Cytosine deamination catalyzed by the SssI DNA methyltransferase

Summary of the Ph. D. Thesis

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Introduction

DNA methylation, along with histone modification, is a major component of epigenetic regulation, and is found in many organisms from bacteria to mammals. N6-methyladenine, N4-methylcytosine and C5-methylcytosine are natural components of the DNA, since the addition of the methyl group does not interfere with the Watson-Crick pairing properties of the bases, but it provides new information for DNA-interacting proteins.

In the genomes of eukaryotes only cytosine-C5 methylation can be found, which in mammals occurs mainly at CpG sequences. Cytosine methylation is catalyzed by DNA-cytosine methyltransferases, which transfer an activated methyl group from the donor S-adenosyl-L-methionine (SAM) to the carbon-5 of the cytosine. The genomic distribution of the methylcytosine residues is not random, it rather forms a tissue specific pattern. Properly established and maintained methylation patterns are essential for mammalian development and for appropriate function of the organism. Abnormal methylation can be linked to aging and to a number of pathological conditions, such as neurodegenerative diseases, diabetes or cancer. Epigenetic research focusing on DNA-methylation is of great importance not only in diagnostics but in the development of potential therapies as well. Thus there are on-going research efforts to study the methylation profile of selected genes and correlate it with tissue-specific gene expression in health and disease. The most widely used method for identification of methylated cytosines employs the treatment of genomic DNA with sodium-bisulfite. Upon this treatment unmethylated cytosines deaminate to uracil through the formation of an unstable dihydro-cytosine residue, while 5-methylcytosines remain unchanged. Due to the conditions used to achieve complete conversion (long incubation of single stranded DNA at high temperature and low pH) the method is prone to certain reaction artefacts that affect its reliability.

It was observed that certain prokaryotic DNA-methyltransferases and the catalytic domain of the mammalian Dnmt3a MTase are able to catalyse deamination of the target cytosine to uracil if the methyl donor SAM is omitted from the reaction. The enzymatic cytosine deamination is assumed to follow a path analogous to that of bisulfite mediated C-to-T conversion and is based on the formation of 5,6-dihydro-cytosine intermediate generated during the methyl-transfer. The prokaryotic DNA-(cytosine-5)-methyltransferase SssI shares the specificity of eukaryotic DNA methyltransferases (CG), and is an important model and experimental tool in the study of eukaryotic DNA methylation. Results in the literature on the

deaminase ability of this enzyme are controversial. Some results showed that M.SssI can deaminate cytosine or even ^{m5}C, whereas another study did not find evidence for M.SssI-mediated cytosine deamination.

Aims of the study

Based on the similarity of the bisulfite mediated and enzymatic cytosine deamination, we intended to explore the possibility of using the deamination side-activity of the M.SssI to distinguish between unmethylated and C5-methylated cytosines in the epigenetically relevant CpG dinucleotides. The use of M.SssI as an enzymatic alternative to bisulfite conversion would allow bypassing some of the technical difficulties associated with this method. We wished to address the following specific questions:

- Can the SssI methyltransferase catalyse deamination of cytosine? This question was justified because of the conflicting data in the literature on this matter.
- Can the SssI methyltransferase catalyse deamination of 5-methylcytosine?
- Can we find reaction conditions where the rate difference between M.SssI catalyzed cytosine and 5-methylcytosine deamination is sufficient for reliable discrimination between cytosine and 5-methylcytosine?
- Can we create conditions where the SssI MTase shows deamination activity *in vivo*, in the presence of SAM?

In the course of our work with the SssI DNA methyltransferase an accidental observation led to the discovery that CG-specific methylation creates new substrate sites for the MvaI restriction enzyme. These new sites differed from the known CCWGG (W can be A or T) MvaI recognition sequence, therefore we set out to characterise the new, methylation-dependent substrate sites and cleavage. This work shed light on a previously unknown activity of the MvaI endonuclease.

Methods

- Recombinant DNA techniques, creation of plasmid constructs
- Site directed mutagenesis, creation of protein variants with amino acid substitutions
- Western blot analysis and affinity chromatography
- *In vivo* and *in vitro* methyltransferase activity assay
- Mutation rate measurements
- DNA sequence analysis
- Radioactive labelling of oligonucleotides and phosphor-image analysis
- Statistical evaluation of experimental data

Results

To detect the C-to-U deamination by M.SssI, we used a sensitive genetic assay based on the reversion to wild-type of the antibiotic sensitive phenotype determined by a mutant kanamycin resistance gene. The pUP41 plasmid carries a mutant kanamycin resistance gene in which an amino acid substitution led to kanamycin sensitive phenotype. The mutant codon is located within a SmaI restriction site CCCGGG. Conversion of the underlined cytosine to thymine reverts the amino acid substitution to wild-type and restores kanamycin resistance. Because the underlined cytosine is in a CG dinucleotide, the substrate site for SssI DNA methyltransferase, pUP41 can be used to assay M.SssI-catalyzed cytosine deamination. Deamination of cytosine first creates a U:G mismatch, which – if left unrepaired – is converted to C-to-T mutation after DNA replication. Reversion to kanamycin resistance by cytosine deamination as described above eliminates the SmaI site and creates a new MvaI site (CCWGG).

Confirming some of the previous results we could show that M.SssI can deaminate cytosines to uracil in a slow reaction, and that the rate of the conversion can be increased by the SAM analogue 5'-amino-5'-deoxyadenosine (AA).

In single-stranded DNA the rate of bisulfite mediated conversion of C-to-U is ~50-fold higher than the rate of ^{m5}C-to-T conversion, which forms the basis of bisulfite-sequencing. We compared the reactivities of cytosine and 5-methylcytosine to M.SssI mediated

deamination in double stranded DNA, and found that in the presence of AA the difference of the reaction rates is at least 100-fold. This suggests that the M.SssI-catalysed conversion is sensitive enough to reliably discriminate between unmethylated and C5-methylated cytosines. However, our results showed that the rate of M.SssI mediated cytosine deamination is still much slower than that obtained with standard bisulfite protocols, making the M.SssI mediated cytosine deamination, at present, impractical as an alternative to bisulfite sequencing.

Besides studying this side activity of M.SssI *in vitro*, we wished to test whether the enzyme can be used to deaminate cytosines *in vivo*, in the presence of SAM. To this end we constructed two mutant enzymes using information from previous work with the HpaII MTase. Amino acids Phe17 and Gly19 of conserved motif I, presumed to be part of the SAM binding pocket, were changed by site directed mutagenesis to Ser and Asp respectively. We expected the amino acid substitutions to weaken the cofactor binding abilities of the enzyme, and thus to mimic conditions of limiting SAM *in vivo*. The cytosine deamination activity of both mutant enzymes (F17S and G19D) was tested in two different *E. coli* hosts. In one of them the *sssIM* allele and the targeted mutant kanamycin gene (*kanS*) gene were on two separate plasmids, while the other host carried the *kanS* gene on the chromosome. The G19D variant caused a ~10-fold increase in the deamination rate in both types of hosts lacking uracil-DNA-glycosylase (Ung) related repair, while the WT enzyme and the F17S mutant had no effect. The elevated C-to-U reversion rate was also demonstrated by fluctuation test. Interestingly, M.SssI(G19D) increased the deamination rate also in Ung^+ hosts, suggesting that the enzyme can block uracil-DNA glycosylase mediated repair. Consistent with the envisioned effect of impaired SAM binding, both mutants had reduced methyltransferase activities *in vivo* and in *vitro* compared to WT.

During our work with the CG-specific DNA cytosine-5 methyltransferase M.SssI, we noticed that *in vivo* or *in vitro* M.SssI methylation leads to the appearance of new substrate sites for the MvaI restriction enzyme. Appearance of these new sites was dependent on CG-methylation and was specific for MvaI, the phenomenon was not shown by its isoschizomer BstNI.

The MvaI restriction endonuclease cuts the CC↓AGG/CC↑TGG double-stranded sequence, as indicated by the arrows, as a monomer. The cognate MvaI MTase modifies the internal cytosine to produce N4-methylcytosine (C^{m4}CAGG/C^{m4}CTGG). C5-methylation of the same cytosine does not protect the site against MvaI cleavage. We have shown that the

MvaI endonuclease, in addition to its double-stranded cleavage activity on the canonical recognition sequence CC↓AGG/CC↑TGG, nicks BcnI sites ($C^{m5}C$↓GGG/CC^{m5}CGG) as indicated, if the inner cytosines are C5-methylated. This nicking activity results in double-strand scissions at CC^{m5}C↓GGG/CC^{m5}C↑GGG (CG-methylated SmaI) sites, where two oppositely oriented methylated BcnI sites overlap. The double-stranded cleavage rate and the stringency of the substrate site recognition are lower at the methylation-dependent site than at the canonical target sites.

MvaI formally combines features of "typical" Type II REases cutting unmethylated sequences, with those of methyl-directed Type II enzymes, which require methylated substrate sites. To our knowledge, MvaI is the first REase, for which such dual specificity has been shown. The new, methylation-dependent activity represents nicking and double-stranded cleavage specificities ($C^{m5}C$↓GGG/CC^{m5}CGG and CC^{m5}C↓GGG/CC^{m5}C↑GGG, respectively) not known before.

Results in brief

- We have shown that in the absence of the methyldonor (SAM) M.SssI can catalyze the deamination of cytosines in CG dinucleotides and the rate of this reaction can be increased by the SAM analogue 5'-amino-5'-deoxyadenosine.

- We have shown that similarly to the bisulfite mediated conversion, cytosine and 5-methylcytosine have different reactivities to the M.SssI catalyzed deamination.

- Our results show that the slow rate of the enzyme catalyzed cytosine deamination reaction makes the M.SssI in its present form impractical to be used as an alternative to bisulfite conversion. Nevertheless, the *E. coli*-KanS strain constructed during this study offers a directed evolution strategy to select M.SssI variants with increased cytosine deaminase activity.

- We have shown that the amino acid substitutions F17S and G19D lead to severe drop in the methyltransferase activity and the G19D mutant enzyme has enhanced C-to-U deaminase activity *in vivo*, where SAM is present. This result raises the possibility of

using the enzyme variant as a CG-specific targetable cytosine deaminase to induce C-to-T transitions at pre-determined CG sites *in vivo*.

- We have shown that the MvaI endonuclease has dual specificity: in addition to its double-stranded activity on the canonical recognition site, it is able to nick another specifically methylated sequence. This inherent property of the MvaI restriction enzyme must be taken into consideration when working with CG-specifically methylated DNA e.g. digestion of mammalian DNA. The methyl-directed activity of the enzyme represents new cleavage specificity, broadening the toolbar of DNA-research.

List of publications

Stier I., Kiss A. (2010): **The Type II restriction endonuclease MvaI has dual specificity** - Nucleic Acids Research 38: 8231-8238.
I.F. 7,836

Stier I., Kiss A. (2013): **Cytosine-to-Uracil Deamination by SssI DNA Methyltransferase** – PLOS One 8(10): e79003. doi:10.1371/journal.pone.0079003
I.F. 3,730