**Identification of the Active Oligomeric State of an Essential Adenine DNA Methyltransferase from Caulobacter crescentus**

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*Caulobacter crescentus* contains one of the two known prokaryotic DNA methyltransferases that lacks a cognate endonuclease. This endogenous cell cycle regulated adenine DNA methyltransferase (CcrM) is essential for *C. crescentus* cellular viability. DNA methylation catalyzed by CcrM provides an obligatory signal for the proper progression through the cell cycle. To further our understanding of the regulatory role played by CcrM, we sought to investigate its biophysical properties. In this paper we employed equilibrium ultracentrifugation, velocity ultracentrifugation, and chemical cross-linking to show that CcrM is dimeric at physiological concentrations. However, surface plasmon resonance experiments in the presence of S-adenosyl-homocysteine evince that CcrM binds as a monomer to a defined hemi-methylated DNA substrate containing the canonical methylation sequence, GANTC. Initial velocity experiments demonstrate that dimerization of CcrM does not affect DNA methylation. Collectively, these findings suggest that CcrM is active as a monomer and provides a possible in vivo role for dimerization as a means to stabilize CcrM from premature catabolism.

Most organisms encode DNA methyltransferases (MTases) to modify their genomic DNA by the catalytic transfer of an activated methyl group using S-adenosyl-l-methionine (AdoMet) as the donor. These enzymes fall into one of three prokaryotic DNA methyltransferases that lacks a cognate endonuclease. This endogenous cell cycle regulated adenine DNA methyltransferase (CcrM) is essential for *C. crescentus* cellular viability. DNA methylation catalyzed by CcrM provides an obligatory signal for the proper progression through the cell cycle. To further our understanding of the regulatory role played by CcrM, we sought to investigate its biophysical properties. In this paper we employed equilibrium ultracentrifugation, velocity ultracentrifugation, and chemical cross-linking to show that CcrM is dimeric at physiological concentrations. However, surface plasmon resonance experiments in the presence of S-adenosyl-homocysteine evince that CcrM binds as a monomer to a defined hemi-methylated DNA substrate containing the canonical methylation sequence, GANTC. Initial velocity experiments demonstrate that dimerization of CcrM does not affect DNA methylation. Collectively, these findings suggest that CcrM is active as a monomer and provides a possible in vivo role for dimerization as a means to stabilize CcrM from premature catabolism.

The DNA MTase modifies either an adenine or cytosine base within a specific sequence of the host DNA, thereby rendering the sequence resistant to endonuclease cleavage. Invading foreign DNA, which lacks a methyl modification within this defined sequence, is rapidly cleaved. Not all MTases exist as part of a restriction-modification system. The most studied example is the widespread adenine DNA MTase, Dam. Dam plays several important regulatory roles in the cell, including chromosome replication, gene expression, and DNA mismatch repair (reviewed in Refs. 11 and 12 and references therein). Additionally, Dam has been shown to be the master regulator that activates the transcription of several virulence genes in *Salmonella typhimurium* and *Escherichia coli* (13).

At least one other DNA MTase has been identified that is not part of a restriction-modification system: a DNA adenine MTase denoted CcrM (cell cycle regulated DNA MTase). CcrM catalyzes the methylation of adenine at the N-6 position within the canonical sequence GANTC (where N can be any nucleobase) (14). Homologs of CcrM are found throughout the α-subdivision of Gram-negative bacteria. Members of this class include the freshwater bacterium *Caulobacter crescentus*, the nitrogen-fixing soil bacterium *Rhizobium meliloti*, the plant pathogen *Agrobacterium tumefaciens*, and the animal pathogen *Brucella abortus* (15). Unlike the nonessential Dam MTase, CcrM activity is absolutely required for viability throughout the α-subdivision (16, 17), and its activity is tightly regulated during the cell cycle (14).

The cell cycle of *C. crescentus* is typified by an asymmetric cell division yielding a motile swarmer cell and a sessile stalked cell (18), with DNA methylation serving as an obligatory signal for proper cell cycle progression. DNA replication is restricted to a stalked cell (19), so the progeny swarmer cell must first differentiate into a stalked cell to initiate chromosome replication (18). Following semiconservative DNA replication, the resulting daughter chromosomes are hemi-methylated. At this point the division plate begins to form as the cell enters the predivisional cell stage. Concomitant with this step, CcrM is synthesized de novo. The DNA is rapidly remethylated restoring full methylation status immediately prior to the completion of cell division (17). Simultaneous with cell division is the rapid and complete degradation of CcrM. It is imperative that the delicate balance of methylation be maintained in order for *C. crescentus* to survive. An increase in CcrM stability leading to its constitutive methylation results in an aberrant cell morphology (14), whereas an abrogation of CcrM activity results in cellular death (15). Strict temporal regulation is achieved by: (a) transcription of the ccrM gene exclusively in the predivisional stage of the cell cycle (17) and (b) catabolism by a Lon-dependent pathway immediately prior to cell division (20). Considering that CcrM is essential among pathogenic α-proteobacteria and that eukaryotes lack adenine DNA MTases,
CcrM may be an ideal target for the design of a new class of anti-microbial drugs (16, 21).

The oligomeric state of DNA-binding proteins involved in gene expression has been shown to be a critical property governing activity. For instance, dimerization of λ cI repressor monomers is required for high affinity binding to bacteriophage λ operator DNA (22). In contrast, dimerization inhibits DNA binding by the TATA-binding proteins (23). DNA MTases have also been shown to exist in alternate oligomeric states and to possess a variable active form. Whereas most DNA MTases are monomeric, M.RsrI (24, 25) and the human placental DNA MTase (26) have been shown to be dimeric in solution. The functional state of M.RsrI has been identified as the monomeric form. However, the human placental DNA MTase is active as a dimer. In this paper, we show that the mechanistic behavior of CcrM is similar to the bacterial DNA MTase M.RsrI (i.e. exists in solution as a dimer at micromolar concentrations and functions actively as a monomer). Additionally, we propose a possible role for dimerization within the context of the C. crescentus cell cycle.

EXPERIMENTAL PROCEDURES

Materials—[3H]AdoMet (82.4 Ci/mmol) was purchased from New England Nuclear (Boston, MA). AdoHcy was obtained from Sigma. Dimethyl suberimidate was obtained from Pierce. Biotin-CPG columns and all other phosphoramidites for DNA synthesis were purchased from Glen Research (Sterling, VA), whereas all other phosphoramidites and ancillary reagents were from PE Biosystems (Foster City, CA). DSS1 and glass fiber filters were obtained from Whatman (Clifton, NJ). All restriction enzymes and T4 DNA ligase used in cloning were acquired from New England Biolabs (Beverly, MA). All other materials were of the highest purity reagent grade commercially available.

DNA Substrates—Synthetic oligonucleotides were generated using an Expedite Biosystems DNA synthesizer. Single-strand oligonucleotide purification was performed as previously described (27). Following purification, the complementary single strands were annealed in the presence of 150 mM sodium chloride. The resulting duplex DNA (Fig. 1) was used for amino acid analysis by method of Edelhoch (28). Typical yields with this protocol are 5–10 mg of pure CcrMlifer of cells grown.

Characterization of CcrM Oligomerization

Several experiments were also performed using ultracentrifugation buffer (reduced and nonreduced) containing a chaotropic agent, 6 M guanidine hydrochloride. To assure dialysis equilibrium was achieved, the protein samples were dialyzed against the appropriate buffer at 4 °C for at least 12 h (2 changes of 2 liters each) immediately prior to analysis. Simultaneous acquisition of a broad concentration range (300 mM to 12.5 M) was accomplished by utilizing a six-channel centerpiece while monitoring at wavelengths (220–300 nm) appropriate for the protein concentration. Native CcrM and nonreduced, denatured CcrM data were acquired at rotor speeds of 14,000, 18,000, 22,000, and 26,000 rpm, whereas reduced, denatured CcrM data were collected at 14,000, 18,000, 24,000, 30,000, and 36,000 rpm. Equilibrium was achieved when two consecutive sets of data taken 2 h apart were achieved until a state of experimental equilibrium was reached.

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Data were edited using the software package Microcal Origin (version 3.78, Microcal Software, Northampton, MA). Local and global nonlinear least squares analyses were employed using the program WinNonlinLR (version 1.05) (29) to extract solution molecular masses. Because of the strong UV absorbance of the oxidized reducing agent, poor noise to signal ratio was observed at low protein concentration in the presence of β-ME making global analysis results unreliable. Therefore, the dissociation constant for reduced CcrM was determined by fitting the solution molecular mass observed for several concentrations above 300 nM (at each rotor speed) to a general hyperbolic curve-fitting equation.

**Sedimentation Velocity Ultracentrifugation and Analysis—**Sedimentation velocity experiments were performed in a two-channel center-piece using a Beckman XL-I Analytical Ultracentrifuge in absorbance mode. Following dialysis into ultracentrifugation buffer in the presence 3 mM β-ME, varying concentrations of CcrM (3–11.5 μM) were centrifuged at a rotor speed of 36,000 rpm at 25 °C. The van Holde-Weischet method (30), within the UltraScanII software package (version 3.0), was utilized to calculate the sedimentation coefficient at each concentration (S20,w). Further verification of sedimentation coefficients was accomplished by employing the transport method (31) and the second moment method (32) contained in the Microcal Origin software package. The S20,w values were converted to the standard reporting value, 20 °C in pure water (S20,wc), by the following equation (33).

\[
S_{20,w} = S_{20,c} \left(1 - \frac{v_{20,w}}{v_{20,c}}\right) \left(1 - \frac{v_{20,b}}{v_{20,c}}\right)
\]

where \(v_{20,w}\) (the partial specific volume of CcrM at 20 °C) is 0.7374 ml/g, \(v_{25,w}\) is 0.7396 ml/g, \(\rho_{20,w}\) (the density of pure water at 20 °C) is 0.9982 g/ml, \(\rho_{20,b}\) (the density of buffer b at 25 °C) is 1.01132 g/ml for ultracentrifugation buffer, \(\eta_{20,w}\) (the viscosity of pure water at 20 °C) is 0.01002 poise, and \(\eta_{20,b}\) is 0.00905 poise for ultracentrifugation buffer. The data base program Sedinterp (version 1.01) provided the means to calculate the partial specific volume of CcrM by the method of Cohn and Edsall (34). The S20,w values were determined by plotting S20,w, versus CcrM concentration and extrapolating to infinite dilution.

**Chemical Cross-linking—**Chemical cross-linking of CcrM was achieved by using the bifunctional amine cross-linking agent, diethyl maleimide suberimidate (DMS). A DMS stock solution of 50 mM was prepared in cross-linking buffer (50 mM potassium phosphate, pH 8.5, 150 mM potassium chloride, 3 mM EDTA, 5 mM β-ME, 10% glycerol) immediately prior to use. Cross-linking reactions were performed as follows: CcrM renaturation was varied from 100 nM (4 μg/ml) to 2 μM (79 μg/ml) in the presence of 10 mM DMS in cross-linking buffer; this mixture was incubated at ambient temperature for 30 min; and quenched by the addition of a final concentration of 100 mM glycine, pH 8. Subsequently, the cross-linked reaction mixtures were separated by electrophoresis on a 7% SDS-PAGE and visualized by Coomassie staining.

**Surface Plasmon Resonance—**CcrM-DNA binding stoichiometry was measured using BIACORE 2000 (Biacore AB, Uppsala, Sweden) and data were acquired by the software package provided by the manufacturer. An N6-45/45(sp)-mer with one methylation site containing a 3′-biotin label on the methylated strand and an abasic substitution for the target adenine on the nascent strand to facilitate tight binding (25, 35–37) was used. The biotinylated DNA was immobilized to a ligand load density of 500 nM (4 μg/ml) on a CMSNH filter. A CcrM titration (concentration range, 0–2.0 μM with a random injection order) was performed in SPR buffer (50 mM potassium phosphate, pH 8.0, 150 mM potassium acetate, 5 mM β-ME, 0.005% surfactant P-20). The activity of CcrM was unaffected by increasing the pH from 7.5 to 8.0 (data not shown), and thus, a pH of 8 was applied to minimize nonspecific interactions between CcrM and streptavidin. The flow rate for each injection was maintained at 25 μl/min, while the association phase was monitored for 3 min, and the dissociation phase was recorded for 15 min. Simultaneous multi-channel injection over a control surface (no DNA) and the experimental surface (containing DNA) provided a real time background subtraction. To avoid sample loss because of adsorption to the storage tubes or the fluids, 3 mM BSA was supplemented in each injection. To enhance sequence selectivity and minimize nonspecific binding, 50 μM S-adenosyl-homocysteine (AdoHcy) was also included. Between each injection, the surface was regenerated with a 30-s injection of SPR buffer containing 2 mM potassium chloride at a rate of 50 μl/min, followed by a 5-min stabilization period. Using BIAl evalution (version 3.1), the background subtracted data were fit to a Langmuir binding model to determine Kd for each injection concentration. By plotting Rmax as a function of CcrM concentration, the equilibrium dissociation constant (Kd) and the maximum analyte response level (Rmax) were obtained according to Equation 2 (38). Given these values, Equation 3 was used to calculate the CcrM-DNA stoichiometry (39).

\[
R_{eq} = \frac{R_{max} \cdot C}{K_d + C}
\]

\[
\text{Stoichiometry} = \frac{\text{analyte response}}{\text{ligand Mn}} \left(\frac{\text{analyte Mn}}{\text{ligand Mn}}\right)
\]

where the analyte molecular mass is approximated as 27,500 Da.

**Kinetic Assays—**Methyltransferase activity of CcrM was measured by monitoring tritium incorporation from [3H]AdoMet into a defined DNA substrate (40) (Fig. 1). Assays were conducted in a buffer consisting of 50 mM potassium phosphate, pH 7.5, 150 mM potassium acetate, 5 mM β-ME with 5 μM DNA, 50 μM AdoMet, and increasing concentrations of CcrM (125 nM to 1 μM). At 1-min intervals, the reaction was quenched by spotting 5-μl aliquots on DE81 filter paper. Filters were prepared as described in Berdis et al. (40). All data were corrected for nonspecific binding of [3H]AdoMet to the DE81 filter paper.

**Results**

**Determination of the Oligomeric State of CcrM**

**Analytical Ultracentrifugation—**Sedimentation equilibrium ultracentrifugation was used to verify the molecular mass of CcrM (amino acid sequence predicted = 39,608 Da). Because of the strong absorbance at short wavelengths (<250 nm) inherent to most oxidized reducing agents, preliminary experiments were performed in the absence of reducing agents. The resulting data were fit to a single ideal species model using WinNonlinLR. The apparent molecular mass of nonreduced CcrM increased in a concentration-dependent manner. However, the molecular mass was observed to dramatically decrease as the rotor speed was increased from 14,000 to 22,000 rpm (data not shown). To test whether this apparent heterogeneity was a result of protein aggregation, denatured CcrM was ultracentrifuged in the presence of 6 M guanidine hydrochloride. The same correlation between molecular mass and rotor speed was observed (data not shown). The occurrence of molecular masses that exceed that of the monomer while in the presence of chaotropic agents, such as guanidine hydrochloride, can only be explained by a covalent interaction between two molecules of CcrM arising from inter-protein disulfide bond formation.

This theory was tested by protein-gel electrophoresis in which CcrM was loaded in the presence or absence of β-ME onto a denaturing gel. In the presence of β-ME, a single band consistent with the amino acid sequence predicted monomeric mass of CcrM was observed, whereas in the absence of reducing agents a higher molecular mass species corresponding to a dimer appeared (data not shown). Taken together, these data are most consistent with the formation of a covalent dimer species in the absence of reducing agents.

As a result of heterogeneity arising from inter-protein disulfides, equilibrium ultracentrifugation was conducted in the presence of the reducing agent β-ME. However, limitations imposed by the poor signal-to-noise ratios only allowed experiments to be performed at CcrM concentrations above 300 nM. The fitted molecular masses increased from 52,000 to 80,000 Da in a concentration-dependent manner, consistent with the formation of a dimeric species. Diagnostic plots of molecular mass versus load concentration (Fig. 2A) and molecular mass versus rotor speed (Fig. 2B) indicate that reduced CcrM displays behavior typical of a mass-action directed association (41). Global analysis of these data by WinNonlinLR to determine the dissociation constant proved unsuccessful. Therefore, the dissociation constant (Kd) was determined by plotting the fitted molecular mass as a function of load concentration at each rotor speed. The data for each speed were fit (Fig. 3) to a general hyperbolic equation by fixing the molecular mass at 27,500 Da (40).
Characterization of CcrM Oligomerization

infinite dilution to that of the monomer. This treatment provided an average $K_d$ of 398.9 ± 73.8 nm. By this method, the molecular mass of the highest oligomeric state was determined to be 81,316 ± 1,669 Da, in excellent agreement with the sequence-predicted dimeric mass of 79,216 Da. As a control, the reduced form of CcrM was denatured by addition of 6 M guanidine hydrochloride and subjected to equilibrium ultracentrifugation. The denatured, reduced CcrM yielded a monomeric molecular mass (40,100 Da ± 2000 Da) that was invariant with increasing rotor speed (data not shown).

Confirmation of CcrM dimerization was sought by employing sedimentation velocity ultracentrifugation. The sedimentation coefficient of CcrM in the presence of 3 mM β-ME was determined over a broad concentration range (3–11.5 µM). The van Holde-Weischet method (30) was used to extract $S_{20,w}$ values, which were converted to $S_{20,w}$ according to Equation 1. Fig. 4 displays the buffer corrected sedimentation coefficients ($S_{20,w}$) as a function of increasing CcrM concentration. Extrapolating the sedimentation coefficients to infinite dilution yields an $S_{20,w}^0$ of 5.1 ± 0.1 S. The theoretical maximum $S_{20,w}^0$ for a CcrM monomer is 3.5 S, with a Stokes radius of 260 nm. These results provide supporting evidence that CcrM is present in a higher oligomeric form. In fact, a $S_{20,w}^0$ of 5.1 ± 0.1 S can be crudely modeled as a prolate ellipsoid with an axial ratio of ~2.75 (oblate ellipsoidal ratio, 2.80) and a Stokes radius of 360 nm.

Chemical Cross-linking of CcrM—The chemical cross-linking agent, dimethyl suberimidate, specifically reacts at pH 8.5 with N-terminal amine and lysine residues to create amidine cross-links between protein binding partners. Given that there are 24 lysine residues/molecule of CcrM, it was thought that DMS would be ideal for trapping the dimeric form of CcrM in solution. To assess this, the concentration of CcrM was varied from 100 nM to 2 µM and treated with DMS for 30 min at ambient temperature. The reactions were quenched with an excess of glycine and visualized by SDS-PAGE (Fig. 5). As the concentration of CcrM was increased, a diffuse band with a molecular mass of ~75,000 Da was observed that was not present in the CcrM sample without exposure to DMS. It was reported that DMS-treated proteins could migrate as a diffuse smear. This smearing arises because of nonuniform cross-linking, which can lead to differential binding of SDS (42, 43). Because of the large number of lysine residues, it is not surprising that more than one cross-linked species would be present. These results further support the previously mentioned evidence that CcrM exists as a dimer in solution. It is worth noting that experiments in the presence of AdoMet resulted in no alteration in the pattern observed in Fig. 5. Therefore, on the basis of chemical cross-linking, AdoMet alone does not appear to affect dimerization.

Assessment of the Active Form of CcrM

Determination of CcrM-DNA Stoichiometry by SPR—As a first step toward identification of the active form of CcrM, we employed SPR. SPR measures a change in the refractive angle arising from a binding event. Typically, experiments focused on DNA-protein interactions require the DNA (ligand) to be immobilized on the surface of the flow cell and the enzyme (analyte) in question to be passed over the surface in increasing concentrations to allow determination of binding constants. Therefore, to facilitate the determination of CcrM-DNA stoichiometry, a DNA substrate was synthesized that contained a
3′-biotin tag, which was immobilized through a biotin-streptavidin interaction on the surface of a streptavidin chip. The strand containing the biotin end label also harbored an N⁶-methylated adenine within the canonical CcrM sequence. The complementary strand included a tetraphydouran derivative in which the target adenine has been removed resulting in an abasic lesion (Fig. 1). This hemi-methylated, abasic duplex DNA has been shown to be an efficient trap of CcrM with a K₅₅ less than 250 nM. The trap substrate was then chosen over the substrate DNA because of the increased lifetime of the specific protein-DNA interaction (25, 35–37), thus simplifying experimental determination of the equilibrium response. CcrM binds a DNA substrate with the canonical sequence located 10 base pairs from the biotin with the same affinity to that of a substrate with the site located an equal distance from either end according to fluorescence anisotropy experiments. Therefore, site placement should not compromise the determination of CcrM-DNA stoichiometry.

This biotinylated DNA substrate was immobilized to a total load density of 187.2 response units. The AdoMet analog, AdoHey, was included in the CcrM titration injection (concentration range, 0–2.0 μM) to enhance the selectivity of the DNA MTase for its target site (44). The background nonspecific binding and bulk contributions at each concentration of CcrM were experimentally determined by simultaneous injection over a surface that lacked DNA. The background subtracted data for one injection series are shown in Fig. 6A.

Sensograms were fit to the Langmuir binding model (a single class of noninteracting binding sites in a 1:1 binding interaction) and the heterogeneous ligand model (two or more populations of noninteracting binding sites). The appropriate model was chosen based on the quality of the fit to the data. Only the Langmuir binding model resulted in a satisfactory fit from which the equilibrium response (Rₑ) for each CcrM concentration was ascertained. The resulting Rₑ value was plotted as a function of injection concentration (Fig. 6B) and fitted to a hyperbolic binding equation (Equation 2) to extract a dissociation constant of 76.9 ± 9.1 nM and the absolute analyte response (plateau; Rₘₐₓ = 269.9 response units). The analyte and ligand response values were inserted into Equation 3, from which a stoichiometry of 1.00 ± 0.02 CcrM:DNA was calculated.

Effect of Dimerization on DNA Methylation and Effect of

Concentration on Initial Velocity—Elucidation of the minimal kinetic mechanism evinces that CcrM follows an ordered bi-bi mechanism in which the DNA is bound first followed by the AdoMet cofactor (40) and subsequent catalytic methyl transfer. Because SPR experiments highly suggest that the functional state of CcrM is the monomer, initial velocity kinetics were used to assess whether dimerization affects DNA methylation by entering into the preferred catalytic cycle of CcrM at different stages. Accordingly, we preformed the CcrM-DNA binary complex followed by initiation with [³H]AdoMet. We also established a direct competition between catalysis and dimerization by simultaneously adding DNA and [³H]AdoMet to initiate the reaction. In the former scenario, it was expected that CcrM would dissociate into monomers and establish an equilibrium partition between dimeric CcrM and DNA bound CcrM. In the latter instance, we expected to have a pronounced bias in the equilibrium partition toward dimeric CcrM. This would ultimately affect the initial velocity of methyl incorporation as the concentration of CcrM is increased and the population shifts toward the dimer.

These experiments employed a standard filter binding assay (described in Ref. 40). Briefly, the amount of [³H]CH₃ catalytically incorporated into a defined DNA substrate (Fig. 1) at a streptavidin chip containing a light load density (187.2 response units) of a biotinylated N⁶-45-mer/45(s). The injections were performed in random order in the presence of 50 μM AdoHey. For clarity, a single titration series is shown, although the injection series was performed in duplicate. B, the equilibrium response (Rₑ) for each concentration is plotted versus the injection concentration. The data were fit to Equation 2 with a correlation coefficient of 0.988. Rₑ, and the ligand immobilization response were substituted into Equation 3 to calculate the CcrM-DNA stoichiometry.

A. J. Berdis, unpublished results.
B. C. J. Haney, unpublished results.
Characterization of CcrM Oligomerization

DISCUSSION

DNA methylation catalyzed by CcrM serves as an obligatory signal for the proper progression of cell-cycle events. Any modification of CcrM-catalyzed methylation has a detrimental effect on bacterial viability throughout the α-class of proteobacteria, which includes several pathogenic species. Within the C. crescentus model system, CcrM activity is tightly coordinated via protein expression and Lon-mediated catabolism. Using a combination of biophysical techniques and initial velocity experiments, we have unveiled an additional layer in the delicate coordination of CcrM control and activity.

We employed equilibrium analytical ultracentrifugation to assess the oligomeric state of CcrM in the absence of substrates and/or cofactors. In the presence of reducing agents, diagnostic plots of molecular mass as a function of increasing CcrM concentration indicate a transition from a monomeric to dimeric species, which does not associate to form a higher oligomeric state. In addition, the molecular mass is invariant with increasing rotor speed. Taken together, these data provide thermodynamic evidence that CcrM dimerization, in the presence of reducing agents, is a fully reversible mass action-mediated process. Dimerization proceeds with a dissociation constant of 398.9 ± 73.8 nM. Further support for the dimerization of CcrM in solution has been provided by velocity ultracentrifugation and chemical cross-linking experiments. In light of the in vitro results in the absence of substrates and/or cofactors, it was of interest to ascertain the likely oligomeric form of CcrM in vivo. During the predivisional phase of the C. crescentus life cycle, CcrM is present at an intracellular concentration of ~4.5 µM (3000 copies/cell (40)). Therefore, CcrM is predicted to exist predominantly as a dimer at physiological concentrations (80–85%).

The preponderance of dimeric CcrM at physiological concentrations raised a fundamental question relating to the active form of CcrM. Surface plasmon resonance experiments performed in the presence of AdoHcy, an AdoMet analog, revealed that CcrM binds to DNA as a monomer. However, initial velocity kinetics demonstrated that dimerization does not affect CcrM activity. Both of these experiments were performed at concentrations of CcrM as high as 1.25 µM, which would correspond to a 67% dimeric population based on the observed dimerization constant for CcrM. This apparent paradox can be rationalized by the mechanism of methylation. Direct methyl transfer from AdoMet, catalyzed by MTases, requires the target nucleotide to be properly aligned in an extrahelical position within the enzyme active site. Accordingly, the reaction requires gross conformational changes in the DNA as well as in the enzyme. Recent evidence provided by M.EcoRV suggests that DNA MTases exist in an open-closed equilibrium in the presence of DNA. The slow observed rate of product release is reported to be the opening of the “closed” complex (45). This is also observed even in the absence of the cofactor, AdoMet. If dimer dissociation were rapid compared with the dissociation of monomeric CcrM from DNA after catalysis, then as monomeric CcrM is taken out of solution due to DNA binding the equilibrium would increasingly favor dimer dissociation. The end result would be an observed first order dependence of initial velocity on total enzyme concentration.

Several DNA MTases are sensitive to the presence of thiol reductants including E. coli Dam (46) and M.EcoRI (47). This effect has been attributed to the failure to maintain an active site cysteine in the proper reduced state. However, a cysteine is not directly implicated in the mechanism of methyl transfer to the exocyclic amino position (48). Furthermore, amino acid sequence alignment of adenine DNA MTase reveals no conserved cysteine residue within the 10 consensus MTase domains (49). Because no thiol is required for catalysis and

TABLE I

Rate of CcrM-catalyzed methylation as a function of substrate order of addition represented in Fig. 7

| Substrate | Order of addition | k_{meth} | min^{-1} |
|-----------|------------------|----------|----------|
| N^6-23/30 | E + DNA → AdoMet | 0.18 ± 0.03 |
| N^6-23/30 | E → DNA + AdoMet | 0.14 ± 0.02 |
| N^6-23/30 | E + DNA → AdoMet (40)% | 0.20 ± 0.10 |

a Berdis et al. (40) data included for sake of comparison.
methyl transfer does not require redox chemistry, there is no obvious reason why methylation of the N-6 amine of adenine should require an external reductant.

We have observed that the absence of reducing agents not only affects the catalytic efficiency of CcrM but also results in the formation of a covalent dimeric species (see above). Although CcrM binds DNA as a monomer, steady state kinetics show that the monomer-dimer equilibrium that exists in solution does not affect catalysis. Dimerization can only be innocuous if dissociation of the dimer is rapid compared with the rate-limiting step for catalysis, which for CcrM is the dissociation from DNA (40). In the absence of reducing agents, CcrM is covalently trapped in the catalytically inactive dimeric form. Therefore, it seems likely that the primary role of reducing agents is to prevent the formation of nonproductive dimeric CcrM species mediated by inter-protein disulphide bonds.

In addition to being sequence-specific, DNA MTases also discriminate between alternate methylation states of the target site. In eukaryotic systems there is a clear distinction between de novo MTases that methylate unmethylated duplex DNA and maintenance MTases in which methylation of the nascent strand is directed by CpG methylation on the template strand (50). Bacterial MTases that possess equal catalytic activity regardless of the methylation state are said to be capable of de novo and maintenance methylation. However, some bacterial MTases are specific for hemi-methylated DNA consistent with a role of maintaining a pattern of genomic methylation; these are termed maintenance MTases (51, 52).

Semiconservative DNA replication during the stalled cell stage of the C. crescentus cell cycle leads to the formation of hemi-methylated genomic DNA. As previously indicated, CcrM activity is temporally compartmentalized to the subsequent predivisional cell. As a direct result of which, CcrM only encounters hemi-methylated DNA in vivo, and thus its physiologic role is that of a maintenance MTase. Consistent with this in vivo role, it has been shown that CcrM demonstrates a tighter binding affinity and enhanced catalytic efficiency toward hemi-methylated DNA substrates (40). Accordingly, all the experiments performed for this paper exploited a hemi-methylated substrate, which would only require the catalytic transfer of one methyl group per binding event. It has been previously suggested that because of an asymmetric nature, this hemi-methylated substrate would be expected to exclusively accommodate a monomeric MTase species (44).

To date, virtually all DNA MTases studied exist as monomers. In this paper we present evidence that CcrM from C. crescentus is dimeric in solution but active as a monomer. Similar to CcrM, the maintenance adenine DNA MTase M.RsrI from Rhodobacter sphaeroides (25) exists as a dimer in solution (24). Prior literature showed that M.RsrI deposits a single methyl group per binding event and that at the concentrations employed in these assays the enzyme is likely to be a monomer in the absence of substrates and cofactors (24). The recently published crystal structure (53) of M.RsrI, however, allows for the possibility of a DNA-induced dimer that would perform said methylation. The controversy was quickly clarified in the accompanying paper to the crystal structure (25) in which active site titration experiments provide evidence that M.RsrI is functional as a monomer. In contrast, the human placental DNA MTase is a de novo MTase and, as such, requires the transfer of two methyl groups per nascent recognition sequence. Furthermore, this MTase requires dimerization to activate the enzyme for catalysis (26). Could it be that de novo MTases are functional dimers and that maintenance MTases are functional monomers? Overall, very little is known about the active oligomeric state of DNA MTases and how this state correlates to the physiologically relevant methylation function. However, with the enormous interest in DNA methylation in cancer and bacterial virulence, more insight may be gained within the not so distant future.

Within C. crescentus, genomic methylation is catalyzed by the tightly regulated nonrestriction-modification MTase, CcrM. First, translation of CcrM initiates in coincidence with the stalked-to-predivisional morphological transition (17) and, second, Lon-mediated proteolysis rapidly eliminates CcrM immediately prior to cell division (20). Although the physiologic role played by CcrM dimerization is unknown at present, it is attractive to speculate that the role for the preponderance of dimeric CcrM at physiological concentrations is to protect the newly synthesized enzyme from premature catalolysis by the ubiquitously present Lon protease. In this manner, C. crescentus can minimize CcrM degradation while simultaneously maintaining CcrM and Lon in the cell prior to genomic remethylation.

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5 The active site titration does not preclude the possibility that M.RsrI remains a dimer and methylates two distinct DNA substrates (one per monomer). However, the crystal structure (53) renders this scenario unlikely. The defined crystallographic dimer interface and the defined target recognition domain overlap in such a manner that this appears to make duplex DNA binding and oligomerization mutually exclusive.
Identification of the Active Oligomeric State of an Essential Adenine DNA Methyltransferase from *Caulobacter crescentus*

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