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Research paper

Investigation of the C-terminal domain of the bacterial DNA-(adenine N6)-methyltransferase CcrM

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ABSTRACT

CcrM-related DNA-(adenine N6)-methyltransferases play very important roles in the biology of *Caulobacter crescentus* and other alpha-proteobacteria. These enzymes methylate GANTC sequences, but the molecular mechanism by which they recognize their target sequence is unknown. We carried out multiple sequence alignments and noticed that CcrM enzymes contain a conserved C-terminal domain (CTD) which is not present in other DNA-(adenine N6)-methyltransferases and we show here that deletion of this part abrogates catalytic activity and DNA binding of CcrM. A mutational study identified 7 conserved residues in the CTD (out of 13 tested), mutation of which led to a strong reduction in catalytic activity. All of these mutants showed altered DNA binding, but no change in AdoMet binding and secondary structure. Some mutants exhibited reduced DNA binding, but others showed an enhanced DNA binding. Moreover, we show that CcrM does not specifically bind to DNA containing GANTC sequences. Taken together, these findings suggest that the specific CcrM-DNA complex undergoes a conformational change, which is endergonic but essential for catalytic activity and this step is blocked by some of the mutations. Moreover, our data indicate that the CTD of CcrM is involved in DNA binding and recognition. This suggests that the CTD functions as target recognition domain of CcrM and, therefore, CcrM can be considered the first example of a δ-type DNA-(adenine N6)-methyltransferase identified so far.

1. Introduction

DNA methylation is present in prokaryotes and eukaryotes and adds additional information to the DNA [1–3]. It can occur at the C5 and the N4 positions of cytosine bases and at the N6 position of adenine bases. The methyl group always points into the major groove of the DNA where it can be recognized by DNA binding proteins and influence DNA recognition [2]. DNA methylation is introduced by DNA methyltransferases (MTases) which usually methylate DNA in a sequence specific manner and employ S-adenosyl-L-methionine (AdoMet) as donor of an activated methyl group. In mammals, DNA methylation is mainly set at the C5 position of cytosine residues within CpG dinucleotides and it is involved in many regulatory processes and plays an important role in several diseases [4,5]. In prokaryotes, all three types of DNA methylation have been observed and DNA methylation participates in restriction-modification systems, post-replicative mismatch repair, gene regulation and cell cycle regulation (among other processes) [2,6,7]. While most bacterial DNA MTases are part of restriction/modification systems, some of them, called orphan MTases, are not accompanied by a corresponding restriction enzyme. The most prominent orphan MTases are the *Escherichia coli* DNA adenine MTase (EcoDam) and the *Caulobacter crescentus* cell cycle-regulated MTase (CcrM). EcoDam and homologues in other γ-proteobacteria methylate adenine residues in GATC sequences and play a role in mismatch repair, DNA replication and gene regulation [6,7]. CcrM was first found in *C. crescentus* and has a central role in the regulation of the complex cell cycle, which results in asymmetric division into morphologically different daughter cells [8–14]. CcrM was shown to be essential, at least under certain growth conditions, for *C. crescentus* and several other α-proteobacteria, including human pathogens, thus making it a potential drug target [8,15–17].

Chemically MTases can be subdivided into two major groups, enzymes that catalyse the formation of a C–C bond by methylating

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the C5 carbon in cytosines and enzymes methylating exocyclic amino groups in adenines or cytosines forming a C–N bond [2,18]. These two groups of enzymes have a similar two-domain structure containing one large catalytic domain responsible for AdoMet binding and catalysis and one smaller target recognition domain (TRD) responsible for DNA recognition, which is not conserved between different enzymes [1,2]. Despite the different chemistry the catalytic domain is structurally related between the two groups of N- and C-MTases. It comprises two subdomains, one mainly involved in AdoMet binding and the other one in binding of the flipped target base and catalysis. DNA binding by DNA MTases is mediated by the catalytic domain and TRD acting in concert [1,2]. In addition to providing the binding pocket for the flipped target base, the catalytic domain forms mainly backbone contacts to the DNA. The TRD, in general, provides the sequence specific contacts, although in the case of EcoDam (for example) an N-terminal tail also mediates an important base specific Lysine-Guanine contact [19,20].

Based on multiple sequence alignments, the group of N-MTases can be further subdivided into subgroups based on the consecutive arrangement of the three conserved subdomains (catalytic subdomain, AdoMet-binding subdomain and TRD) [18] (Fig. 1A). While 6 subgroups, called α, β, γ, δ, ε, and ζ, are possible in principle [18,21], only α, β and γ DNA MTases have been described so far, and CcrM had been classified as a member of the β-group.

DNA methyltransferases represent an example of a closely related group of enzymes which massively diversified to recognize different DNA sequences. The specific evolutionary pathways for these transitions are only rarely known [20,22,23] mainly because the mechanism of DNA recognition by many enzymes is not understood at molecular level due to a lack of structural and biochemical data. Here, we investigated the mechanism of DNA recognition by *C. crescentus* CcrM and show that its C-terminal domain (CTD), which is conserved in CcrM-related enzymes interacting with GANTC sites, but absent in other MTases, is essential for catalytic activity. By mutating conserved amino acids within this domain to alanine, followed by purification of the enzyme mutants and investigation of their methylation activity, secondary structure, DNA binding, and AdoMet binding, we demonstrate that the CTD has an important role in DNA binding and recognition. Our data suggest that the CTD represents the TRD of CcrM which would lead to a re-classification of CcrM as the first member of the δ group of DNA-(adenine N6)-MTases.

2. Materials and methods

2.1. Site-directed mutagenesis, protein expression and purification

PCR-based megaprimer site-directed mutagenesis was carried out with Pfu DNA Polymerase (Thermo Scientific) basically as described [24]. His_6-tagged CcrM deletion mutants consisting of only the N-terminal parts with stop codons at positions alanine 257 (N-terminal domain 257, NTD257) or glycine 265 (NTD265) were prepared and sequenced. The C-terminal part of the CcrM gene (starting from aa 256) was cloned as GST fusion into a pGEX-6P-2 at *Nde*I and *Not*I restriction sites. Wild type and mutants of His_6-tagged *C. crescentus* CcrM were expressed and purified in *E. coli* HMS174 (DE3) (Novagen) cells for initial methylation activity screening and CD measurements as described [25,26]. The GST tagged domain was purified following standard procedures recommended by the supplier (Amersham Bioscience).

Protein purification could be improved by expressing wild type and mutants of *C. crescentus* CcrM in *E. coli* ER2566 (NEB). Cells were grown to OD_{600} of 0.5–0.6 at 37 °C, and then transferred to 25 °C and induced for 4 h with isopropyl-β-D-thiogalactopyranoside (IPTG, 0.5 mM). Cells were harvested by centrifugation and stored at −20 °C. The pellets were suspended in lysis buffer (50 mM HEPES, pH 7.5, 500 mM NaCl, 0.1 mM DTT, 10% glycerol, 20 mM imidazole) and lysed by sonication. The cell lysates were cleared by centrifugation (40,000 × g, 1 h) and loaded onto Ni-NTA agarose columns (Qiagen). The columns were extensively washed with lysis buffer and CcrM proteins were eluted with 50 mM HEPES, pH 7.5, 500 mM NaCl, 0.1 mM DTT, 10% glycerol, 220 mM imidazole. The eluates were dialyzed consecutively against dialysis buffer I and II (dialysis buffer I: 50 mM HEPES, pH 7.5, 200 mM NaCl, 0.1 mM DTT, 10% glycerol, dialysis buffer II: 50 mM HEPES, pH 7.5, 200 mM NaCl, 0.1 mM DTT, 60% glycerol).

2.2. Circular dichroism spectrophotometry

CD measurements were made with a Jasco J-815 spectrophotometer at 20 °C using protein concentrations of 12 μM in a cell with a 0.1 mm path length in dialysis buffer II. CD spectra were collected in the wavelength range of 195 nm–250 nm with a 0.5 nm step size and 1 nm bandwidth. Recorded spectra were scaled by using Microsoft Excel Solver Add-In for comparison of mutant *C. crescentus* CcrM proteins with the wild type enzyme.

2.3. Methylation activity assay

A biotin-avidin microplate assay [27] was used to study the methylation activities of the different *C. crescentus* CcrM mutants. As a substrate, a biotinylated double stranded 23mer oligodeoxynucleotide with one hemimethylated GANTC site was used. The substrate was prepared by heating a mixture of complementary oligodeoxynucleotides (20 μM each) to 95 °C for 5 min followed by slow cooling to ambient temperature (Bt-d(GGC AGC TAC GAA TCG CAA CAG CT), d(AGC TGT TGC GA^{m}T TCG TAG CTG CC)). The quality of the annealed oligonucleotides was evaluated by polyacrylamide gel electrophoresis.

The methylation reactions were performed in reaction buffer (50 mM HEPES pH 7.0, 50 mM NaCl, 1 mM EDTA, 0.5 mM DTT, and 5 μg/mL BSA) under single turnover conditions with 1 μM enzyme, 0.5 μM DNA substrate, and 760 nM ^{3}H-labeled AdoMet (Perkin Elmer, 2.942 TBq/mmol). The catalytic activity was determined by linear regression of the initial data points. All kinetic experiments were carried out in at least two repeats.

2.4. AdoMet binding

Binding of S-adenosyl methionine (AdoMet) to *C. crescentus* CcrM wild type and mutants was investigated by a fluorescence based competition assay using 1-anilinonaphtahalene-8-sulfonic acid (1,8-ANS) as fluorophore [28,29]. The basis of this assay is that ANS binds to the AdoMet binding pocket of MTases, where the stacking interactions with aromatic amino acid residues lead to an increase in ANS fluorescence. AdoMet binds more strongly to the pocket and it can displace ANS leading to a decline in fluorescence. *C. crescentus* CcrM wild type or mutants (1 μM) and 1,8-ANS (50 μM) were incubated in buffer (100 mM sodium phosphate buffer pH 7, 10 mM KCl, 1 mM $MgSO_4$) supplemented with different concentrations of AdoMet at ambient temperature for 10 min before fluorescence measurements. Fluorescence was measured with a JASCO FP-8300 Spectrofluorometer (excitation: 395 nm, emission: 400 nm–600 nm, excitation and emission bandwidth: 2.5 nm). For quantitative analysis, fluorescence intensities between 480 nm and 490 nm were averaged. The Microsoft Excel solver module was used for fitting the data to a competition binding equilibrium described by the Equation (1).