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Sequence-specific and mechanism-based crosslinking of Dcm DNA cytosine-C^5 methyltransferase of *E.coli* K-12 to synthetic oligonucleotides containing 5-fluoro-2′-deoxycytidine

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ABSTRACT

The product of the *dcm* gene is the only DNA cytosine-C^5 methyltransferase of *Escherichia coli* K-12; it catalyses transfer of a methyl group from S-adenosyl methionine (SAM) to the C-5 position of the inner cytosine residue of the cognate sequence $CC^A/_TGG$. Sequence-specific, covalent crosslinking of the enzyme to synthetic oligonucleotides containing 5-fluoro-2′-deoxycytidine is demonstrated. This reaction is abolished if serine replaces the cysteine at residue # 177 of the enzyme. These results lend strong support to a catalytic mechanism in which an enzyme sulfhydryl group undergoes Michael addition to the C^5-C^6 double bond, thus activating position C-5 of the substrate DNA cytosine residue for electrophilic attack by the methyl donor SAM. The enzyme is capable of self-methylation in a DNA-independent reaction requiring SAM and the presence of cysteine at position # 177.

INTRODUCTION

Methylation of 2′-deoxyuridine-5′-monophosphate (dUMP) to deoxythymidine-5′-monophosphate (dTMP), catalysed by thymidilate synthetase, is the paradigmatic case of a reaction that proceeds *via* a covalent adduct, formed reversibly by Michael addition of the enzyme to the C^5-C^6 double bond of its pyrimidine substrate; for a review see Pogolotti and Santi (1). The attacking nucleophilic centre of the enzyme was identified as the sulfhydryl function of a particular cysteine residue (1–3). Nature utilizes this route also for other reactions of nucleic acids metabolism, such as tRNA uracil-C^5 methylation (4), tRNA/aminoacyl tRNA synthetase interaction (5) and possibly several others (5). In 1983, D.V.Santi and colleagues conjectured that the same basic mechanism may be underlying postreplicative DNA cytosine-C^5 methylation (6). This idea immediately offered an explanation of the inhibitory *in vivo* effect of nucleoside analogues such as 5-azacytidine (7, 8) or 5-fluoro-2′-deoxycytidine (8) on DNA methylation. In analogy to the blockage of thymidilate synthetase by 5-fluoro-2′-dexoyuridine-5′-monophosphate—the active form of the tumor-inhibitory drug 5-fluorouracil (9)—one could now envisage covalent and irreversible adduct formation between any given DNA cytosine-C^5 methyltransferase and DNA carrying at one of its target sites of methylation a 5-azacytosine or 5-fluorocytosine (5-FC) residue (see Figure 1).

This proposal was further supported by a number of investigations in which *in vitro* DNA/protein crosslinking could be demonstrated after one or the other of the nucleoside analogues mentioned above was incorporated into DNA, either *in vivo* (10, 11) or by DNA polymerase reaction *in vitro* (12). While these observations were clearly compatible with the assumption of the enzyme being trapped after Michael addition to the modified cytosine residue, they did not furnish structural proof and, in particular, since site-specific analogue incorporation was not possible, they did not provide information pertaining to sequence selectivity of the crosslinking reaction. This, however, is an important point, since one has to demand sequence specificity of the irreversible adduct formation, if it is to be accepted as a true mechanistic model of the first steps of enzymatic methyltransfer.

Recently, synthesis of DNA containing 5-fluorocytosine residues at specific positions was independently accomplished by three different laboratories, once by a combination of standard oligonucleotide synthesis and site-specific enzymatic 5-FC incorporation (13), twice by direct chemical synthesis of 2′-deoxyoligonucleotides containing 5-fluoro-2′-deoxycycytidine (14, 15). In two cases (13, 16), crosslinking of a DNA cytosine-C^5 methyltransferase to such DNA substrates was demonstrated. Until present, however, no proof of sequence specificity and hence of a truly mechanism-based reaction has been furnished. With the experiments described here we provide unequivocal evidence of sequence-specific crosslinking of the *Escherichia coli* K-12 DNA cytosine-C^5 methyltransferase (Dcm) to a 5-FC residue replacing the inner cytosine of the cognate sequence $CC^A/_TGG$.

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MATERIALS AND METHODS

Oligonucleotide synthesis

2′-Deoxyoligonucleotides were synthesized by the phosphoramidite method (17) with cyanoethyl as the phosphate protecting group (18), implemented on automated DNA synthesizers (Pharmacia GeneAssembler and Applied Biosystems 380B). Oligonucleotides containing 5-fluorocytosine residues, were prepared as described earlier (15). Oligonucleotides were purified by H.P.L.C. essentially as described (19). The following 2′-deoxyoligonucleotides (all 15mers, sequences reading 5′ to 3′ left to right) were synthesized (F = 5-FC):

GTATCCCAGGAATCG (**CCUP**), GTATCCFAGGAATCG (**CFUP**), GTATCFCAGGAATCG (**FCUP**), GTATCCCGAGAATCG (**CTUP**), CGATTCCTGGGATAC (**CCLO**), CGATTCFTGGGATAC (**CFLO**), CGATTFCTGGGATAC (**FCLO**), CGATTCTCGGGATAC (**CTLO**).

Enzymes and chemicals

Restriction endonucleases were from Boehringer Mannheim or Gibco/BRL; T4 polynucleotide kinase was purchased from Boehringer Mannheim; [γ-^{32}P] ATP (3000 Ci/mmol) was from Amersham Buchler and [^{3}H]-SAM (73 Ci/mmol, terminal methyl group of methionine side chain labelled) was from NEN (Du Pont). SDS was from BioRad, S-adenosyl methionine (SAM) from Boehringer Mannheim. Sinefungin was purchased from Sigma, all other chemicals from Merck.

Bacterial strains and plasmids

E.coli strain RP4182 (source: J.S.Parkinson):Δ(*supD-dcm-fla*), *trp*, *gal*, *rpsl*, was applied for gene expression. Expression vectors pMc5edcm and pMa5edcmS are members of the pMa/c family of phasmids (20) and were constructed as described elsewhere (Hanck and Fritz, manuscript in preparation). pMc5edcm contains the *dcm* gene of *E.coli* K-12 under control of a phage lambda promoter; pMa5edcmS is a derivative of pMc5edcm with a serine codon at codon position #177 of the *dcm* gene instead of the wild-type cysteine codon.

Buffers

DCM reaction buffer: 50 mM Tris−HCl, 5 mM EDTA, 10 mM β-mercaptoethanol, 100 mM NaCl, pH 7.8; kinase buffer: 40 mM Tris−HCl, 10 mM $MgCl_2$, 1 mM DTT, 5% glycerol, pH 9.0; loading buffer: 5%(v/v) glycerol, 10 mM DTT, 0.05% (w/v) bromophenol blue; sample buffer: 62.5 mM Tris−HCl, pH 6.8, 10% (v/v) glycerol, 70 mM SDS, 5% (v/v) β-mercaptoethanol, 0.0025% (w/v) bromophenol blue; TE: 10 mM Tris−HCl (pH 8), 0.1 mM EDTA; TBE: 90 mM Tris, 90 mM boric acid, 2.5 mM EDTA.

Radioactive labelling of synthetic oligonucleotides and construction of oligonucleotide duplexes

5 pmol of oligonucleotide were incubated in kinase buffer with 60 μCi [γ-^{32}P]-ATP (3000 Ci/mmol) and 8 units polynucleotide kinase at 37°C for 30 min. Excess [γ-^{32}P]-ATP was removed by passing the reaction mixture through a NAP-5 column (G25-Sephadex, Pharmacia) with water as the eluent. Oligonucleotide duplexes were constructed by mixing 80 fmol of the radioactive labelled oligonucleotide (corresponding to 20 nCi) with a 20-fold excess of the unlabelled, complementary oligonucleotide in TE. The samples were incubated at 80°C for 3 min and at room temperature for 10 min. Non-radioactive oligonucleotide duplexes were prepared the same way by mixing 2 pmol each of the complementary strands.

Preparation of Dcm and Dcm/Cys177Ser protein

Dcm DNA cytosine-C^5 methyltransferase and the mutant Dcm/Cys177Ser enzyme were produced by overexpression of the respective cloned structural genes in *E. coli* RP4182 and purified to near homogeneity as described elsewhere (Hanck and Fritz, manuscript in preparation).

In vitro methylation of C^5-unmethylated DNA

3 μg double stranded pMc5-8 DNA (20), prepared from transformed RP4182 and therefore C^5-unmethylated, was incubated at 37°C for 30 min with 1 μg purified Dcm or Dcm/Cys177Ser protein in 50 μl DCM reaction buffer containing 80 μM SAM. The volume was adjusted to 400 μl with TE (pH 8), extracted with phenol/chloroform/isoamyl alcohol (50:48:2) and DNA was precipitated by addition of ethanol. The DNA was sedimented by centrifugation and redissolved in 20 μl TE buffer.

Determination of the specific activity of Dcm DNA cytosine-C^5 methyltransferase

12 μg C^5-unmethylated, double stranded pMc5-8 DNA (20) (corresponding to 5 pmol phasmid DNA or 80 picoequivalents of DNA cytosine residues susceptible to methyltransfer by Dcm) were mixed with 50 ng Dcm (1 pmol) or 500 ng Dcm/Cys177Ser (10 pmol) and 150 pmol [^{3}H]-SAM (10 μCi, 73 Ci/mmol) in 110 μl DCM reaction buffer (total volume). The reaction mixture was incubated at 37°C. Aliquots of 10 μl each were withdrawn from the mixture at various time points and quenched by adding 10 μl ice-cold 20% (v/v) aqueous trichloro acetic acid (TCA). Precipitated high molecular weight material was retained on glass fibre filters (GF50, Schleicher and Schuell). Filters were washed with ice-cold 10% (v/v) TCA solution, dried, and transferred to scintillation cocktail ('Ready Save', Beckman). Radioactivity was measured in a liquid scintillation counter (TRI-CARB 1900 TR, Packard). Specific activity was calculated from the slope of the linear incorporation of radioactivity observed during the first 25 min of the reaction. One unit of enzyme transfers 1 pmol [^{3}H]-labelled methyl group per minute to TCA-precipitable material (21).

Gel electrophoretic retardation assay of non-covalent DNA/protein association

The procedure was adapted from literature (22). Duplex DNA, prepared and [^{32}P]-5′-endlabelled as described above, was mixed with 10 μg Dcm or Dcm/Cys177Ser protein in 20 mM Tris−HCl, 1 mM EDTA, 10 mM β-mercaptoethanol, 50 mM NaCl, pH 7.5 in a total volume of 20 μl. The mixture was incubated at room temperature for 20 min. An equal volume of loading buffer was added. Samples were electrophoresed through a pre-cooled (4°C) 5.1% polyacrylamide gel (30 : 0.8 acrylamide : bisacrylamide, 200×250×1.6 mm, 1×TBE), at 4°C, 7. 5 V/cm for 6 h (1×TBE as running buffer). The gel slab was transferred to a sheet of blotting paper (Schleicher & Schuell, GB 003) and dried. For autoradiography of the electrophoretic pattern, Kodak-XOMAT-S film was exposed with an intensifier screen (Amersham Buchler) at −80°C overnight.

Gel electrophoretic analysis of covalent DNA/protein crosslinking

[^{32}P]-5′-endlabelled or non-radioactive duplex DNA, prepared as described above, was mixed with 10 μg Dcm or Dcm/Cys177Ser protein in DCM reaction buffer. Optionally, 80 μM SAM or 1 μCi [^{3}H]-SAM (73 Ci/mmol) and 80 μM

sinefungin were added; the volume was adjusted to 20 μl. The samples were incubated at 37°C for 30 min; an equal volume of 2×sample buffer was added and the samples were kept at 100°C for 10 min. Samples were electrophoresed through a 10% polyacrylamide gel (30 : 0.8 acrylamide : bisacrylamide, 200×250×1.6 mm) containing 2% SDS according to Laemmli (23). Running conditions: 20 V/cm for 3 h. [^{3}H] label was visualized by fluorography as follows. After fixing the gel for 45 min in 10% (v/v) acetic acid, 30% (v/v) EtOH, the gel was soaked in Entensify Part A (Du Pont) for 45 min and in Entensify Part B (Du Pont) for another 45 min. Transfer to filter paper and film exposure (up to 1 week) were as described above.

RESULTS AND DISCUSSION

Qualitative assay of sequence-specific methyltransfer activity

Both purified proteins, Dcm and the Dcm/Cys177Ser (residue #177: serine instead of cysteine), were tested for their ability to methylate C^5-unmethylated DNA, prepared from a Dcm$^-$ strain. C^5-unmethylated DNA was incubated with Dcm or Dcm/Cys177Ser and S-adenosyl methionine at 37°C for 30 min. DNA was isolated from the reaction mixtures and checked for its susceptibility to cleavage by restriction endonuclease *Eco*RII, which fails to cleave CC^A/$_T$GG sites methylated at the inner cytosine residue. From the results displayed in Figure 2, it is evident that the reaction with the wild-type enzyme renders the DNA resistant to challenge by *Eco*RII cleavage (see Figure 2, lane 6), whereas incubation with the Dcm/Cys177Ser mutant protein does not (lane 8). The catalytic inactivity of the mutant protein cannot be attributed to a defect in overall folding since Dcm and Dcm/Cys177Ser show indistinguishable DNA binding properties (see below: Figure 4). Thus, residue Cys^{177} is identified as essential for catalysis. In addition, the assay provides confirmatory evidence that the overproduced Dcm gene product indeed catalyses the methylation of the expected target cytosine residue.

Determination of the specific activity of Dcm DNA cytosine-C^5 methyltransferase

The time course of enzymatic methyltransfer from [^{3}H]-SAM to high-molecular-weight DNA was followed by a TCA-precipitation assay (Figure 3). The incorporation catalysed by Dcm (open circles) was linear for the first 25 min of the reaction. The slope indicates a specific activity of 18,000 units/mg; for comparison, the specific activity of purified M.*Eco*RII has been determined to 21,100 units/mg (24). In first approximation, the Dcm/Cys177Ser protein is a null mutant; there is, however a small but significant increase with time of TCA-precipitable radioactive material (Figure 3, filled circles) corresponding to a formal specific activity about 1000 times lower than that of the wild-type enzyme. If this increase is indeed brought about by a specific methyltransfer reaction, it could be caused either by residual activity of the Ser^{177}-form of the enzyme or, more likely, by a contamination of the mutant protein by ca. 0.1% wild-type enzyme, due to translational misincorporation at position #177 of cysteine instead of serine. Such mechanisms

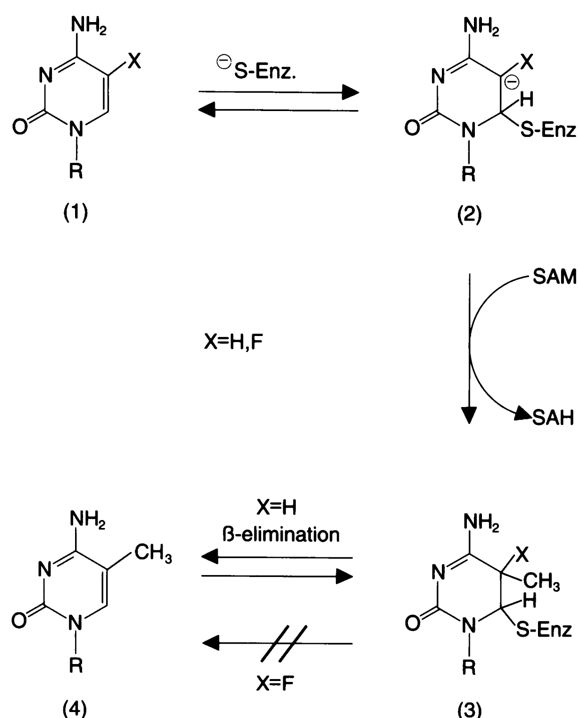

Figure 1. Proposed mechanism of enzymatic DNA cytosine-C^5 methylation. A cysteine residue of the catalytic centre of the DNA cytosine-C^5 methyltransferase undergoes Michael addition to the C^6/C^5 double bond of the target cytosine residue activating its C^5 atom as a nucleophilic centre [(1) → (2)]. Methyltransfer [(2) → (3)] is followed by regeneration of the catalyst *via* β-elimination [(3) → (4)]. If a 5-fluorocytosine residue is attacked by the enzyme (X = F), the final step of the reaction is blocked and a stable, covalent adduct of enzyme and substrate DNA results.

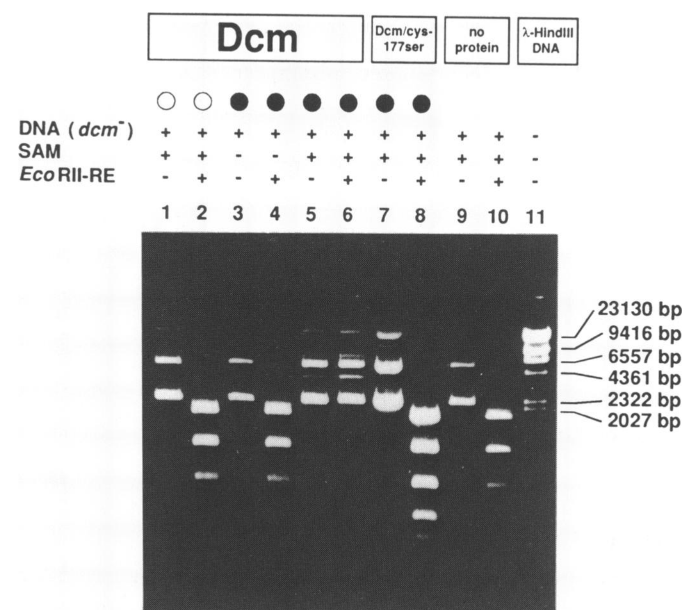

Figure 2. Qualitative assay of methyltransfer activity of Dcm and Dcm/Cys177Ser. C^5-unmethylated, double stranded phasmid DNA was incubated with S-adenosyl methionine (SAM) and Dcm or Dcm/Cys177Ser as described under Materials and Methods. Subsequently, the DNA was exposed to *Eco*RII restriction endonuclease; samples were electrophoresed through a 1% agarose gel containing 0.5 mg/l ethidiumbromid. *Eco*RII-RE : *Eco*RII restriction endonuclease ○: enzyme kept at 100°C for 10 min; ●: untreated enzyme. Plus and minus symbols indicate presence/absence of the respective reagent in the assay mixture. Lane 11: Mixture of DNA fragments obtained by cleaving phage λ DNA with *Hind*III.

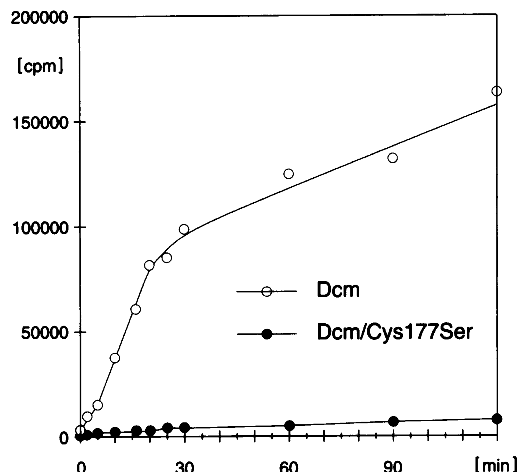

Figure 3. Time course of methyltransfer reaction. C^5-unmethylated phasmid DNA was incubated with S-adenosyl-[^{3}H]-methionine and Dcm or Dcm/Cys177Ser; aliquots of the reaction mixture were removed at the time points indicated and DNA precipitated with 20% aqueous TCA. Precipitated material was collected on glass fibre filters and radioactivity measured by liquid scintillation counting as described under Materials and Methods. Note that a ten-fold higher amount of Dcm/Cys177Ser was used relative to Dcm. [cpm]: counts per minute.

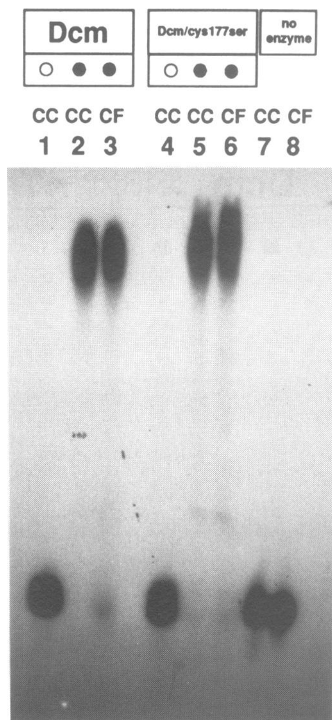

Figure 4. Electrophoretic mobility shift of oligonucleotide duplexes containing a target sequence of Dcm DNA cytosine-C^5 methyltransferase by wild-type Dcm and its Cys177Ser mutant. Purified proteins (wild-type Dcm or Dcm/Cys177Ser) were incubated with radioactively labelled DNA duplexes. Mixtures were electrophoresed through a native 5.1% polyacrylamide gel (see Materials and Methods). **CC:** Hybrid of oligonucleotides CCUP and CCLO (normal target sequence). **CF:** Hybrid of oligonucleotides CFUP and CFLO (with inner cytosine residue of target sequence replaced by 5-fluorocytosine). ○: protein boiled for 5 min. ●: untreated protein.

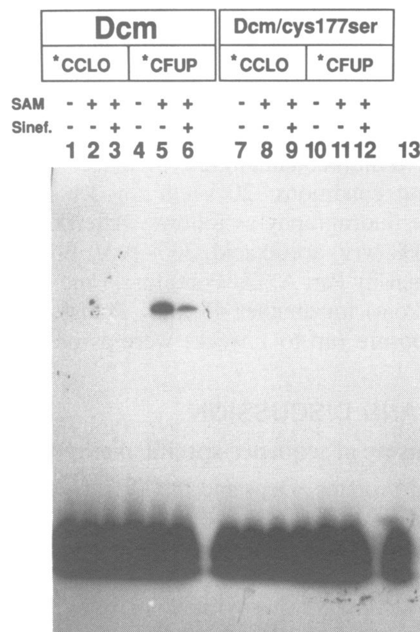

Figure 5. Demonstration of a covalently crosslinked adduct between Dcm enzyme and an oligonucleotide containing a 5-FC residue at the site of methylation. Purified Dcm enzyme (wild-type or Cys177Ser mutant) was incubated at 37°C for 30 min with hybrids of oligonucleotides CFUP and CCLO, 5′-endlabelled with [^{32}P] alternatively in the upper, 5-FC containing strand (***CFUP**) or the lower, unmodified strand (***CCLO**). Optionally, SAM and/or sinefungin (80 μM) were added as indicated. Mixtures were electrophoresed through a 10% PA-gel containing 2% SDS (see Materials and Methods). Lane 13 shows a marker of *CFUP/CCLO.

have been documented (25) and were discussed earlier in the context of protein engineering (26). Misincorporation may be especially pronounced under conditions of forced and massive gene overexpression. On the basis of the data presented here, we cannot distinguish between these two most plausible explanations for the residual activity observed with Dcm/Cys177Ser.

Both wild-type Dcm and the Dcm/Cys177Ser mutant bind to cognate DNA

The Cys177Ser mutant of the Dcm enzyme is deficient in methyltransfer (see above). Since we want to address the question of involvement of cysteine residue # 177 of the enzyme in the molecular mechanism of methyltransfer catalysis, it is necessary to demonstrate that the loss of catalytic activity of the Cys177Ser mutant is not due to a folding defect. This is possible by exploiting the fact that the function of sequence-specific DNA methyltransferases can be dissected into two separate aspects: binding of the enzyme to the target sequence and subsequent catalysis of the methyltransfer reaction. With Figure 4 we demonstrate that the wild-type Dcm enzyme and the Cys177Ser mutant are equally competent in binding to oligonucleotide duplexes containing a recognition sequence ($CC^A/_TGG$).

Radioactively labelled duplex DNA molecules were prepared from oligonucleotides CCUP and CCLO or, respectively, from CFUP and CFLO as described under Materials and Methods. They were incubated with either wild-type Dcm enzyme or its Cys177Ser mutant; incubation with heat-denatured proteins

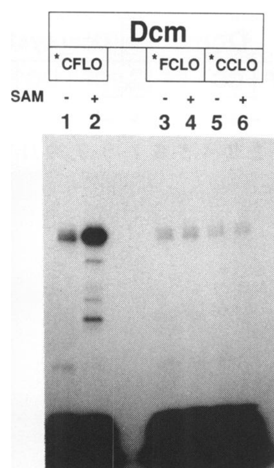

Figure 6. Demonstration of sequence specificity of covalent adduct formation. Experimental conditions were as described in the legend to Figure 5. DNA duplexes were prepared from oligonucleotide CCUP, hybridized to either CCLO, CFLO or FCLO as indicated. In all three duplexes, a [^{32}P] 5′-endlabel was present in the lower strand (asterisk).

served as a control. Reaction mixtures were subjected to electrophoresis through polyacrylamide gel under non-denaturing conditions. In all four experiments, the radioactive band was shifted to a position of lower mobility (Figure 4, lanes 2, 3, 5, 6; controls: lanes 1 and 4). Results obtained for the wild-type and for the mutant protein are virtually indistinguishable. Titration of the radioactively labelled DNA duplexes with increasing amounts of wild-type Dcm enzyme or its Cys177Ser mutant confirmed near identity of the DNA binding properties of the two forms of the enzyme in quantitative detail (data not shown). We regard this result as strong evidence against a possible structural defect of the Dcm/Cys177Ser mutant. The data shown in Figure 4 further demonstrate that replacement of the inner cytosine residue of the Dcm target sequence by 5-fluorocytosine does not interfere with protein binding. Analogous replacements in other DNA cytosine-C^5 methyltransferases of cysteine residues for serine have lead to loss of methyltransfer activity; the folding status of the corresponding stable gene products, however, was not characterized (27, 28).

Dcm forms a detergent-stable adduct to DNA that contains a 5-fluorocytosine residue at the substrate position

A duplex DNA molecule was constructed by hybridizing oligonucleotides CFUP and CCLO. In this duplex, the Dcm recognition sequence is tagged by a cytosine-5-fluoro function at the second position in the upper strand, C[5-FC]AGG, whereas the lower strand carries the unmodified sequence CCTGG. The upper or lower strand, alternatively, of this DNA duplex was 5′-endlabelled with [^{32}P] as indicated by asterisks in the header of Figure 5. Incubation of the CFUP/CCLO duplex with both wild-type Dcm enzyme and S-adenosyl methionine (SAM) leads to formation of a DNA/protein adduct that is stable to 10 min boiling in 2% SDS and subsequent electrophoresis through a polyacrylamide gel containing 2% SDS indicating presence of a covalent bond between DNA and protein. The crosslink occurs specifically to the DNA strand containing the 5-fluorocytosine residue (slow moving band in Figure 5, lane 5 and absence of this band in lane 2).

The crosslinking reaction depends on addition of the methyl donor SAM (Figure 5, lane 4) and is partially suppressed by addition of sinefungin (lane 6), a competitive inhibitor of DNA methyltransferases (29). Replacement of the cysteine sulfhydryl function at position # 177 of the enzyme by a serine hydroxyl group abolishes the reaction (Figure 5, lanes 7–12). Clearly, these data lend strong support to the reaction mechanism outlined in Figure 1 and they also identify the sulfhydryl group of cysteine residue # 177 as the function that undergoes Michael addition to the C^5-C^6-double bond of the DNA cytosine residue.

The DNA/protein crosslinking reaction is sequence specific

Strong but circumstantial evidence for the DNA/protein crosslinking reaction being mechanism-based was already provided by the SAM-dependence we and others (13, 16) observed and by the suppressing effect of sinefungin described above. It was, however, still conceivable that the addition reaction of the enzyme to a DNA could occur in a sequence-unspecific way with any cytosine residue the enzyme makes contact with (for example while sliding along the DNA in search of its specific substrate sequence). Linkage of the addition reaction to the molecular mechanism of catalysis therefore depends on demonstration of sequence specificity. Evidence pertaining to this point is presented in Figure 6.

Oligonucleotides CFLO, FCLO and CCLO, each 5′-endlabelled with [^{32}P], were hybridized to unlabelled oligonucleotide CCUP. The three resulting duplex DNA molecules were separately subjected to reaction conditions that lead to covalent DNA / protein crosslinking as demonstrated above. Similar to the results obtained with duplex CFUP/CCLO (Figure 5), DNA duplex CCUP/CFLO undergoes SAM-dependent crosslinking (Figure 6, lane 2). Swapping of the modified and the unmodified cytosine residues within the Dcm recognition sequence (CCUP/FCLO), however, abolishes the reaction (lane 4; compare lane 6 for result obtained with completely unmodified duplex). The positive result documented by lane 2 also shows that the reaction does not depend on the special sequence context of the Dcm recognition site provided by the upper strand (compare experiments documented by Figures 5 and 6) and, in addition, that the reaction is not restricted to the C[5-FC]AGG half-site but also works with C[5-FC]TGG.

Minute amounts of slower moving material are also visible independent of SAM addition and presence of a 5-fluorocytosine residue at the correct position within the Dcm recognition sequence (lanes 1, 3–6). This may be due either to the covalently but reversibly crosslinked intermediate formed prior to methyltransfer (Figure 1, structure 2) or to an unspecific side-reaction (*e.g.* presence in the synthetic oligonucleotides of trace quantities of reactive molecular species of unknown structure and capable of unspecific crosslinking cannot strictly be ruled out).

The stable DNA/protein adduct contains the methyl group donated by SAM

Three molecules are involved in the Dcm-catalysed methylation reaction, substrate DNA, the enzyme itself and SAM, the methyl donor. The observed dependence of the covalent crosslinking reaction on presence of SAM suggests that the DNA / protein adduct is stably locked only after methyltransfer has occurred (Figure 1, structure 3). This aspect of the reaction was tested by following the fate of the transferred methyl group after reaction of [^{3}H]-SAM. Oligonucleotide duplexes indicated in the header

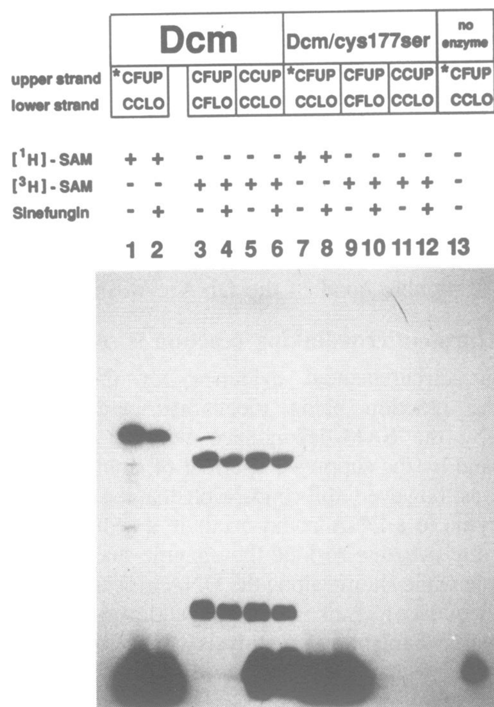

Figure 7. Presence in the trapped reaction intermediate of a ^{3}H-methyl group donated by [^{3}H]- SAM. Purified Dcm enzyme (wild-type or Cys177Ser mutant) was incubated with various oligonucleotide hybrids as indicated (asterisks stand for presence of a [^{32}P] 5'-endlabel). Optionally, [^{1}H]-SAM (80 μM), [^{3}H]-SAM (1μCi, 7.5 μM) or Sinefungin (80 μM) was added as indicated. Mixtures were electrophoresed through 10% polyacrylamide gel containing 2% SDS (see Materials and Methods). Bands visible in lanes 1, 2, 7, 8 and 13 are due to [^{32}P] 5'-endlabels introduced into the synthetic oligonucleotides as indicated; bands in the other lanes are due to ^{3}H introduced into the reactions as tritiated methyl group of SAM. Autoradiography/fluorography conditions were as described under Materials and Methods.

of Figure 7 were prepared as described above, they were either [^{32}P]-5'-endlabelled in one strand (Figure 7, lanes 1, 2, 7, 8, 13) or unlabelled (lanes 3–6, 9–12). Duplexes were incubated with Dcm or Dcm/Cys177Ser under standard reaction conditions of methylation with [^{3}H]-SAM, [^{1}H]-SAM and sinefungin added as indicated.

Reaction of tritiated SAM with unlabelled DNA yields three radioactive products (Figure 7, lane 3). Of these, the slowest moving one corresponds to the covalent DNA / protein adduct, as evident from its position in the gel (compare lanes 1 and 2, which again display the product of the reaction illustrated in Figure 5) and also by the effect of sinefungin addition (compare lanes 3 and 4). The two faster moving bands of lanes 3 and 4 are independent of the presence in the substrate DNA of a 5-fluorocytosine residue (lanes 5 and 6) but completely dependent on the presence of a sulfhydryl function at position # 177 of the enzyme (lanes 7–12). Thus it seems that these two species are the product of methylation of the protein by SAM. Furthermore, partial suppression of this reaction by sinefungin (compare lane 3 with lane 4 and lane 5 with lane 6) suggests that the reaction proceeds *via* SAM bound to the active site of the enzyme. It is not possible at present to decide whether the Cys^{177} sulfhydryl group is itself the ultimate recipient of the methyl group or whether it is required for catalysis of automethylation of the

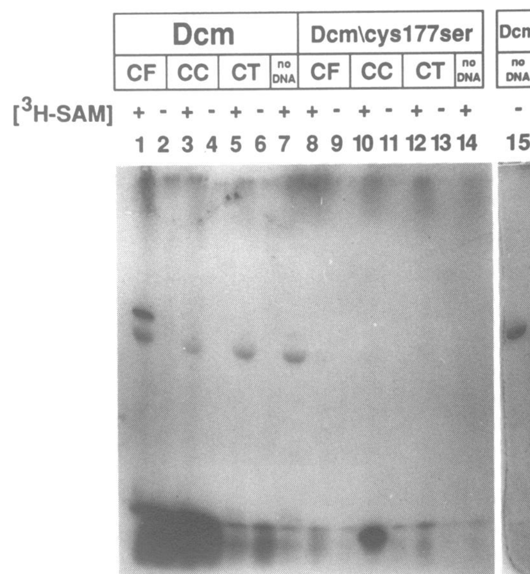

Figure 8. Methylation of the enzyme by SAM. Purified Dcm or Dcm/Cys177Ser protein was incubated with non-radioactive DNA duplexes and analysed by electrophoresis through a 10% polyacrylamide gel containing 2% SDS. **CF:** Hybrid of oligonucleotide CFUP and CFLO (with 5-FC residue at inner cytosine position of target sequence; lanes 1, 2, 8, 9); **CC:** Hybrid of oligonucleotide CCUP and CCLO (normal target sequence; lanes 3, 4, 10, 11); **CT:** Hybrid of oligonucleotide CTUP and CTLO (scrambled target sequence, nor recognized by Dcm; lanes 5, 6, 12, 13); **+:** 2 μCi [^{3}H]-SAM added (lanes 1, 3, 5, 7, 8, 10, 12, 14). Lane 15 was cut off the gel before fluorography and instead stained with Coomassie Blue.

enzyme at some other site. The faster moving of the two bands presumably corresponds to a proteolysis product of Dcm; in later experiments with a fresh batch of enzyme it was no longer observed (compare Figure 8). The fastest moving material in lanes 5 and 6 is methylated oligonucleotide; since the film is saturated in that area, no influence of sinefungin addition on methylation is discernible.

Methylation of the enzyme by SAM does not require presence of substrate DNA

For M.*Hha*I DNA cytosine-C^5 methyltransferase, an ordered mechanism was deduced from kinetic data in which the enzyme first binds DNA, then SAM (30). Since methylation of Dcm seems to proceed through binding of SAM to the active site (see above) it seemed plausible that this reaction may be stimulated by the presence of DNA.

Dcm DNA cytosine-C^5 methyltransferase (wild-type or Cys177Ser mutant) was incubated under standard conditions with [^{3}H]-SAM and a variety of different oligonucleotide duplexes or with [^{3}H]-SAM alone as indicated in the header of Figure 8. Again, the reaction is totally dependent on presence of the sulfhydryl group at position # 177 (compare lanes 1–7 with lanes 8–14). Lanes 1, 3 and 5 demonstrate, that there is no influence of the nucleotide sequence on protein methylation; DNA can even be totally omitted from the reaction mixture with no loss of band intensity. Note that formation of the covalent DNA/protein adduct is possible only with duplex CFUP/CFLO (lane 1, slowest moving band). Lane 15 shows a marker of Dcm enzyme; this part of the gel had been cut off before fluorography and stained separately with Coomassie blue. The band visible at the bottom

of lane 10 is caused by methylated oligonucleotide and reflects the small but significant activity of the mutant enzyme discussed in detail above. In contrast, amounts of methylated oligonucleotide in lane 3 are so large as to cause an extended area of blurred, saturated blackening of the film.

These results confirm the notion that, under the reaction conditions applied, Dcm is methylated by SAM to a significant extent either directly at its active residue Cys^{177} or in a reaction requiring Cys^{177} for catalysis. Methyltransfer from SAM to a protein cysteine residue that is part of a ProCys motif—as is Cys^{177} of Dcm—has been observed earlier with various other DNA- or protein methyltransferases, either as a photo-affinity labelling reaction or in a self-catalysed fashion; cases in point are M.*Eco*RII (31), CheR (32) and—in a more general way—Ada; in the latter case the methyl donor is a DNA O^6-methylguanine residue (33). The functional significance, if any, of the protein methylation reaction we observe with Dcm is unclear at present.

CONCLUSION

Residue Cys^{177} of DNA cytosine-C^5 methyltransferase forms a covalent crosslink with DNA 5-fluorocytosine residues in a sequence-specific and mechanism-based reaction. Therefore, the resulting DNA / protein adduct, which carries the methyl group donated by SAM, is a *bona fide* model of the key intermediate in the catalytic process (Figure 1, structure 3). Ready availability both of Dcm and of chemically synthesized 2'-deoxyoligonucleotides containing 5-fluorocytosine residues (15), open the door to a full structural description of the adduct and hence to an understanding of the stereochemical course of the methyltransfer reaction. This information may be of rather general nature, because all DNA cytosine-C^5 methyltransferases are structurally related (28, 34, 35); conservation of the active-site ProCys motif also extends to thymidilate synthetase (36). DNA-independent methylation of Dcm by SAM in a reaction depending on active-site residue Cys^{177} is an interesting reaction of yet unknown functional potential.

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