Turkish Journal of Biology

Volume 25 | Number 2 Article 9

1-1-2001

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HATİCE PINARBAŞI

ERGÜN PINARBAŞI

DAVID P. HORNBY

Turk J Biol
25 (2001) 177-184

Substitution of the Conserved Cysteine With Glycine (Cys82Gly) of *Agmenellum quadruplicatum* Methylase *Aqu*I (M. *Aqu*I) is not Cytotoxic to *E. coli*

Hatice PINARBAŞI
Cumhuriyet University, Faculty of Medicine, Department of Biochemistry, 58140, Sivas-TURKEY

Ergün PINARBAŞI
Cumhuriyet University, Faculty of Medicine, Department of Medical Biology and Genetics, 58140, Sivas-TURKEY

David P. HORNBY
Krebs Institute for Biomolecular Research, Department of Molecular Biology, Universiyt of Sheffield, Western Bank, Sheffield, S10 2TN, UK

Received: 09.02.2000

Abstract: Cytosine 5 DNA methyltransferases share ten conserved motifs. Motif IV contains an absolutely conserved proline-cysteine dipeptide. The cysteine residue of this motif is involved in catalysis by forming a covalent bond with the 6-position of cytosine prior to methyl group transfer. AquI DNA methyltransferase recognising the sequence CCCGGG is a heterodimer unlike other C5 DNA methyltransferases. We changed the conserved cysteine (Cys82) of M.*Aqu*I to serine and glycine. The presence of mutations was confirmed by automated DNA sequencing. Mutants were tested by in vivo plasmid protection assay and also by transformation into *mcr*A+BC+ strain of E. coli. Replacement of the conserved cysteine with serine led to an apparent loss of the methyltransferring ability of the enzyme. Interestingly, it was found that substitution of cysteine with glycine is not cytotoxic to *E. coli* in the case of M.*Aqu*I.

Key Words: M.*Aqu*I, mcr, DNA Methyltransferase, site-directed mutagenesis

Agmenellum quadruplicatum Metiltransferazı *Aqu*I (M.*Aqu*I) Korunmuş Sisteininin Glisinle Değiştirilmesi (Cys82Gly) *E. coli* İçin Sitotoksik Değildir

Özet: Sitozin 5 DNA metiltransferazlar evrim süreci içinde korunmuş on motif içerirler. Motif IV kesinlikle korunmuş Prolin-Sistein dipeptid içerir. Bu motifdeki sistein amino asidi metil grup transferi için sitozinin 6. karbonu ile kovalent bağ oluşturarak katalizde görev alır. CCCGGG DNA dizisini tanıyan M.*Aqu*I diğer metiltransferazlardan farklı olarak bir heterodimerdir. Biz M.*Aqu*I yapısındaki korunmuş sisteini (Cys82) serin ve glisin ile değiştirdik. Mutasyonların varlığı otomatik dizi analizi ile doğrulandı. Mutantlar *in vivo* olarak plasmidin restriksiyon endonükleaz etkisinden korunması yöntemi ve mcr A^+BC^+ *E. coli* suşuna transformasyon ile test edildi. Korunmuş sisteinin serinle yer değiştirmesi enzimin metiltransfer özelliğini kaybetmesine neden oldu. İlginç olarak M.*Aqu*I da sisteinin glisin ile değiştirilmesinin *E. coli* için toksik olmadığı bulundu.

Anahtar Sözcükler: M.*Aqu*I, *mcr*, DNA Metiltransferaz, bölge yönlendirilmiş mutasyon

Introduction

Methylation is the most common modification of DNA. Most bacteria and vertebrates have 5-methyl cytosine (5meC), N6-methyl adenine (6meA) and N4-methyl cytosine (N4meC). There is a considerable variation in the level and type of methylation between species (1). In prokaryotes, DNA methylation is part of the restriction-modification (R-M) system. It is thought that their function is to act as a prokaryotic immune system (2). Cells harbouring the R-M system are resistant to the entry of foreign DNA from bacteriophage.

C5 DNA methyltransferases (MTases) can be found in both eukaryotes and prokaryotes (1). 5-methyl cytosine is the only methylated base found in vertebrates. The biological function of DNA methylation has been linked to a large variety of cellular processes including gene expression, differentiation and development, carcinogenesis, X-chromosome inactivation, genomic imprinting and regulation of chromatin structure (3). Currently fifty C5 MTase sequences are known. Comparative analysis has shown that these proteins share an ordered set of sequence motifs which alternate with nonconserved region (4). Up to ten motifs can be identified in the majority of the known sequences, including the C terminal 500 amino acids of the eukaryotic CpG MTase (5,6). The sequence alignments suggest that six motifs are highly conserved (I, IV, VIII, IX and X). The variable region located between conserved motifs VIII and IX shows the greatest heterogeneity in size, sequence and composition among C5 MTases. Within the variable region, a small subregion termed the target recognition domain (TRD) is directly involved in sequence specific recognition. Two motifs have been assigned functional roles in the common chemistry of these enzymes. Motif I (FXGXG) of the C5 MTases is the cofactor binding site (7). Motif IV contains a conserved Pro-Cys (P-C) dipeptide that is known to be a part of catalytic site. It contains nucleophilic thiol proposed by Wu and Santi (8). In the proposed scheme, carbon 6 of the target cytosine undergoes nucleophilic attack by the thiolate group of the active site cysteine. This leads to the formation of Michael adduct between carbon 5 of the cytosine ring and the cysteine and also activation of C5 by conversion to a resonance-stabilised carbanion. Electrophilic attack by the carbanion on the methyl group of S-Adenosyl-L-Methionine leads to methyl addition at C5 of the cytosine and generates the intermediate and S-Adenosyl L-Homocysteine. β elimination releases the enzyme and produces the product 5-methyl 2' deoxycytidine. This proposed mechanism has been confirmed by analysis of the crystal structure of M.HhaI and M.HaeIII (9, 10). The cysteine residue of motif IV in these enzymes is involved in the catalysis reaction by forming a covalent bond with the 6 position of the target cytosine prior to methyl transfer. It was first observed for the SPR multispecific methyltransferase (found in many *Bacillus subtilis* bacteriophages) that replacement of the cysteine residue in P-C motif by serine abolished catalytic activity (11). Studies on M.*Eco*RII revealed that substitution of conserved cysteine with glycine, valine, tryptophan or serine abolished the methyltransfer ability of the enzyme (12, 13). M.*Aqu*I from *Agmenellum quadruplicatum* recognising the DNA sequence of CCCGGG is a C5 DNA MTase belonging to type II R-M system. Although other C5 DNA Mtases are monomeric enzymes, M.*Aqu*I is a

heterodimer. According to the crystal structure of M.*Hha*I (9, 15), the α subunit corresponds to the catalytic domain, while the β subunit corresponds to the DNA-binding domain. We have previously subcloned the genes encoding the α and β subunits separately to expression vector pET14b, and expressed the recombinant polypeptides in *E. coli*. In this paper we describe the substitution of Cys residue (Cys82) in P-C motif to serine and glycine.

Materials and Method

Bacterial strain and plasmid

Escherichia coli BL21(DE3) (F- *omp*T (*lon*) *hsd*SB (r^-B m^-B) strain was used throughout this study. Mutagenesis studies were done on the monomeric form of M.*Aqu*I. A plasmid pETAB encoding the monomeric form of M.*Aqu*I (αβ) was constructed in our laboratory by fusing the intact β ORF to the 3' end of the α subunit inframe with the hexahistidine residue of pET14b (Figure 1).

Digestions with restriction endonucleases, ligations, transformations and DNA agarose gel electrophoresis were performed essentially as described by Maniatis et al. (16).

Site-Directed Mutagenesis

A PCR based method was used (17). This procedure requires one mutagenic primer and two universal primers and makes use of two subsequent amplification rounds, the first with a

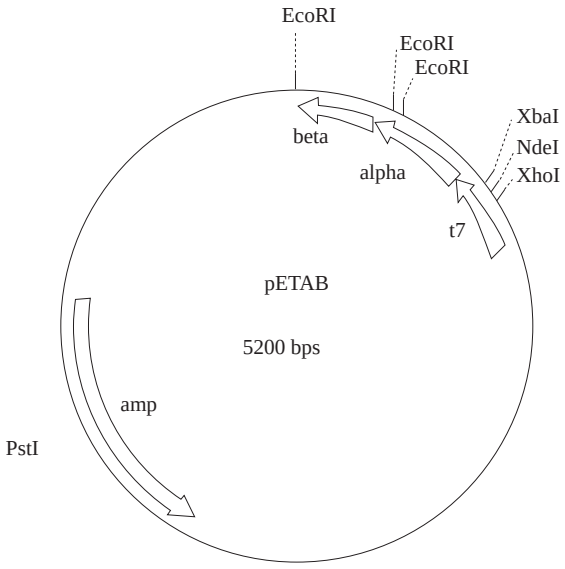

Figure 1. Schematic representation of the plasmid pETAB encoding the monomeric form of M.*Aqu*Iαβ.

mutagenic oligonucleotide and a universal primer and the second one using the purified first fragment as a primer together with the second reverse primer. This resultant PCR product is then digested with appropriate restriction enzymes and cloned. The strategy for mutagenesis is outlined in Figure 2.

Substitution of catalytic Cys with Ser in αβ

In order to replace the cysteine residue (Cys82) with serine in P-C motif in αβ, two primers were synthesised: a mutagenic primer (MS) was used as the 3' primer and the 5' primer contained *Nde*I site (α5'N2) to simplify cloning of the PCR product. The primer sequences were as follows:

5' ACCTGCTAGGCTGAAACTTTGT**GAA**GGGGGCCCACCAATAAC 3' (MS)

5' GAGACTGCCATATGGAAAAAAAACTGATAAGCCT 3' (α5'N2)

*Nde*I

10 ng of plasmid DNA (pETAB) was used as a template in a total volume of 50 µl, containing 1 unit of Vent DNA polymerase, polymerase buffer, 200 µM of dNTPs and 10 pmoles of each primer (MS, α5'N2). The PCR was performed for 30 cycles, with each cycle consisting of a denaturation step at 94 °C for 1 min, a primer annealing step at 55 °C for 1.5 min and an

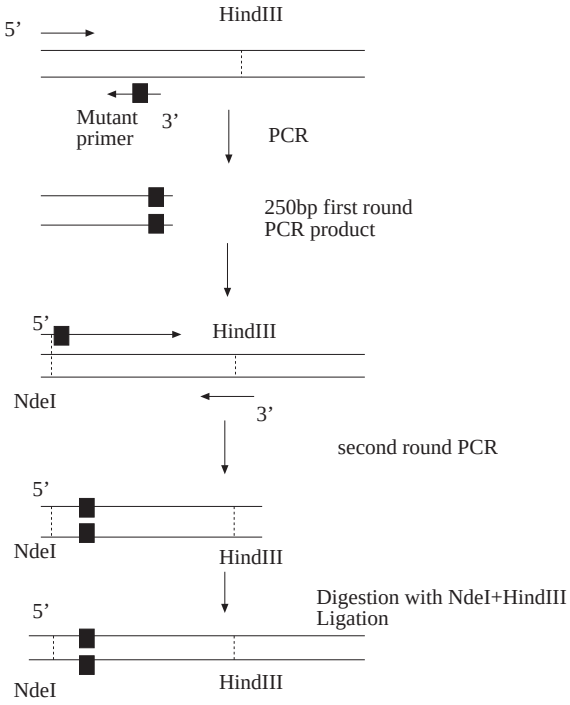

Figure 2. Schematic illustration of site directed mutagenesis employed for the substitution of cysteine of P-C motif to Ser and Gly in pETAB

A primary mutant fragment generated in the first PCR stage is used as a primer on the wild-type template to generate the full-length mutant product into second PCR stage.

extension step at 72 °C for 30 seconds. This was followed by a single cycle consisting of a further elongation step at 72 °C for 5 min followed by cooling to 4°C. Production of the 250 bp PCR product was confirmed by agarose gel electrophoresis, electro-eluted and then used in the second round of PCR reaction as a 5' primer along with the 3' primer HP2 containing a unique *Hind*III restriction enzyme site

5'GATCCATGATTCTTACTCGTAGCGGTACCGCTGCCGCGCGGCACAAGCTTGC

CAAAA 3'. (HP2)

The second round PCR was performed as described for the first step. The 750 bp second round PCR product was gene cleaned from a 1% agarose gel and digested with *Nde*I and *Hind*III restriction enzymes and was ligated into pETAB which had been cleaved with the same enzymes to produce pETABser.

The mutagenesis procedure and PCR program employed for replacement of the catalytic cysteine with Gly was the same as used for substitution with Ser except that the 3' primer used for first PCR step was MG

(5' ACCTGCTAGGCTGAAACTTTGT**CCA**GGGGGCCCACCAATAAC 3'). The altered sequence is highlighted. The resultant PCR product was digested with *Nde*I and *Hind*III and ligated into the *Nde*I/*Hind*III site of pETAB to produce pETABgly.

DNA Sequencing

Plasmid DNAs were sequenced on an Applied Biosystems 373A DNA sequencer by the Biomolecular Synthesis Laboratory of the Krebs Institute. A Qiagen kit was used to purify plasmid DNA by following the manufacturer's instructions.

Results

In order to determine that the correct mutations had been introduced into $\alpha\beta$, the corresponding genes in pETABser and pETABgly were sequenced. The required mutations leading to conversion of Cys82 to Ser and Gly were found to be present (Figures 3A and 3B respectively). The altered codons are underlined.

Two tests were used to assay the activity of the mutant methyltransferases. First, the plasmid pETAB expressing the wild-type enzyme and pETABser and pETABgly, expressing the Ser and Gly mutants of M.*Aqu*I respectively, were transformed into *E.coli mcr* A^+BC^+ strain BL21(DE3). If the MTase is active then it will methylate the recognition sites within the bacterial genome. These sites will then be recognized by the *mcr* endonucleases as foreign and the DNA will be hydrolysed. This effectively kills the cells and no colonies will appear following transformation. If the MTase is inactive then the genomic DNA will not be hydrolysed and transformants will appear. Competent BL21(DE3) (*mcr*A^+BC^+) cells were transformed with pETAB, pETABser and pETABgly. No colonies were observed following transformation of

pETAB while the transformations of both Ser and Gly mutants were efficient, suggesting that Cys to Ser and Gly mutations abolished the methyltransfer activity of the enzyme.

Second, plasmid DNA from cells expressing the wild type or Ser mutant methyltransferase were isolated and their sensitivity to digestion by *Sma*I restriction enzyme (CCCGGG) was determined in vitro. Active methyltransferase should methylate the CCCGGG sequences in the plasmid in vivo, thereby protecting it from digestion by *Sma*I in vitro. Figure 4 shows that plasmid DNA (pETAB) from cells expressing the wild-type monomeric M.*Aqu*I gene was completely protected from *Sma*I digestion (Figure 4, lane 2), whereas plasmid DNA (pETABser) expressing the Ser mutant was linearised by *Sma*I (Figure 4, lane 3). Since there is a unique *Sma*I site on the plasmid, this result indicates that Cys to Ser mutation abolished the activity of the enzyme.

Efficient transformation of pETABgly into *E. coli* BL21(DE3) cells suggests that the Cys82 to Gly mutation of M.*Aqu*I is not cytotoxic to *E. coli*.

Discusion

We have shown that the substitution of the conserved cysteine (Cys82) in the monomeric form of M.*Aqu*I with serine and glycine results in the loss of methyltransferase activity in the cell and a lack of protection of these sites from endonuclease attack.

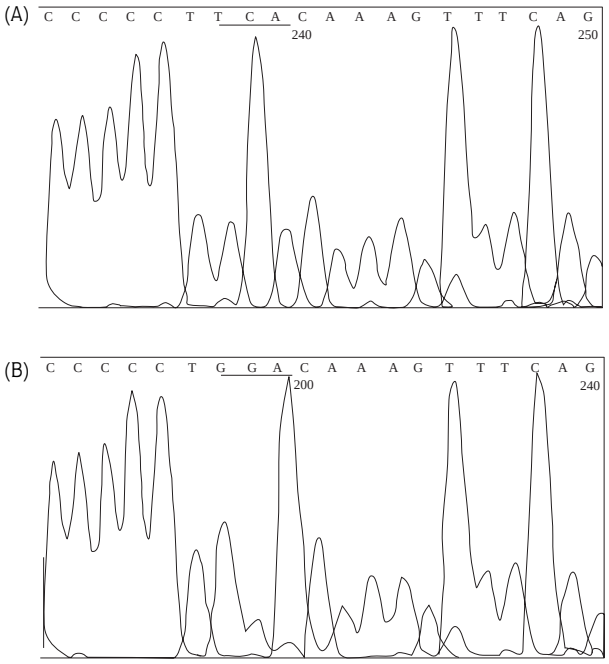

Figure 3. DNA sequence data showing the presence of the Cys to ser (A) and Gly (B) mutations in M.*Aqu*I$\alpha\beta$. The altered codons are underlined.

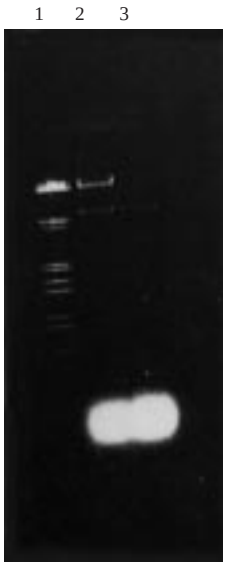

Figure 4. In vivo plasmid protection assay result.
Lane1: *EcoR*I/*Hind*III digested λ DNA
Lane 2: pETAB cut with *Sma*I
Lane 3: pETABser cut with *Sma*I

The C5 methyltransferases share a common architecture and contain a motif with absolutely conserved Pro-Cys residue. Substitution of cysteine of P-C motif with Gly, Ser, Val and Trp results in loss of methyltransferase activity of M.*EcoR*II (12). However, substitution of the cysteine with glycine results in the inhibition of cell growth and the mutant allele can be maintained in the cells only when it is poorly expressed (13). The Cys to Ser mutation abolishes the activity of SPR methyltransferase (11). Mi and Roberts (18) replaced cysteine 81 in the P-C motif with Arg, His, Ser and Gly and showed that M.*Hha*I exhibits similar properties. They found that Gly81 mutation causes cytotoxicity due to abnormally tight DNA binding by the mutant methyltransferase, which probably interferes with replication or transcription. In addition, the *Schizosaccharomyces* pombe pseudo methyltransferase pmtI^{+} has been activated by deletion of a serine residue from the naturally occurring active site P-S-C motif (19). Wyszynski et al. (13) showed that conserved cysteine is not essential for the specific stable binding of the enzyme to its substrate. However substitution of the Cys with tryptophan does reduce DNA binding. In our study substitution of catalytic Cys to Ser resulted in a loss of activity consistent with these results mentioned above. However replacement of catalytic Cys by Gly also abolished the activity. This is in contrast to the result obtained with M.HhaI and also M.EcoRIIgly mutants, because the Gly82 mutant of M.AquI is not cytotoxic to *E. coli*.

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