Function of Pro-185 in the ProCys of conserved motif IV in the *Eco*RII [cytosine-C5]-DNA methyltransferase

Valeri G. Kossykh*, Samuel L. Schlagman, Stanley Hattman

Department of Biology, University of Rochester, Rochester, NY 14627, USA

Received 22 May 1995

Abstract ProCys in the conserved sequence motif IV of [cytosine-C5]-DNA methyltransferases is known to be part of the catalytic site. The Cys residue is directly involved in forming a covalent bond with the C6 of the target cytosine. We have found that substitution of Pro-185 with either Ala or Ser resulted in a reduced rate of methyl group transfer by the *Eco*RII DNA methyltransferase. In addition, we observed an increase in the K_m for substrate *S*-adenosyl-L-methionine (AdoMet), but a decrease in the K_m for substrate DNA. This is reflected in minor changes in k_{cat}/K_m for DNA, but in 10- to 100-fold reductions in k_{cat}/K_m for AdoMet. This suggests that Pro-185 is important to properly orient the activated cytosine and AdoMet for methyl group transfer by direct interaction with AdoMet and indirectly via the Cys interaction with cytosine.

Key words: Amino acid homology; DNA methylation; 5-methylcytosine; Catalysis

1. Introduction

DNA methyltransferases (MTases) recognize specific nucleic acid sequences and transfer the methyl group from AdoMet to adenine or cytosine residues within these sequences. MTases that are components of bacterial restriction–modification systems protect the cellular DNA against degradation by methylation of the sequence recognized by the cognate restriction enzyme. There are three major classes of MTases, which differ in the resulting modification product: viz. N^6-methyladenine (m^6A), N^4-methylcytosine (m^4C) or C^5-methylcytosine (m^5C).

The deduced amino acid (aa) sequences have been determined for more than 90 cloned MTase genes [1]. Comparison of the sequences has revealed a remarkable similarity among the m^5C MTases [2–4], which are readily distinguishable from the other two classes of MTases. The latter groups, however, also share similarities with each other, but these are not as pronounced as those found among the m^5C MTases [5–8]. The m^4C and N^6A MTases methylate an exocyclic amino nitrogen, whereas m^5C MTases methylate a ring carbon. Thus, the two groups use distinct reaction mechanisms, which most probably contribute to their structural differences. Of the ten conserved motifs observed in m^5C MTases, motif IV [PxxxGxPCQxxSxxG], containing ProCys, is thought to be part of the catalytic site [9]. The Cys contains a nucleophilic thiol that interacts with Cyt in the recognition sequence. Mutagenesis studies performed to determine the role of this Cys showed that its substitution by other amino acids resulted in a complete loss of function by the MTases, M.*Eco*RII, M.*Hha*I, M.*Hae*III, M.*Dcm* [10–13]. Furthermore, introduction of 5-fluorocytosine in the DNA target resulted in the formation of trapped intermediates [13–15]. The covalent bond between Cys and C6 of Cyt was clearly visible in the M.*Hha*I-DNA structure, where 5-fluorocytosine-containing DNA was used to make the co-crystal [16]. From the 3D structure of M.*Hha*I, it has been suggested that the motif IV Pro makes a hydrophobic contact with substrate AdoMet [16,17]. These data prompted us to use the technique of site-directed mutagenesis to study the role of the Pro-185 residue in the *Eco*RII MTase motif IV.

*Corresponding author. Fax: (1) (716) 275-2070.
E-mail: vksy@dbv.cc.rochester.edu

Abbreviations: aa, amino acid; MTase, methyltransferase; AdoMet, *S*-adenosyl-L-methionine; Cyt, cytosine.

2. Materials and methods

2.1. Chemicals and resins

[*methyl*-^{3}H]AdoMet (73 Ci/mmol and 15 Ci/mmol) was from New England Nuclear. Unlabeled AdoMet (90% pure; from Sigma) was purified by HPLC on a C18-reversed-phase column (25 cm × 4.6 mm, fitted with a 4.5 cm × 4.6 mm guard C18-column) using isocratic elution in 50 mM ammonium acetate, pH 4.5, with 5% methanol. The AdoMet was dried in vacuo, dissolved in 5 mM HCl, 5% ethanol and stored at −20°C (or at 4°C if it was used within one week). The final preparation was 99.6% pure. The concentration of AdoMet was calculated using a molar extinction coefficient at 256 nm of 14,700 (in acid) [18]. Phosphocellulose and DE81 ion-exchange filter paper were purchased from Whatman. Hydroxyapatite and heparin agarose were from Bio-Rad.

2.2. Cloning, mutagenesis and sequencing

The *Eco*RII MTase gene was amplified using linearized plasmid pVK33 [19] as a template with primers containing *Bgl*II site according to the PCR protocol of Perkin Elmer-Cetus. The PCR-generated *Eco*RII MTase gene containing fragment was cloned into *Bgl*II site of vector pGC2 [20]. The new plasmid, designated pGC2RIIM, was then mutagenized to obtain mutants at Pro-185. Mutagenesis was performed by a slight modification of the method of Kunkel et al. [21]; viz. Sequenase 2.0 (US Biochemical) replaced *Escherichia coli* PolIk and only one primer was used. The *Eco*RII MTase gene was excised on a *Nde*I-*Bam*HI fragment and cloned into the vector, pJW2 [22]. DNA sequence analysis was by the method of Sanger et al. [23].

2.3. Bacterial growth

E. coli B834 [24] cells containing an appropriate plasmid were grown in ampicillin-containing LB broth [25] at 30°C to an OD_{600} = 0.8–1.0. The temperature was raised to 42°C and incubation continued an additional 3 h. The cells were collected by low-speed centrifugation and stored frozen at −20°C.

2.4. Purification of wt and mutant Eco*RII-MTases*

Frozen cells were thawed and suspended in 20 mM potassium phosphate, pH 7.4, 1 mM EDTA, 7 mM 2-mercaptoethanol (= PEM buffer), containing 0.4 M NaCl, 0.1% NP40, 0.1 mM phenylmethylsulfonyl fluoride and 0.5 mg/ml lysozyme. Cells were disrupted by sonication and cellular debris was removed by centrifugation at 100,000 × *g* for 1.5 h. The supernatant was diluted two-fold in PEM buffer and applied to a phosphocellulose column. Fractions containing MTase activity were pooled and purified on an hydroxyapatite column. Active

SSDI 0014-5793(95)00795-4

fractions were dialysed against PEM buffer with 0.1 M NaCl and purified further by chromatography on heparin agarose.

2.5. Steady state kinetic determinations

Apparent K_m(DNA), K_m(AdoMet) and k_{cat} values were determined by monitoring [^{3}H]CH_3 transfer from labeled AdoMet to unmethylated substrate DNA (phage $\lambda \cdot dcm^- dam^-$). Reaction mixtures (50 μl) contained 100 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 200 μg/ml of bovine serum albumin and variable concentrations of enzyme, DNA or AdoMet (see Table 2). Under the conditions employed, methyl transfer was linear for at least 30 min. After incubation at 37°C for 10 min, 40 μl was removed and spotted onto DE81 filters. These were then washed three times with 50 mM KH_2PO_4, twice with 80% ethanol, once with 95% ethanol, dried and the radioactivity counted in a liquid scintillation spectrometer. The number of methyl groups transferred to the DNA was calculated from the radioactivity bound to the filter [minus that in the zero time aliquot] divided by the specific activity. Kinetic parameters were obtained using the Enzyme Kinetics program from Trinity Software.

2.6. Other analytical procedures

SDS-polyacrylamide gel electrophoresis was carried out according Laemmli [26]. Protein concentrations were determined by the method of Bradford [27] with bovine serum albumin as the standard.

3. Results

3.1. Production of aa replacements at Pro-185 in motif IV

We used oligonucleotide site-directed mutagenesis of the *Eco*RII MTase gene to change Pro-185 located in the ProCys of motif IV [3]. Following DNA sequence analysis, an *Nde*I-*Eco*RI fragment containing the mutation was subcloned into the corresponding sites of plasmid pJW2 [22]. The standard MTase assay (see section 2) was then used to determine enzyme activity in crude extracts containing either the wt or mutant enzymic form. The results summarized in Table 1 show that each of the eight Pro-185 substitutions resulted in a sharp reduction of MTase activity. It should be noted that these assays were performed in 1.0 μM AdoMet, which corresponds to a concentration 3-fold higher than the K_m for wt MTase. As observed later, hoverever, two mutant forms taken for further analysis exhibited increased K_ms for AdoMet. Hence, the activities shown in Table 1 are lower limits for those two mutants, and possibly others as well. Two of the replacements, P185A and P185S, were studied further. The wt and mutant *Eco*RII-MTases were overproduced and purified, as described in section 2. The protein was about 55 kDa (data not shown), consistent with the size predicted from the *Eco*RII MTase open reading frame [28].

3.2. Steady state kinetic analysis

Table 2 summarizes the results of steady state kinetic analyses. The k_{cat} and the apparent K_ms for AdoMet and DNA were derived from initial velocity experiments by varying AdoMet and DNA concentrations. We observed that the k_{cat} was 2- to 8-fold reduced, the K_m for AdoMet was 5- to 13-fold increased, whereas the K_m for DNA was 2- to 4-fold reduced in the mutants compared to the wt. This is reflected in minor changes in k_{cat}/K_m for DNA, whereas 10- to 100-fold reductions in k_{cat}/K_m for AdoMet were observed.

Table 1
Activity in cell extracts of wt and mutant *Eco*RII-MTases

Enzyme	% activity
wt	100
P185A	8.0
P185S	1.5
P185C	1.0
P185F	1.0
P185V	1.0
P185R	< 1.0
P185H	< 1.0
P185L	< 1.0

Cell extracts were prepared as described in section 2. Standard asssay reaction mixtures (50 μl) contained 100 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 200 μg/ml bovine serum albumin, 25 μg/ml $\lambda \cdot dam^- dcm^-$ DNA, 1.0 μM AdoMet and different dilutions of cell extracts.

4. Discussion

In our study with the *Eco*RII-MTase, site-directed mutagenesis was performed to produce amino acid substitutions of Pro-185 in the motif IV ProCys. Two mutant proteins, P185A and P185S, were overexpressed and purified. They were different from the wt enzyme with respect to k_{cat} and K_m for substrates DNA and AdoMet (Table 2). Interestingly, these substitutions resulted in 2- to 4-fold decrease in K_m for DNA, and 5- to 13-fold increases in K_m for AdoMet. The ProCys dipeptide plays a crucial role in catalysis by stabilizing a transient ternary complex through interaction with AdoMet and the DNA Cyt to be methylated. The formation of the ternary complex precedes the activation of Cyt, when methyl group transfer occurs. All these steps depend on the establishment of stereo-specific coupling between AdoMet, Cyt and the ProCys dipeptide. Our results indicate that Pro-185 mutations affect the interactions of *Eco*RII MTase with both substrates. Substitutions at Pro-185 could stabilize or destabilize the transition steps of the DNA:enzyme:AdoMet intermediate, which results in a decreased rate of catalysis. However, some capacity to coordinate the two substrate sites in the methyl transfer reaction is still retained. Because of its unique properties, Pro is regarded as a key residue in protein folding. It has less conformational freedom than any other aa residue, especially in unfolded structures (such as the one in which motif IV is located), since the Pro side chain is fixed by a covalent bond to the backbone. Therefore, we propose that the main role of the Pro-185 is to

Table 2
Catalytic and substrate binding parameters of wt and mutant *Eco*RII-MTases

MTase	k_{cat} (s^{-1})	K_m [AdoMet] (10^{-6} M)	k_{cat}/K_m [AdoMet] (10^6 $M^{-1} \cdot s^{-1}$)	K_m [DNA] (10^{-12} M)	k_{cat}/K_m [DNA] (10^{12} $M^{-1} \cdot s^{-1}$)
wt	0.042	0.32	0.131	215.0	0.00019
P185A	0.025	1.70	0.015	128.7	0.00019
P185S	0.005	4.20	0.001	58.0	0.00009

The apparent steady state kinetic parameters were obtained at 37°C with 0.003–1.0 nM $\lambda \cdot dam^- dcm^-$ DNA, 0.05–7.6 μM AdoMet in 100 mM Tris, pH 8.0, 1 mM EDTA, 200 μg/ml bovine serum albumin, 1 mM DTT and 0.003 or 0.06 nM MTase.

properly orient the activated Cyt and AdoMet for methyl group transfer by direct interaction with AdoMet and indirectly via the Cys interaction with Cyt. The role(s) of the other amino acids within and flanking motif IV remains unknown.

Acknowledgements: We thank to Dr. E. Rukavtsova for performing of PCR. This work was supported by a National Institutes of Health Grant GM29227.

References

[1] Wilson, G.G. (1991) Nucleic Acids Res. 19, 2539–2565.
[2] Chandrasegaran, S. and Smith, H.O. (1988) in: Structure and Expression. From Proteins to Ribosomes (Sarma, M.H. and Sarma, R.H. eds.) vol. 1, pp. 149–156, Adenine Press, Guilderland/New York.
[3] Pósfai, J., Bhagwat, A.S., Pósfai, G. and Roberts, R.J. (1989) Nucleic Acids Res. 17, 2421–2435.
[4] Lauster, R., Trautner, T.A. and Noyer-Weider, M. (1989) J. Mol. Biol. 206, 305–312.
[5] Hattman, S., Wilkinson, J., Swinton, D., Schlagman, S., MacDonald, P.M. and Mosig, G. (1985) J. Bacteriol. 164, 932–937.
[6] Lauster, R. (1989) J. Mol. Biol. 206, 313–321.
[7] Lauster, R., Kriebardis, A. and Guschlbauer, W. (1987) FEBS Lett. 220, 167–176.
[8] Klimasauskas, S., Timinskas, A., Menkevicius, S., Butkiene, D., Butkus, V. and Janulaitis, A. (1989) Nucleic Acids Res. 17, 9823–9832.
[9] Wu, J.C. and Santi, D.V. (1987) J. Biol. Chem. 262, 4778–4786.
[10] Wiszynski, M., Gabbara, S. and Bhagwat, A.S. (1993) Nucleic Acids Res. 20, 319–326.
[11] Mi, S. and Roberts, R.J. (1993) Nucleic Acids Res. 21, 2459–2464.
[12] Wiszynski, M., Gabbara, S., Kubareva, E.A., Romanova, E.A., Oretskaya, T.S., Gromova, E.S., Shabarova, Z.A. and Bhagwat, A.S. (1993) Nucleic Acids Res. 21, 295–301.
[13] Hank, T., Schmidt, S. and Fritz, H.-J. (1993) Nucleic Acids Res. 21, 303–309.
[14] Friedman, S. and Ansari, N. (1992) Nucleic Acids Res. 21, 3241–3248.
[15] Chen, L., MacMillan, M., Chang, W., Ezaz-Nikpay, K., Lane, W.S. and Verdine, G.L. (1991) Biochemistry 30, 1108–11025.
[16] Cheng, X., Kumar, S., Postfai, J., Pflugrath, J.W. and Roberts, R.J. (1993) Cell 74, 299–307.
[17] Klimasauskas, S., Kumar, S., Roberts, R.J. and Cheng, X. (1994) Cell 76, 357–369.
[18] Shapiro, S.K. and Ehninger D.J. (1966) Anal. Biochem. 15, 323–333.
[19] Kossykh, V.G., Glinskaite, I.V., Buryanov, Ya.I. and Bayev, A.A. (1983) Doklady Biol. Sci. 265, 375–378.
[20] Myers, R.M., Lerman, L.S. and Maniatis, T. (1985) Science 229, 242–247.
[21] Kunkel, T.A., Bebenek, K. and McClary, J. (1991) Methods Enzymol. 206, 125–139.
[22] Wang, H., McConnell, D.J. and O'Mahony, D.J. (1990) Nucleic Acids Res. 18, 1070.
[23] Sanger, F. Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[24] Wood, W.B. (1966) J. Mol. Biol. 16, 118–133.
[25] Miller, J.H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[26] Laemmli, U.K. (1970) Nature 227, 680–685.
[27] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[28] Som, S., Bhagwat, A. and Friedman, S. (1987) Nucleic Acids Res. 15, 313–332.
[29] Som, S. and Friedman, S. (1991) J. Biol. Chem. 266, 2937–2945.