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Purification, crystallization and preliminary X-ray analysis of the M.*Bse*CI DNA methyltransferase from *Bacillus stearothermophilus*

ALEKOS ATHANASIADIS,[a,b]† YANIS PAPANIKOLAU,[a,b]† MARIA RINA,[b] MARIA PAPADOVASILAKI,[b] ZBIGNIEW DAUTER,[c] KYRIACOS PETRATOS,[b] VASSILIS BOURIOTIS[a,b] AND MICHAEL KOKKINIDIS[a,b]* *at* [a]*University of Crete, Department of Biology, PO Box 1470, GR-71110 Heraklion, Crete, Greece,* [b]*Institute of Molecular Biology and Biotechnology (IMBB), PO Box 1527, GR-71110 Heraklion, Crete, Greece, and* [c]*European Molecular Biology Laboratory, c/o DESY, Notkestrasse 85, D-22603 Hamburg, Germany. E-mail: kokkinidis@venus.imbb.forth.gr*

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Abstract

The DNA methyltransferase M.*Bse*Cl from *B. stearothermophilus* methylates the N6 atom of the 3′ adenine in the sequence 5′-ATCGAT-3′. The 579-residue protein has been isolated and crystallized using seeding and microdialysis techniques. The crystals are monoclinic, space group $P2_1$ with cell dimensions $a = 53.7$, $b = 85.7$, $c = 151.8$ Å and $\beta = 95.1°$, two molecules in the asymmetric unit and diffract to at least 2.5 Å resolution.

1. Introduction

DNA-methyltransferases (MTases) are a family of enzymes that occur in virtually every major biological group (Adams, 1990). They catalyze the transfer of CH_3 from the cofactor *S*-adenosyl-L-methionine (AdoMet) to specific positions on bases in a target sequence of double-stranded DNA which may comprise two to eight base pairs. MTases fall into two major classes defined by the position methylated. The members of one class methylate a pyrimidine ring carbon yielding C5-methylcytosine (m^5C-MTases, C-MTases). Members of the other class methylate exocyclic amino N atoms (N-MTases), forming either N4-methylcytosine (m^4C-MTases) or N6-methyladenine (m^6A-MTases). In bacteria all three types of MTases have been found; their most common role is to protect host DNA from the cell's restriction enzymes (Wilson & Murray, 1991) and in mismatch repair (Modrich, 1991). In eukaryotes only m^5C-MTases have been observed; they have been implicated in the control of gene expression (Cedar, 1988), developmental regulation (Antequera, Macleod & Bird, 1989), genomic imprinting (Reik, Collick, Norris, Barton & Surani, 1987; Swain, Stewart & Leder, 1987) and mutagenesis (Cooper & Youssoufian, 1988).

Comparative sequence analyses of m^5C-MTases (Lauster, Trautner & Noyer-Weidner, 1989; Posfai, Bhagwat, Posfai & Roberts, 1989) reveal a homogeneous class of molecules built upon a set of ten motifs, some of which are implicated in cofactor binding and catalysis. N-MTases show four conserved segments (Timinskas, Butkus & Janulaitis, 1995) and represent a more diverse class of enzymes. Presently, three MTase structures are known: The m^5C-MTases M.*Hha*I (Cheng, Kumar, Posfai, Pflugrath & Roberts, 1993; Klimasauskas, Kumar, Roberts & Cheng, 1994) and M.*Hae*III (Reinisch, Chen, Verdine & Lipscomb, 1995), and the m^6A-MTase M.*Taq*I methyltransferase (Labahn *et al.*, 1994) are bilobal with similarly folded catalytic domains (harboring the catalytic and cofactor binding sites), and different DNA-recognition domains. The complexes of M.*Hha*I and M.*Hae*III with DNA reveal that the substrate nucleotide is flipped out of the double helix during the modification reaction. The common fold observed in the catalytic domains is probably universal for AdoMet-dependent methyltransferases (Schluckebier, O'Gara, Saenger & Cheng, 1995).

In order to gain further insight into the structural basis of N6-adenine methylation, we have initiated the crystallographic study of the M.*Bse*CI DNA methyltransferase from *Bacillus stearothermophilus*. M.*Bse*CI is a m^6A-MTase which methylates N6 of the 3′ adenine in the sequence 5′-ATCGAT-3′ with an optimum temperature for activity 323–328 K and an optimum pH of 7.4 (Rina & Bouriotis, 1993). Its sequence comprises 579 amino acids (66.7 kDa), lacks a detailed homology to other MTases (with the exception of the isomethyleric M.*Ban*III from *B. aneurinolyticus*), and exhibits the conserved motifs of m^6A-MTases (Rina, Markaki & Bouriotis, 1994).

2. Materials and methods

2.1. *Purification of M.BseCI*

The growth of bacterial cells, the original purification scheme and the assay of M.*Bse*CI have been fully described (Rina & Bouriotis, 1993). Crystallizability was however drastically improved with a new purification protocol. Frozen cell paste [*E.coli* (pBseCIM8)] was thawed in 100 ml buffer containing 50 m*M* Tris–HCl, pH 7.5, 200 m*M* NaCl, 5%(*v*/*v*) glycerol, 1 m*M* DTT, 1 m*M* EDTA and subsequently incubated at 273–277 K for 1 h with 300 μg ml^{-1} lysozyme and protease inhibitors [20 μg ml^{-1} leupeptine, 1 μg ml^{-1} phenylmethyl sulfonyl fluoride (PMSF), 150 μg ml^{-1} benzamidine]. The lysate was treated for 30 min with DNAase (10 μg ml^{-1}) in 11 m*M* $MgCl_2$. After removing the precipitate by centrifugation (17 000*g* for 1 h), the supernatant was diluted 1:4 with Tris–HCl buffer, pH 8.5 (to a concentration of 50 m*M* Tris–HCl) and loaded onto a Q-Fast Flow column (100 ml) pre-equilibrated in 50 m*M* Tris–HCl buffer, pH 8.5. The column was first washed with 3 column volumes of 50 m*M* Tris–HCl buffer, pH 8.5, then with 10 column volumes of 50 m*M* Tris–HCl, pH 7.5 (buffer *A*) and a linear gradient 0–1.0 *M* of NaCl in buffer *A* (1 l) was applied. Fractions exhibiting M.*Bse*CI activity eluted at approximately 0.25 *M* NaCl, and were pooled, diluted with an equal volume of 40 m*M* KH_2PO_4/NaOH buffer, pH 7.0 containing 0.2 *M* NaCl and loaded onto a 70 ml phosphocellulose column previously equilibrated with 20 m*M* KH_2PO_4/NaOH, pH 7.0 and 0.1 *M* NaCl (buffer *B*). The column was washed with buffer *B* until no absorption at

† A. Athanasiadis and Y. Papanikolau have equally contributed to this work.

280 nm was evident in the effluents, and a linear gradient 0.1–1.0 *M* of NaCl in buffer *B* (500 ml) was applied. The protein elutes at approximately 0.3 *M* NaCl and the fractions with M.*Bse*CI activity were pooled, concentrated to 4 ml and loaded onto a 450 ml Sephacryl S-200 High Resolution (Pharmacia) gel-filtration column pre-equilibrated with 100 m*M* MES buffer, pH 6.6, 200 m*M* ammonium sulfate and 1 m*M* dithiothreitol (DTT). M.*Bse*CI elutes after 200–250 ml of this buffer. The first two chromatography steps performed at room temperature on an FPLC (fast protein liquid chromatography) system (Pharmacia) while gel filtration was performed at 273–277 K. Typical yields are 60 mg of homogeneous M.*Bse*CI from approximately 100 g cell paste. The new protocol differs from the one initially developed (Rina & Bouriotis, 1993) both in the method used for cell disruption and in the columns used (the old scheme uses phosphocellulose as the first column, followed by heparin–Sepharose and hydroxylapatite). The advantages of the new purification procedure over the old one are described in §3.

2.2. *Crystallization and X-ray analysis*

The purified protein [in 100 m*M* 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 6.6, 200 m*M* ammonium sulfate, 1 m*M* DTT] was concentrated to approximately 20 mg ml^{-1} using Amicon Centriprep 30 and Centricon 30 microconcentrators. Initial crystallizations were performed using the hanging-drop vapour-diffusion method (Ducruix & Giegé, 1992) and various crystal forms were obtained from a variety of polyethylene glycols (PEG) in the pH range 6–8. Large crystals were grown with 8–10% PEG 8000 or 20000 at pH 6–6.5 (MES buffer), but exhibited irregular shapes, considerable mosaicity, frequent twinning and sensitivity to X-irradiation. For better crystals, a more controlled strategy was developed, based on observations of the effects of PEG on protein solubilities in electrolyte solutions (Papanikolaou, 1995; Papanikolaou & Kokkinidis, 1997): PEG and salts have opposite effects on M.*Bse*CI solubilities; at low ionic strengths PEG reduces the solubility of the protein, but this effect can be reversed by an increase in electrolyte concentration. M.*Bse*CI solubility can be thus fine-tuned by properly balanced PEG and salt concentrations, a property that can be utilized in a more rational approach to crystallization. Subsequent experiments consisted of a microseeding (*A*) and a macroseeding (*B*) step (Stura & Wilson, 1992) and were performed in microdialysis (Ducruix & Giegé, 1992) cells. A typical cell (Fig. 1*a*) contained approximately 50 µl protein solution (concentration 10 mg ml^{-1}) with 12% PEG, 50 m*M* MES buffer pH 6.6 and 300 m*M* ammonium sulfate, to which a stock of microscopic seeds obtained by crushing an old crystal (*e.g.* grown in hanging drops) had been transferred. A reduction of ammonium sulfate concentration across the dialysis membrane leading to a decrease in protein solubility and favourable growth conditions for the seeds was achieved by immersing the microdialysis cell in a small Petri dish containing 3 ml of 12% PEG, 50 m*M* MES buffer, pH 6.6 and 270 m*M* ammonium sulfate, and sealing the dish with Parafilm (step *A*). Small crystals which served as seeds for the subsequent macroseeding step, usually appeared after 24 h. After reaching a size of approximately 0.1 mm, their quality was assessed under a polarizing microscope (Fig. 1*a*). The best crystals were thoroughly washed with reservoir buffer and transferred individually into new microdialysis cells (starting conditions in the cells: 50 µl protein solution 10 mg ml^{-1}, 12% PEG, 50 m*M* MES, pH 6.6, 300 m*M* ammonium sulfate), where the ammonium sulfate concentration was gradually reduced to 240 m*M* over a period of several weeks (step *B*, Fig. 1*b*). Slow crystal growth continued for approximately one month, usually resulting in prism or parallelepiped-shaped crystals, up to a size of 0.8–1.5 mm

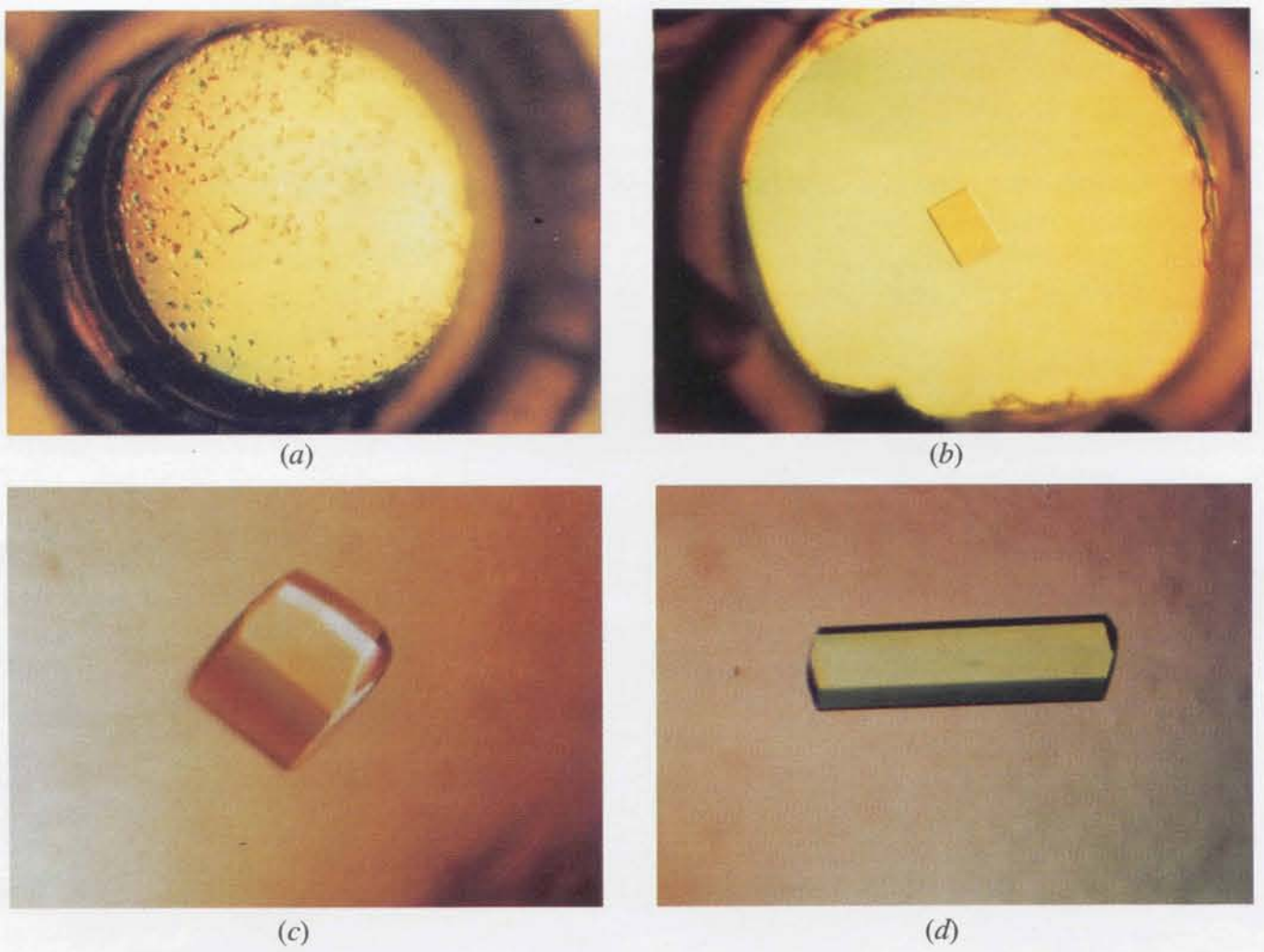

Fig. 1. Crystallization of M.*Bse*CI. (*a*) Growth of a seed stock in microdialysis cells. (*b*) Growth of a crystal in a cell to which an initial seed has been transferred. (*c*, *d*) Fully grown crystals with maximum dimensions of approx. 1.5 mm.

along the longest edge (Figs. 1*c* and 1*d*). Alternatively, the initial seeds were obtained in a nucleation step performed in microdialysis cells containing a 50 µl protein solution (10 mg ml^{-1}), 10% PEG, 50 m*M* MES buffer pH 6.6 and an initial concentration of 250 m*M* ammonium sulfate which was gradually reduced to 180 m*M*. Small crystals grown in this step, were then treated as in step *B*. All crystallizations were conducted at 290–291 K. Crystals were mounted in glass capillaries for preliminary X-ray analysis and collection of X-ray diffraction data, both performed at the EMBL/DESY synchrotron radiation beamline X11 ($\lambda = 0.927$ Å) using a MAR Research image plate ($T = 283$ K). Diffraction data were processed using *DENZO* (Otwinowski, 1991) and the *CCP*4 (Collaborative Computational Project, Number 4, 1994) program suite.

3. Results and discussion

The new purification protocol improves drastically the purity of M.*Bse*CI compared with the previous scheme (Rina & Bouriotis, 1993), mainly because of the efficient removal of proteolytic contaminants. Furthermore, M.*Bse*CI proteolysis which represented the most serious problem at all stages of the old protocol with respect to the crystallizability of the protein, is reduced to practically non-detectable levels after the first two columns.

Pure M.*Bse*CI in 200 m*M* ammonium sulfate retains its crystallizability for several weeks. M.*Bse*CI crystallizes in space group $P2_1$ with the cell dimensions $a = 53.7$, $b = 85.7$, $c = 151.8$ Å and $\beta = 95.1°$. Assuming two molecules per asymmetric unit, a Matthews coefficient (Matthews, 1968) $V_m = 2.6$ Å^3 Da^{-1} corresponding to a solvent content of 53% is obtained. Interestingly, two molecules per asymmetric unit occur frequently in DNA methyltransferases (Reinisch *et al.*, 1995; Kumar, Cheng, Pflugrath & Roberts, 1992), although the active form of the enzymes is the monomer. The present resolution limit is beyond 2.5 Å. A set of native diffraction data extending to 2.5 Å [259851 observed, 46250 unique reflections with $\langle I/\sigma(I)\rangle \simeq 5.0$, $R_{\rm merge} = 4.7\%$, completeness 97%] was collected at the EMBL/DESY synchrotron radiation beamline X11, and a self-rotation function computed in terms of spherical polar angles, was calculated using the program *POLARRFN* (W. Kabsch, Collaborative Computational Project, Number 4, 1994) in order to identify local symmetries in the asymmetric unit. The rotation function (calculated in the range 10–3 Å with a radius of integration of 23 Å and a rotation space sampling step of 5°) indicates the presence of a non-crystallographic twofold axis. We are currently applying the multiple isomorphous replacement method to determine the crystal structure.

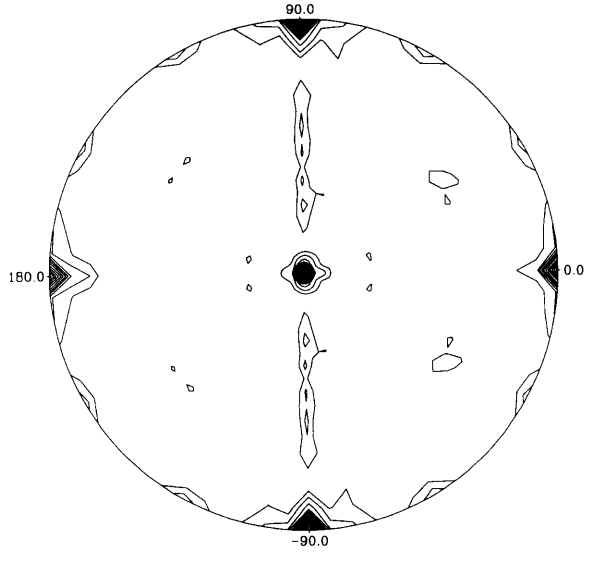

Fig. 2. Stereographic projection of the rotation function for $\kappa = 180°$.

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