is highly basic (pI 8.7) whereas polypeptide β is slightly acidic (pI 6.8).

The catalytic efficiency (k_{cat}/K_m) of *M.EcoHK31I* towards pWM2372 was $2.7 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$. For comparison, the k_{cat}/K_m for *M.EcoRI* on pBR322 is $4.1 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ [20]; the k_{cat}/K_m values for *M.HhaI* on poly(dG·dC) and on a 37mer DNA duplex are 9.6×10^6 and $7.7 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively [21,22]. Therefore although *M.EcoHK31I* is made up of two polypeptides, it performs with an efficiency similar to other mC5-MTases.

Reconstitution of *M.EcoHK31I* *in vitro* by mixing the two purified polypeptides resulted in a methylase that had about 12 % of the activity of the reconstituted enzyme *in vivo*. It is likely

that only some of the subunits were reconstituted properly in the cell-free environment. To confirm this, induced cells containing polypeptides α and β respectively were mixed and the methylase was purified on Affi-gel heparin agarose and FPLC-MonoS columns. *M.EcoHK31I* reconstituted by this means had a specific activity similar to the enzyme reconstituted *in vivo*.

Previously we found that expression of the gene encoding polypeptide α alone modified DNA *in vivo* and there was a 2-fold increase in methyl group transfer to substrate DNA *in vitro* [13a]. A 2-fold increase of methyl group transfer was also observed when purified polypeptide alone was tested *in vitro* (Table 3). The existence of low methylation activity in polypeptide α is consistent with our model in which polypeptide α contains all the essential components for substrate recognition and catalysis, and the role of polypeptide β is for proper docking of DNA.

Crystal structures of two mC5-MTases *M.HhaI* and *M.HaeIII* and a mA6-MTase *M.TaqI* have been reported [23–26]. *M.TaqI* is folded differently from the other two MTases. Nevertheless all three structures can be divided into two domains and the N-terminal domains contain all the elements required for catalytic activity. *M.HhaI* and *M.HaeIII* have highly conserved N-terminal domains for binding AdoMet and for catalysis, which involves the extrusion of substrate cytosine from the DNA helix. In contrast, the C-terminal domains that make specific contact with DNA fold substantially differently.

Sequence comparison of polypeptides α and β and other mC5-MTases suggests that polypeptide α also contains a large N-terminal domain and a small C-terminal domain. The large domain (residues 1–169 and 274–301) contains motifs I–VIII, and X should fold in a similar way to *M.HhaI* and *M.HaeIII* owing to the high degree of similarity between these sequences.

However, there is insufficient information to suggest how the small domain of *M.EcoHK31I* folds, as it contains the variable region of polypeptide α and polypeptide β . Nevertheless the resemblance of DKQGQGER in residues 189–198 of polypeptide α and GKGGQGER in residues 233–240 of *M.HhaI* suggests that at least one of the DNA recognition loops of *M.EcoHK31I* is structurally conserved and the loop bridging the two domains in *M.EcoHK31I* is probably much shorter than that of *M.HhaI*. With the production of about 10 mg/l purified *M.EcoHK31I* in our expression system, we are now in a position to generate crystals to elucidate the structure of this enzyme, which may act as a model for other mC5-MTases with detached parts.

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