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Biochemical characterization of ParI, an orphan C5-DNA methyltransferase from *Psychrobacter arcticus* 273-4

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ABSTRACT

Cytosine-specific DNA methyltransferases are important enzymes in most living organisms. In prokaryotes, most DNA methyltransferases are members of the type II restriction-modification system where they methylate host DNA, thereby protecting it from digestion by the accompanying restriction endonucleases. DNA methyltransferases can also act as solitary enzymes having important roles in controlling gene expression, DNA replication, cell cycle and DNA post-replicative mismatch repair. They have potential applications in biotechnology, such as in labeling of biopolymers, DNA mapping or epigenetic analysis, as well as for general DNA-protein interaction studies.

The *par*I gene from the psychrophilic bacterium *Psychrobacter arcticus* 273–4 encodes a cytosine-specific DNA methyltransferase. In this work, recombinant ParI was expressed and purified in fusion to either an N-terminal hexahistidine affinity tag, or a maltose binding protein following the hexahistidine affinity tag, for solubility improvement. After removal of the fusion partners, recombinant ParI was found to be monomeric by size exclusion chromatography, with its molecular mass estimated to be 54 kDa. The apparent melting temperature of the protein was 53 °C with no detectable secondary structures above 65 °C. Both recombinant and native ParI showed methyltransferase activity *in vivo*. In addition, MBP- and His-tagged ParI also demonstrated *in vitro* activity. Although the overall structure of ParI exhibits high thermal stability, the loss of *in vitro* activity upon removal of solubility tags or purification from the cellular milieu indicates that the catalytically active form is more labile. Horizontal gene transfer may explain the acquisition of a protein-encoding gene that does not display common cold-adapted features.

1. Introduction

The methylated DNA bases N4-methylcytosine, N6-methyladenine and C5-methylcytosine are considered additional bases of the genetic code that carry epigenetic information not encoded in the DNA sequence itself (reviewed in Refs. [1] and [2]). Generally, DNA methylation has an important role in protein-DNA interaction by either enhancing or disrupting binding of proteins to DNA. The enzymes responsible for these modifications are DNA methyltransferases (DNA-MTases), which catalyze the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to the three above-mentioned positions in DNA [3]. In contrast to the role of eukaryotic DNA-MTases, most prokaryotic DNA-MTases are members of a host protection system, the restriction-modification (RM) system [4,5]. The main function of DNA-MTases in the RM system is methylation of host DNA which confers protection from digestion by restriction endonucleases (REases) that recognize the same specific DNA sequence [6]. Some DNA-MTases are not accompanied by an REase and are so-called orphan MTases [7]. The roles of orphan MTases have been proposed to be within gene regulation, DNA replication, cell cycle and directing post-replicative mismatch repair on newly synthesized DNA strands by *de novo* methylation [2,8–12].

In contrast to eukaryotic DNA 5-cytosine methyltransferases (C5-DNA-MTases), being multi-domain proteins and complexes, the prokaryotic enzymes are single-domain proteins [13]. When three-dimensional structures of C5-DNA-MTases are compared, both prokaryotic

Abbreviations: TEV, tobacco etch virus; DSC, differential scanning calorimetry; CD, circular dichroism; DNA-MTase, DNA methyltransferase; C5-DNA-MTase, DNA 5-cytosine methyltransferase; MBP, maltose binding protein

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and eukaryotic catalytic DNA-MTase domains are similar in structural organization. The catalytic domains are organized into a large and a small sub-domain separated by a marked cleft [14]. Despite their structural homology, the sequence similarity among C5-DNA-MTases is low with the exception of ten conserved motifs, named with roman numerals I-X [3]. In addition to these motifs, all C5-DNA-MTases possess a variable region; the Target Recognition Domain (TRD), which is involved in sequence recognition [3,9]. Most of the conserved motifs are located in the large sub-domain, while the TRD comprises most of the small sub-domain. The best conserved motifs (I, IV, VI, VIII, IX and X) are either structural (motif IX), or involved in SAM binding (motifs I and X), DNA binding (motifs VI, VIII and TRD) or catalysis (motif IV) [3,14–16].

Psychrobacter arcticus 273–4 is a gram-negative bacterium discovered in 20–30 thousand year old permafrost soil in Kolyma, Siberia [17]. *P. arcticus* 273-4 has been genome sequenced [18], grows at temperatures from −10 to 28 °C and has generation time of 3.5 days at temperatures below zero [17–20]. At the protein level, *P. arcticus* 273–4 possesses many common features for psychrophilic bacteria, such as reduced use of proline, arginine and acidic amino acids, an increased lysine content, as well as encoding several cold shock proteins [18–21].

In the present study, a C5-DNA-MTase from *P. arcticus* 273–4, ParI, was characterized on the basis of its potential to possess features relevant for biotechnological applications, such as labeling of biopolymers, DNA mapping or epigenetic analysis [22–24].

2. Materials and methods

2.1. Bioinformatic analyses

A multiple protein sequence alignment where ParI was compared to bacterial C5-DNA-MTase homologs [HhaI from *Haemophilus parahaemolyticus* (GI: 127455**);** M. SssI from *Spiroplasma* sp. (GI: 417325); AbaI from *Acinetobacter baumannii* UH5107 (GI: 446969424)] and the catalytic domain of human DNMT1 (GI: 12231019) was made using the T-Coffee web server [25]. The sequence alignment was rendered with the ESPript server [26]. The sequence alignment was annotated with secondary structure of HhaI (PDB ID: 1MHT) and ParI secondary structure predictions from the PsiPred server [27]. The genomic context of *ParI* in *P. arcticus* 273–4 was analysed by the PHAST server [28]. Promoter prediction was performed with **BPROM** [29], while the Rho-independent terminators were predicted by **ARNold** [30,31].

2.2. Cloning, expression and purification

The gene encoding ParI, *parI* [GenBank ID: 71038525] was amplified from *P. arcticus* 273–4 (DSMZ) with primers synthesized by Sigma-Aldrich and Phusion polymerase (NEB) in a PCR reaction following the protocol for Phusion polymerase. The amplified gene, including a primer-encoded TEV-cleavage site, was cloned into either the pDest17 vector which encodes a His tag (resulting in pHis-ParI), or the pHMGWA vector encoding an N-terminal hexahistidine (His) tag and maltose binding protein (MBP). Both constructs were cloned using the Gateway technology (Thermo Fisher Scientific), according to the protocol provided by the manufacturer [32]. All primers used are listed in Table S-1. Newly cloned constructs were transformed by the heat-shock method into the McrBC-negative *E. coli* T7 Express strain (NEB) for recombinant expression and purification (genotype *fhuA2 lacZ::T7 gene1 [lon] ompT gal sulA11 R(mcr-73::miniTn10–*TetS*)2 [dcm] R(zgb-210::Tn10–*TetS*) endA1 Δ(mcrC- mrr)114::IS10*). The protein was expressed in 1L cultures of Lysogeny broth (LB) media and induced with isopropyl-β-D-1-thiogalactopyranoside (IPTG, VWR) at a final concentration of 0.5 mM. Cells for both His-ParI and His-MBP-tagged ParI were harvested after 7 h expression at 20 °C, by centrifugation at 7500 × *g* for 30 min at room temperature and resuspended in lysis buffer (50 mM Tris pH 8.0, 750 mM NaCl, 5% (v/v) glycerol, 10 mM $MgCl_2$) supplemented with 1x Complete protease inhibitor cocktail (Roche). The cells were disrupted by a cell disruptor (Constant Systems, Ltd.) using a pressure of 1.35 kbar in four cycles. The lysate was cleared by centrifugation at 20000 × *g* for 30 min at 4 °C after which it was incubated with HL/SAN DNase (ArticZymes) for 1 h at 4 °C. Affinity purification of recombinant His-ParI or His-MBP-ParI was carried out on a 5 ml HisTrap HP column (GE Healthcare) equilibrated with buffer A (50 mM Tris pH 8.0, 750 mM NaCl, 5% (v/v) glycerol and 10 mM imidazole) using an ÄKTA purifier (GE Healthcare). The bound protein was eluted across a gradient of 0–100% buffer B (50 mM Tris pH 8.0, 750 mM NaCl, 5% (v/v) glycerol and 500 mM imidazole). The purity of the protein was evaluated by SDS-PAGE. In the case of His-MBP-tagged ParI, the removal of the fusion partner was carried out in buffer C (50 mM Tris pH 8.0, 200 mM NaCl, 5% (v/v) Glycerol, 1 mM DTT and 0.5 mM EDTA) supplemented with TEV protease in a 1:10 mg/mg ratio to ParI and incubated overnight at 4 °C. Buffer exchange was done using a HiPrep 26/60 desalting column (GE Healthcare). The recombinant ParI protein was recovered from the flow-through after a second step of HisTrap affinity purification, while the His-MBP portion remained bound to the column. To determine the oligomeric state of the protein, untagged ParI was separated on a HiLoad 16/60 Superdex 200 prep grade gel filtration chromatography column (GE Healthcare) in buffer C. The protein concentration was determined by measuring the absorbance at 280 nm using a Nanodrop spectrophotometer (NanoDrop Technologies). The theoretical extinction coefficient for the protein is 63995 $M^{-1}cm^{-1}$ as calculated by the ProtParam tool.[1] Pure protein was concentrated by centrifugation through 10 kDa MWCO Amicon Ultra Centrifugal filters (Merck Millipore). The identity of recombinant protein was confirmed by MS/MS by the Proteomics facility (UiT, The Arctic University of Norway).

2.3. Thermofluor assay

To assess the stability of ParI, a fluorescence-based thermal shift (Thermofluor) assay was used [33]. A buffer screen consisting of 24 different buffers at various pHs was performed (Bicine, pH 8; Bicine, pH 9; Hepes, pH 7; Hepes, pH 7.5; Hepes, pH 8; Imidazole, pH 8; MES, pH 6; MES, pH 6.2; MES, pH 6.5; Potassium phosphate, pH 5; Potassium phosphate, pH 6; Potassium phosphate, pH 7; Sodium acetate, pH 4.5; Sodium acetate, pH 5; Sodium cacodylate, pH 6.5; Sodium citrate, pH 4.7; Sodium citrate, pH 5.5; Sodium phosphate, pH 5.5; Sodium phosphate, pH 6.5; Sodium phosphate, pH 7.5; Tris, pH 7.5; Tris, pH 8; Tris, pH 8.5). The final concentration of protein in the reaction was 0.5 mg/ml. SYPRO® Orange Protein Gel Stain (Sigma-Aldrich) was diluted 1:75 from the starting concentration. The assay was performed in a volume of 25 µl and was run in a MiniOpticon real-time PCR system (BioRad) in a temperature range from 5 °C to 95 °C with 1 °C increment every 3 s.

2.4. Differential scanning calorimetry

Thermal denaturation curves were recorded at temperatures between 5 °C and 95 °C using a scan rate of 1 °C/min in an N-DSC III calorimeter (Calorimetry sciences corp.). ParI was dialyzed against DSC-buffer (50 mM HEPES pH 8.0, 200 mM NaCl) and used at a final concentration of 1.4 mg/ml. The DSC-buffer from dialysis was used as a reference.

2.5. Circular dichroism

ParI was dialyzed overnight at 4 °C against CD-buffer (10 mM Tris pH 8.0 and 100 mM NaF). The samples were filtered through a 0.45 µm pore size filter (Spin X Costar) to remove precipitate and diluted to a final concentration of 0.15 mg/ml. SAM was added at a final

[1] Expasy server: http://web.expasy.org/protparam/.

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