Mutational Separation of DNA Binding from Catalysis in a DNA Cytosine Methyltransferase

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Despite the substantial progress made toward understanding sequence-specific recognition of DNA by regulatory proteins,[1] the details of catalysis by DNA-modifying proteins remain poorly understood.[2] The inherently transient nature of catalytic protein–DNA complexes presents problems for their characterization by X-ray crystallography or NMR spectroscopy. Catalytic DNA-binding proteins thus present the challenge of discovering how to stall or subvert the catalytic process so as to obtain a stable macromolecular complex.[3] In this communication, we report the use of site-directed mutagenesis to separate catalysis from DNA binding in a DNA (cytosine-5)-methyltransferase (DC-Mtase) enzyme.

5-Methyl-2′-deoxycytidine (m^5dC), the sole methylated nucleoside found in eukaryotes and one of several in prokaryotes,[4] is generated by DCMtase-mediated donation of a methyl group from the cofactor *S*-adenosyl-L-methionine (AdoMet) to cytosine residues in duplex DNA. DCMtases operate by the mechanism shown in Figure 1a, involving nucleophilic attack of a Cys thiolate on carbon-6 to form a covalent protein–DNA intermediate, probably an enamine,[5] followed by transfer of the methyl group to carbon-5 and finally β-elimination to regenerate the free DCMtase and liberate m^5dC-containing DNA.[6] An intriguing aspect of DCMtase catalysis is that the chemical steps proceed through several discrete intermediates, one or more of which might be trapped by subversion of the normal catalytic pathway. Indeed, we[7] and Santi[8] have shown that the analog 5-fluoro-dC blocks progression through the normal catalytic pathway by forming an irreversibly linked DCMtase–DNA complex (Figure 1b). This covalent trapping reaction enabled us to identify the catalytic nucleophile of the DCMtase from *Haemophilus eagyptius*, M.*Hae*III, as the residue Cys_{71}.[7]

In the present study we have mutated Cys_{71} of M.*Hae*III in an attempt to separate catalysis from recognition of the DNA substrate. Mutant proteins having Cys_{71} changed to Ser and Ala (C71S and C71A, respectively) were generated, overproduced, and purified by standard methods.[9] The wild-type and mutant proteins were tested for formation of an irreversible covalent protein–DNA complex with FdC-containing DNA.[9] As shown

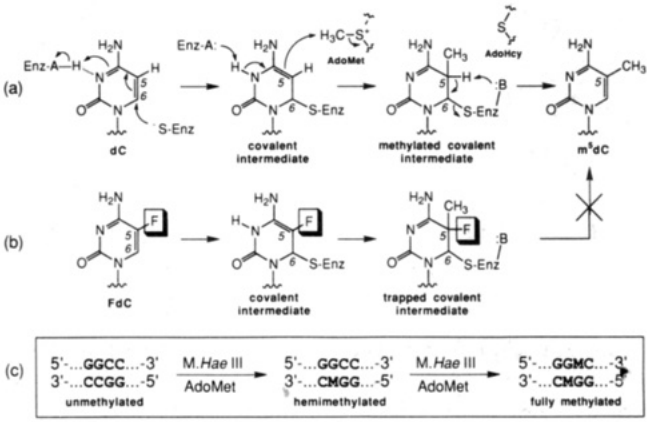

Figure 1. Proposed mechanism of (a) methylation of dC residues in DNA and (b) mechanism-based inactivation by FdC. (c) Sequential DNA methylation reactions catalyzed by M.*Hae*III. **M** = 5-methyl-dC; **F** = 5-fluoro-dC.

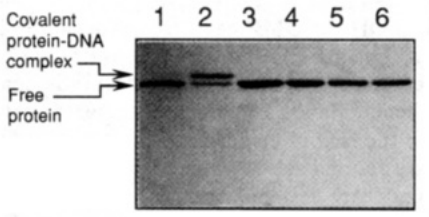

Figure 2. Denaturing SDS-PAGE assay for suicidal inactivation of wild-type M.*Hae*III (lanes 1 and 2) and the mutant proteins C71S (lanes 3 and 4) and C71A (lanes 5 and 6). The proteins were incubated with a duplex FdC-containing 16-mer (sequence as in Table I) in the presence (lanes 2,4,6) or absence (lanes 1,3,5) of AdoMet and then processed as described.[7]

in Figure 2, only the wild-type protein formed a DNA complex that was stable to denaturation; the formation of this irreversible complex, as reported previously, is dependent on the presence of AdoMet.[7] This finding confirms that Cys_{71} is the active-site nucleophile of M.*Hae*III. It is noteworthy that no complex was observed with C71S M.*Hae*III, even though this protein possesses a potentially nucleophilic hydroxyl group.

Although the FdC trapping results are consistent with the lack of an active-site nucleophile in the mutant proteins, these data cannot exclude the possibility that the mutant proteins possess a defect in binding rather than catalysis. To address this issue, we carried out equilibrium DNA-binding experiments[1] using the electrophoretic mobility shift assay (EMSA).[10,11] The thermodynamic dissociation constants (K_ds) of the DNA complexes formed with wild-type M.*Hae*III and the C71A mutant were virtually identical, whereas that for the C71S was slightly higher.[12] This result allows us to conclude that formation of a covalent bond between protein and DNA is not required for either strong binding or discrimination of specific versus nonspecific DNA sequences.

M.*Hae*III carries out two sequential methylation reactions (Figure 1c), first converting a nonmethylated site to a hemimethylated site, which is then converted to a fully methylated site. Each of these methylation states has a distinct biological context: unmethylated sites represent viral (nonself) DNA, hemimethylated sites represent genomic (self) DNA immediately

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(9) Complete experimental details are available in the supplementary materials, which may be obtained directly from the authors by FAX: (617) 495-8755. Alternatively, see paragraph at the end of this communication.
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(11) Equilibrium binding reactions were performed in 20 μL of buffer containing 50 mM NaCl, 50 mM Tris·HCl (pH 7.9), 10 mM EDTA, 5 mM DTT, 0.1 mg/mL BSA, DCMtase, and 5′ end-labeled DNA probe, in the absence or presence of cofactor. The reactions were incubated at 30 °C for 30 min and then electrophoresed on a 4% nondenaturing polyacrylamide gel. The ratio of protein-bound to unbound DNA was measured by Fuji Phosphor Image Analyzer. Details of the data analysis are provided in the supplementary materials.

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Table I. Equilibrium Binding Data for the C71A Mutant of M.*Hae*III[a]

oligonucleotide	K_d (nM) $H_3C-S^+(met)(Ado)$	$S(met)(Ado)$	no cofactor
5'-CGCATAGGCCATGACG-3' 3'-GCGTATCCGGTACTGC-5'	0.36	n.d.[c]	1.7
5'-CGCATAGGCCATGACG-3' 3'-GCGTATC**M**GGTACTGC-5'	0.02	0.35	1.1
5'-CGCATAGG**M**CATGACG-3' 3'-GCGTATC**M**GGTACTGC-5'	~12.5[b]	~12.5[b]	~12.5[b]

[a] Each value represents the mean of at least three independent measurements and is accurate to within ±20%, except for the value for binding to hemimethylated DNA in the presence of AdoMet, which is accurate to within ±50%. [b] This value is indistinguishable from that obtained for binding to a duplex DNA molecule of the same size but which contains no specific binding site for M.*Hae*III. [c] Not determined.

after replication, and fully methylated sites represent mature genomic DNA. Since methylation of viral DNA would allow it to escape inactivation (by a restriction endonuclease), evolution would be expected to select for DCMtases that act on hemimethylated sites rather than on unmethylated sites. The availability of M.*Hae*III variants that retained sequence-specific binding yet lacked catalytic function allowed us to examine whether selection for hemimethylated sites could be observed at the level of DNA binding. At the same time, we investigated the effect of the cofactor on DNA binding.

Thermodynamic binding data on C71A M.*Hae*III are presented in Table I. In all cases, the C71A protein bound fully methylated DNA no more tightly than nonspecific DNA (K_d ~ 12.5 nM). This is consistent with the fully methylated site being a product rather than a substrate. In the presence of AdoMet (Table I), the C71A mutant bound an unmethylated site ~35-fold more tightly than nonspecific DNA (0.36 *vs* 12.5 nM) and a hemimethylated site ~600-fold more tightly than nonspecific DNA (0.02 *vs* 12.5 nM). Comparing the K_d values for binding to a nonmethylated versus hemimethylated site (0.36 *vs* 0.02 nM),[13] it can be calculated that the presence of a single methyl group increases the affinity of the protein for the substrate by 1.7 kcal/mol.[14] Thus, the methyltransferase shows strong discrimination on the basis of methylation state, and the rank order of affinities correlates with expectation based on biology.

The affinity of C71A M.*Hae*III for its specific site in DNA is also strongly influenced by the presence of the cofactor. For example, the protein binds a hemimethylated site (Table I) ~20-fold more tightly in the presence of AdoMet than AdoHcy (0.02 *vs* 0.35 nM) and ~55-fold more tightly with AdoMet than with no cofactor (0.02 *vs* 1.0 nM). Whether this enhancement of binding is due to an allosteric effect[17] or direct DNA binding by the cofactor[18] remains uncertain. Two further points are noteworthy: (i) the protein exhibits strong discrimination for hemimethylated sites only in the presence of AdoMet and (ii) the protein discriminates strongly for cofactor only in the presence of hemimethylated sites.

In this study, we have shown that catalysis can be separated from DNA recognition in a DCMtase enzyme by mutating the protein's active site nucleophile.[19] Binding of the mutant proteins to DNA is strongly activated by hemimethylation of the recognition site and by the methyl donor AdoMet. The M.*Hae*III mutants bind DNA with an affinity and specificity rivaling that of any known DNA-binding protein, despite the fact that the former make specific contacts to a much smaller site and bind DNA as a monomer,[7] foregoing the thermodynamic advantages of multimerization so commonly exploited by DNA-binding proteins. This unusually tight binding may ordinarily be used to drive unfavorable processes accompanying the catalytic cycle, such as the formation of bent or twisted DNA.[22] Whatever the functional origins of their exceptional stability, the structures of these mutant proteins bound to DNA should yield new insights into allosterism and the architecture of protein–DNA interfaces.[23]

Supplementary Material Available: Experimental details; gels illustrating the binding behavior of M.*Hae*III DCMtase, C71A, and C71S; plots showing examples of the calculation of K_ds (13 pages). Ordering information is given on any current masthead page.

(12) Using the hemimethylated substrate shown in Table I, the K_ds for wild-type M.*Hae*III, C71A, and C71S were 0.34, 0.35, and 0.48 nM, respectively, in the presence of 80 μM AdoHcy.

(13) Analogous results were obtained for the C71S mutant. For example, using the hemimethylated substrate shown in Table I, the K_ds were 1.4, 0.48, and 0.08 nM with no cofactor, AdoHcy, and AdoMet, respectively.

(14) This value is comparable to the highest free energy change measured for interactions involving the methyl group of thymidine in DNA (1.8 kcal/mol).[15] It is smaller, however, than the largest free energy change attributed to binding of a methyl group (3.2 kcal/mol).[16]

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(19) While this manuscript was in preparation, the results of two studies related to the present work were published. Hanck *et al.*[20] showed that active-site mutants of the DCMtase DCM failed to form a covalent complex with FdC-containing DNA; Wyszynski *et al.*[21] reported similar DNA methylation and cofactor-dependent binding behavior in mutants of *Eco*RII DCMtase. In addition to having provided independent results in a different protein system, our work complements that of Hanck *et al.* and Wynszynski *et al.* by quantifying the thermodynamics of these effects.

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