Reviving a Dead Enzyme: Cytosine Deaminations Promoted by an Inactive DNA Methyltransferase and an *S*-Adenosylmethionine Analogue[†]

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ABSTRACT: The enzymes that transfer a methyl group to C5 of cytosine within specific sequences (C5 Mtases) deaminate the target cytosine to uracil if the methyl donor *S*-adenosylmethionine (SAM) is omitted from the reaction. Recently, it was shown that cytosine deamination caused by C5 Mtases M.*Hpa*II, M.*Sss*I and M.*Msp*I is enhanced in the presence of several analogues of SAM, and a mechanism for this analogue-promoted deamination was proposed. According to this mechanism, the analogues protonate C5 of the target cytosine, creating a dihydrocytosine intermediate that is susceptible to deamination. We show here that one of these analogues, 5′-aminoadenosine (AA), enhances cytosine deamination by the Mtase M.*Eco*RII, but it does so without enhancing protonation of C5. Further, we show that uracil is an intermediate in the mutational pathway and propose an alternate mechanism for the analogue-promoted deamination. The new mechanism involves a facilitated water attack at C4 but does not require attack at C6 by the enzyme. The latter feature of the mechanism was tested by using M.*Eco*RII mutants defective in the nucleophilic attack at C6 in the deamination assay. We find that although these proteins are defective in methyl transfer and cytosine deamination, they cause cytosine deaminations in the presence of AA in the reaction. Our results point to a possible connection between the catalytic mechanism of C5 Mtases and of enzymes that transfer methyl groups to N^4 of cytosine. Further, they provide an unusual example where a coenzyme activates an otherwise "dead" enzyme to perform catalysis by a new reaction pathway.

The most commonly found methylation of DNA is at C5 of cytosine and is introduced postreplicatively by a group of enzymes called DNA (cytosine-5-)-methyltransferases (C5 Mtases).[1] These enzymes are related to each other by primary sequence, three-dimensional structure, and catalytic mechanism (*1*−*3*). The methyl donor in the reaction is *S*-adenosylmethionine (SAM) and the other reaction product is *S*-adenosylhomocysteine (SAH). The mechanism of methyl transfer is outlined in Figure 1 (left panel).

When SAM is omitted from the reaction, the enzymes initiate the catalytic cycle by attacking C6 of target cytosine but cannot proceed further. Protonation of the resulting anion can cause a side reaction: hydrolytic deamination of target cytosine to uracil (Figure 1, right panel; *4, 5*). Although the rate of the latter reaction is many orders of magnitude lower than that of methyl transfer in the presence of SAM, it can be easily detected by genetic assays that score the thymine that is generated by the replication of uracil. C5 Mtases M.*Eco*RII, M.*Hha*I, M.*Hpa*II, and M.*Sss*I cause deamination of cytosines in this manner (*4*−*7*). Presence of either SAM or SAH in the reaction inhibits cytosine deamination by M.*Eco*RII and M.*Hpa*II (*4, 5*). When SAM is present in the reaction, the reaction proceeds to methylate the cytosine, while the mechanism by which SAH inhibits the reaction is less clear.

Remarkably, some analogues of SAM overcome this inhibition of deamination by SAM and SAH and accelerate the reaction (*8*). Such promoters of deamination include a known inhibitor of DNA methylation, sinefungin, and a derivative of adenosine, 5′-aminoadenosine (AA). These analogues have been shown to overcome the inhibition of M.*Hpa*II and M.*Sss*I by SAM or SAH (*8*). Furthermore, although M.*Msp*I does not promote deaminations by itself (i.e., in the absence of coenzyme or coenzyme analogues), it does so in the presence of AA or sinefungin in the reaction (*9*). It should be noted that not only do sinefungin and AA help overcome the inhibition of enzyme-catalyzed deamination of cytosine, they actively promote it. Thus the level of mutations caused by the enzymes is substantially higher when sinefungin or AA is added to the reaction in the absence of SAM or SAH (*8*).

Zingg et al. (*8*) suggested that sinefungin and AA exert their effects by promoting the formation of a protonated form of the cytosine substrate (structure III), which is susceptible to hydrolysis (Figure 1, right panel). Furthermore, they suggested that amino groups within these analogues were the proton donors that were responsible for the formation of structure III. In other words, the analogues accelerated the reaction by merely enhancing the ability of C5 MTases to protonate C5.

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[1] Abbreviations: C5 Mtase, DNA (cytosine-5-)-methyltransferase; SAM, *S*-adenosylmethionine; AA, 5′-aminoadenosine; SAH, *S*-adenosylhomocysteine.

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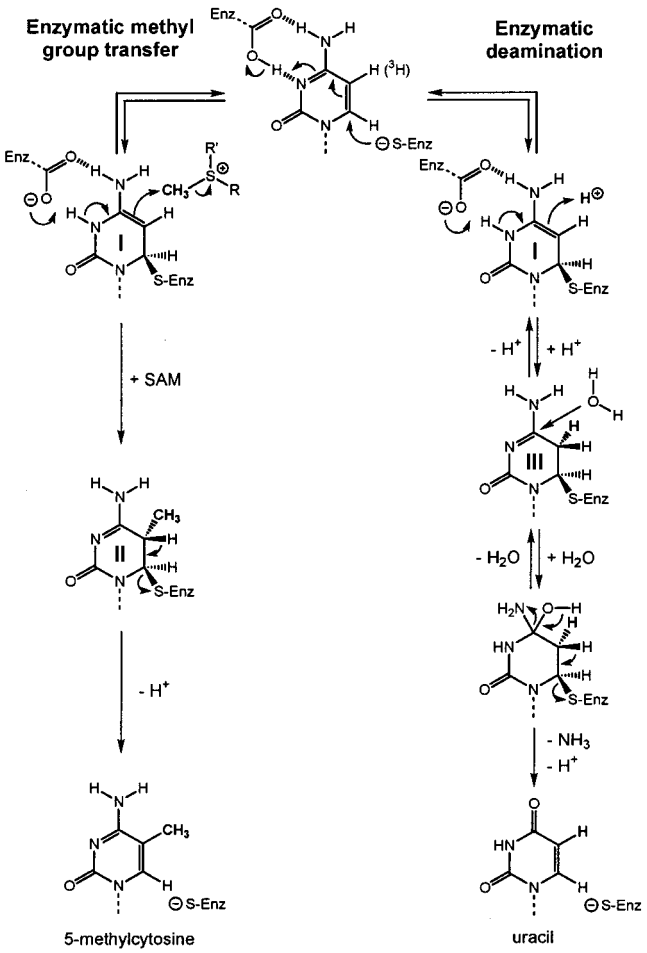

FIGURE 1: Mechanisms of enzyme-catalyzed methyl transfer and deamination. (Left panel) The likely mechanism by which M.*Eco*RII transfers a methyl group to C5 is shown. The thiolate attacking C6 is in the side chain of Cys-186 (*24*, *30*), while the general acid donating a proton to N3 is expected to be Glu-233 (*21*, *23*). The sterochemistry of the reaction is based on the structure of M.*Hha*I ternary complexes (*15*). The identity of the general base that promotes β-elimination is unknown. (Right panel) The scheme for M.*Eco*RII-catalyzed deamination of cytosine is based on a proposal by Selker (*17*), and the role of Cys-186 in this reaction has been confirmed by site-directed mutagenesis (*4*).

We have previously shown that the *Escherichia coli* C5 MTase M.*Eco*RII promotes deamination of cytosine and that this reaction is inhibited by SAM and SAH (*4*). We were curious to know if cytosine deamination by M.*Eco*RII is similarly stimulated by SAM analogues, and if so, whether this occurred by the mechanism outlined in Figure 1 (right panel). We found that AA does promote cytosine deamination in the presence of M.*Eco*RII, but the mechanism of this reaction is likely to be very different than what has been proposed.

EXPERIMENTAL PROCEDURES

Strains and Plasmid. Escherichia coli strain GM31 (*dcm-6 thr-1 hisG4 leuB6 rpsL ara-14 supE44 lacY1 tonA31 tsx-78 galK2 galE2 xyl-5 thi-1 mtl-1*) is from our collection. BH156 (=GM31*ung*) was obtained from M. Lieb (University of Southern California School of Medicine). The plasmid pUP31 contains the *kanS-94D* allele under the transcriptional control of a hybrid UP−*tac* promoter and has been described previously (*10*).

Chemicals and Enzymes. S-Adenosylmethionine (SAM) was purchased from New England Biolabs. *S*-Adenosylhomocysteine and 5′-aminoadenosine were purchased from Sigma−Aldrich Biochemicals (St. Louis, MO) and sinefungin was purchased from Calbiochem (La Jolla, CA). Purification of M.*Eco*RII (*11*) and of its C186S and C186A mutants (*12*) has been described previously.

Construction of DNA Containing Tritiated Cytosine. DNA containing [5-^{3}H]dC was synthesized by the procedure described previously (*12*). Briefly, two DNA oligonucleotides were used to construct a template primer hybrid: The primer

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5' GTCTGCGACAGATTC
3' CAGACGCTGTCTAAGGACCCTATTC
                    |
                    me
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strand was extended with Sequenase (U.S. Biochemical Corp.) in the presence of 200 μM dATP, dTTP, and dGTP (Pharmacia Biochemicals) and 10 μM [5-^{3}H]dCTP [specific activity: 23 Ci/mmol; Moravek Biochemicals]. The reactions were carried out at 25 °C for 20 min in the buffer recommended by the manufacturer. The reaction mixture was deproteinized by extracting it once with phenol/chloroform and once with chloroform and was desalted on a Sephadex G-50 column.

Assay for Enzyme-Catalyzed Deamination of Cytosine. One microgram of pUP31 DNA purified from GM31 was incubated with 0.9 μg of M.*Eco*RII in Mtase buffer (100 mM Tris-HCl, pH 7.8, 20 mM EDTA, pH 8.0, and 0.4 mM dithiothreitol). Reactions were incubated at 37 °C for 30 min and were quenched by the addition of SDS to 0.5%. The reactions were extracted once with phenol/chloroform and once with chloroform. The DNA was precipitated with ethanol and the precipitate was dried and dissolved in 10 μL of TE buffer. One and one-half microliter of this DNA was electroporated into *E. coli* cells by use of the Bio-Rad *E. coli* pulser (Hercules, CA). Cells were plated onto LB plates with 50 μg/mL carbenicillin or kanamycin, and the reversion frequency was defined as the ratio (number of kanamycin-resistant transformants)/(number of carbenicillin-resistant transformants).

Tritium Exchange Assay. One hundred fifty picomoles of tritium-labeled duplex DNA was incubated with 1.5 pmol of M.*Eco*RII in 80 μL of Mtase buffer to start the reaction. The reactions were incubated at 37 °C for 30 min and were quenched by the addition of 10 μL of 10 mg/mL herring sperm DNA and 10 μL of 100% trichloroacetic acid. The reaction mixture was chilled on ice for 30 min to allow precipitation of proteins and DNA and was spun in a microcentrifuge for 15 min at 11000*g*. The pellet was discarded, 10 μL of 10 mg/mL herring sperm DNA was added to the supernatant, and the precipitation and centrifugation steps were repeated. The amount of radioactivity in the supernatant was quantified with a Beckman LS3801 scintillation counter.

RESULTS AND DISCUSSION

5′-Aminoadenosine Assists M.EcoRII in Deamination of Cytosine. We used a genetic reversion assay involving a defective kanamycin-resistance gene (*kanS-D94*; *4*) to assess if AA can promote C to U deaminations. The deaminations

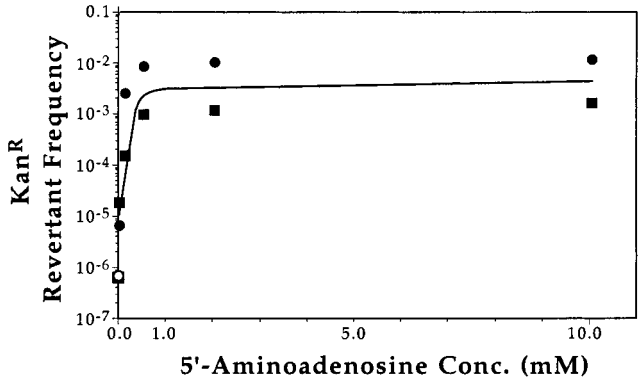

FIGURE 2: Concentration dependence of 5′-aminoadenosine-promoted cytosine deamination. Frequency of C to T mutations caused by the combined action of M.*Eco*RII and AA is shown as a function of AA concentration in the reaction. Results from two sets of experiments (circles and squares) are shown. Closed symbols represent reactions done in the presence of the enzyme; open symbols represent reactions done in the absence of the enzyme.

create U•G mismatches that are replicated in uracil-DNA glycosylase-deficient (*ung*) cells, causing C to T mutations and restoring resistance to kanamycin (Kan^R phenotype). Consequently, the frequency of Kan^R revertants is directly related to the frequency of C to U deaminations. Restriction mapping and DNA sequencing have confirmed that the obtained revertants contain the expected C to T mutation at codon 94 (*4*, *7*, *13*).

Among the SAM analogues that have been shown to promote C to T mutations, we chose AA for our studies because of its ready availability and simple structure. When AA was included in reactions of M.*Eco*RII with DNA containing *kanS-D94*, there was a concentration-dependent increase in C to T mutations (Figure 2). Under the conditions used, the increase leveled off around 500 μM AA, and hence we used this concentration in subsequent experiments. It should be noted that AA had no direct effect on mutations, i.e., incubation of DNA with AA without the enzyme had no detectable effect on the mutation frequency (see below and data not shown).

To confirm that the increase in mutation frequency was due to the deamination of cytosine to uracil, we transformed the treated DNA into *ung* and *ung*$^+$ cells and compared the frequencies of Kan^R revertants. The presence of uracil-DNA glycosylase in cells completely eliminated the increase in revertant frequency caused by the combined action of the enzyme and the analogue (Figure 3). This is the expected result if U is the intermediate in the mutational pathway. It should be noted that, in previous studies with AA and other analogues (*8*, *9*), cytosine deamination was inferred from the scored C to T mutations and was not directly shown.

AA Does Not Enhance Protonation of C5. Zingg et al. (*8*) suggested that the analogues that promote cytosine deamination donate a proton to the target cytosine, creating a 5,6-dihydrocytosine intermediate (structure III, Figure 1, right panel). This suggestion is consistent with the structure of AA. Where SAM has sulfur in its structure, AA has nitrogen, and hence the carbon in the methyl group attached to the sulfur has a roughly equivalent position to the proton in AA. Thus the protonation of C5 by AA may be considered to be similar to the methyl transfer step when SAM is present in the reaction.

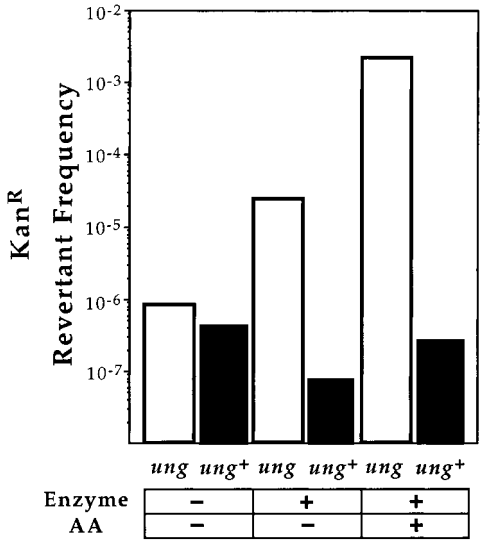

FIGURE 3: Mutations caused by AA and WT M.*Eco*RII in *ung*$^+$ and *ung* cells. Following treatment of pUP31 DNA under conditions indicated below the bar graph, the DNA was electroporated into *ung*$^+$ or *ung* cells and Kan^R revertants were scored.

We used a tritium exchange assay (*14*) to assess the protonation at C5 of cytosine by AA. The principle of this assay is illustrated in Figure 4. On the basis of the crystal structure of the ternary complex, M.*Hha*I is thought to perform a trans addition of a methyl group and a cis elimination of the 5-proton with respect to the active-site thiol (*15*). If the stereochemistry used by M.*Eco*RII is the same as that of M.*Hha*I, then each addition−elimination cycle of M.*Eco*RII should release one tritium from C5 (Figure 4). As a result, the rate of release of ^{3}H by M.*Eco*RII should be a direct measure of C5 protonation by the enzyme. It should be noted that the methyl transfer reaction also eliminates the C5 proton (Figure 1, left panel), and hence SAM is a promoter of ^{3}H-exchange.

As expected, SAM promoted the tritium exchange reaction, increasing the initial velocity of the reaction (Figure 5). But surprisingly, AA did not significantly affect the reaction velocity (Figure 5). To confirm this, we measured the initial reaction velocity of tritium release in the presence of SAM or AA and compared it with the velocity in the absence of any coenzyme. While SAM nearly doubled the initial velocity [(177.2 ± 31.0)%; number of experiments $n = 5$)], AA had little effect on the velocity [(80.8 ± 10.7)%; $n = 4$)]. On the basis of these results, we conclude that it is unlikely that the promotion of cytosine deamination by AA is the result of increased protonation at C5.

Alternate Mechanism for AA-Promoted Cytosine Deamination. At least three mechanisms have been proposed for the deamination of cytosine to uracil. The first of these is an addition−elimination mechanism that is responsible for the mutagenic properties of bisulfite (*16*) and the C5 Mtase-catalyzed deamination in the absence of analogues (*17*). One of the mechanisms by which hydrolytic deamination of cytosine occurs also involves the addition−elimination of water molecules across the 5,6 bond of cytosine (*18*). The results described above show that it is unlikely that AA promotes cytosine deaminations by this mechanism.

The second mechanism involves mutagens such as nitrous acid and nitric oxide, which react directly with the exocyclic

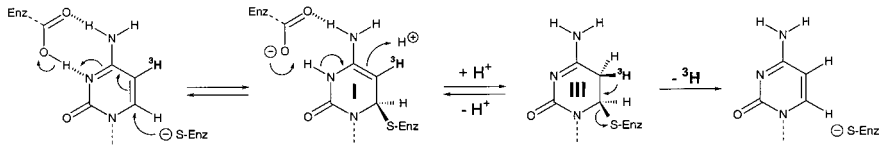

FIGURE 4: Principle of the 5-^{3}H exchange assay. The reactions occur in the absence of SAM or its analogues. Structure I forms also during methyl transfer and cytosine deamination (Figure 1).

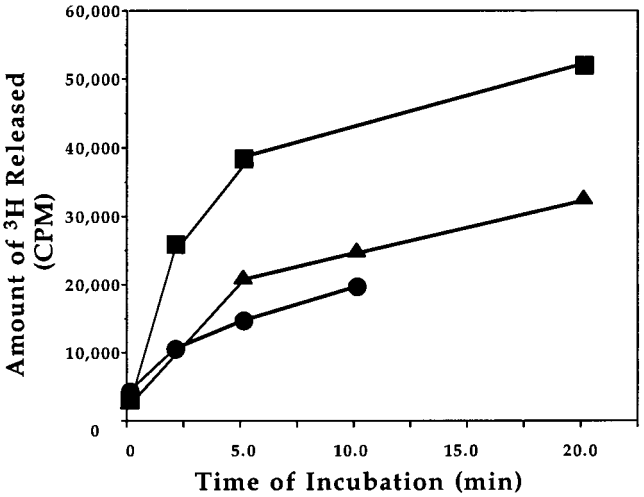

FIGURE 5: Kinetics of 5-^{3}H exchange: time-dependent release of ^{3}H from labeled cytosine by M.*Eco*RII in the absence of coenzyme (●), in the presence of SAM (■), and in the presence of AA (▲). The maximum possible amount of 3H that could be released from this substrate was ~110 000 cpm.

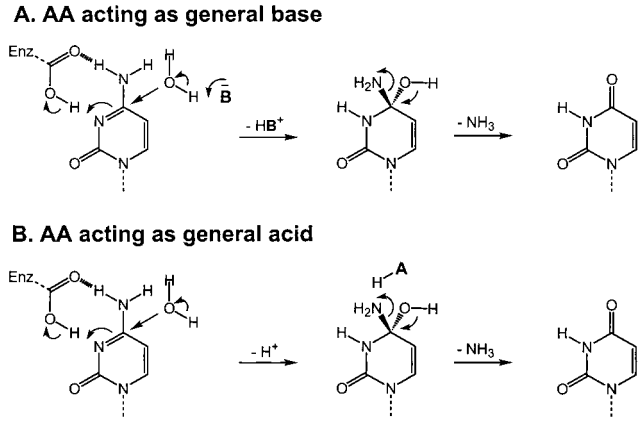

FIGURE 6: Proposed mechanisms of AA-promoted cytosine deamination. (A) AA is shown as a general base B^- (i.e., amino group as NH_2). (B) AA is shown as a general acid A-H (amino group as $^+NH_3$).

amino group of cytosine, forming a diazoate intermediate that converts to a diazonium ion upon dehydration (*19*). The latter species is readily hydrolyzed to generate uracil and nitrogen gas. AA is not expected to generate nitrous acid and hence this mechanism is also not relevant here.

The third mechanism of cytosine deamination involves a direct attack at C4 of cytosine by water molecule (*18*). This requires protonation of N3 and is facilitated by the protonation of N4. We propose two alternate ways in which AA could accelerate this reaction (Figure 6). In one, AA activates a water molecule for attack at C4 (Figure 6A), while in the other it functions as a general acid and protonates N^4 (Figure 6B). In the first pathway AA accelerates the reaction by producing a reactive hydroxyl in the vicinity of C4, while in the other it facilitates the elimination of the amino group as ammonia. We refer to these two mechanisms as direct attack mechanisms.

The steps envisioned in these mechanisms are consistent with our current understanding of the structure and mechanism of C5 Mtases. The crystal structures of ternary complexes of M.*Hha*I containing SAH show several water molecules close to the edge of the cytosine ring that are held in place through hydrogen bonds with conserved glutamine and asparagine residues (*15*, *20*). It is likely that the positions of water molecules are altered when AA is in the complex and that this results in the reorientation of one of the water molecules for attack at C4.

The protonation of N3 is also required for cytosine deamination and this is likely to be done by the enzyme (Figure 6). It is known that a conserved glutamic acid in both M.*Hha*I and M.*Hae*III (Glu-233 in M.*Eco*RII) is in hydrogen-bond contact with N3 and N^4 (*21*, *22*) and is thought to act as a proton donor for N3 (*23*). The same is likely to be true for M.*Eco*RII.

Test for the New Mechanisms. A key feature of the direct attack mechanisms is that they do not require enzyme attack at C6. Consequently, mutants of C5 Mtases defective in the nucleophilic attack at C6 should be competent for cytosine deamination by the direct attack mechanisms. We decided to test this prediction using such mutants of M.*Eco*RII.

We chose mutants in which the cysteine that attacks C6 (Cys186) is replaced with serine (C186S) or alanine (C186A), because these mutants are defective in catalysis but are proficient in DNA binding. These proteins are severely deficient in their ability to form a covalent complex with DNA (*24*) and are very poor at DNA methylation (*12*). In particular, the specific activity of the C186A mutant is at least 6 orders of magnitude lower than that of the wild-type (WT) enzyme (*12*). Equally important, they bind DNA in a sequence-specific manner and their specific complexes with DNA are more stable in the presence of SAM or SAH (*12*, *24*). Consequently, we could reasonably expect that these mutant proteins would form ternary complexes in the presence of DNA and AA.

When C186S protein was incubated with DNA without coenzyme, the frequency of C to T mutations increased slightly. In contrast, C186A caused no detectable increase in mutations (Figure 7). In comparison, the WT enzyme caused an 11-fold increase in mutation frequency under these conditions (Figure 7). These results are consistent with our previous observation (*12*) that the C186S mutant has residual catalytic activity for methyl transfer and ^{3}H exchange and that C186A is completely inactive.

Remarkably, AA promoted C to T mutations in the presence of all three proteins (Figure 7 and Table 1). While the overall increase was greatest in the presence of the WT enzyme (Figure 7), AA also caused a substantial increase in mutations in the presence of either C186S or C186A mutants, and the enhancement in mutations was reproducible (Table

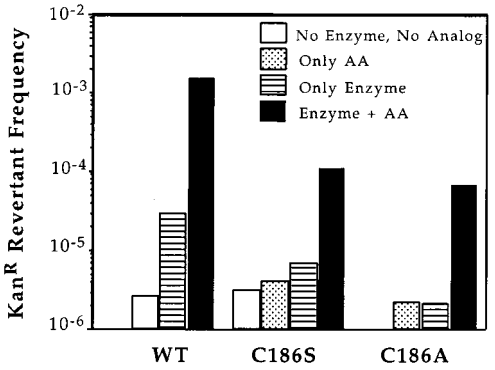

FIGURE 7: C to T mutations caused by M.*Eco*RII mutants in the presence of AA. The frequency of mutations caused by WT enzyme and its two mutants in one experiment is shown. The reaction conditions used for different reactions are explained in the figure.

Table 1: Effects of 5′-Aminoadenosine and M.*Eco*RII Mutants on C to T Mutations

incubation with	C186S[a]	C186A[a]
enzyme[b]	3.3 ± 1.3	1.1 ± 0.2
enzyme + AA[c]	13.7 ± 8.5	41.2 ± 8.7
enzyme + AA[d]	42.2 ± 23.6	43.4 ± 8.7

[a] Results from six experiments. [b] *x*-Fold increase in Kan^R frequency compared to DNA without enzyme or AA. [c] *x*-Fold increase in Kan^R frequency compared to DNA incubated with the corresponding mutant enzyme but without AA. [d] *x*-Fold increase in Kan^R frequency compared to DNA incubated without enzyme or AA.

1, row 2). Furthermore, the mutation frequency in the presence of AA and either mutant was about 40-fold higher than that seen with DNA alone (Table 1, row 3).

We interpret these data in the following way: AA promotes deamination of cytosine in the presence of either mutant by a mechanism that does not involve attack at C6 by cysteine-186. In the presence of AA, the two mutants are much more effective at deaminating cytosines by the new pathway than by the addition−elimination pathway (Figure 1). As a result, the level of mutagenesis caused by the mutants in the presence of AA is similar. In case of the WT enzyme, both the deamination pathways are active, resulting in a much higher level of mutations in the presence of the analogue.

To confirm that uracil was an intermediate in the mutational pathway involving AA, we electroporated DNA treated with the mutants in the presence of AA into *ung* and *ung*$^+$ cells and compared the Kan^R revertant frequencies. As expected, while the combined treatment of DNA with one of the mutant proteins and AA increased mutations in *ung* cells, significant increase in mutations was not seen in *ung*$^+$ cells (Figure 8). On the basis of these results, we conclude that AA promotes C to T mutations by causing deamination of cytosine to uracil.

CONCLUDING REMARKS

While investigating the mechanism by which AA enhances C to T mutations caused by C5 Mtases, we uncovered a new mechanism by which this class of enzymes can cooperate with AA to deaminate cytosines. This mechanism resembles one of the pathways by which "spontaneous" water-mediated deamination of cytosine is thought to occur (*18*) but is unique

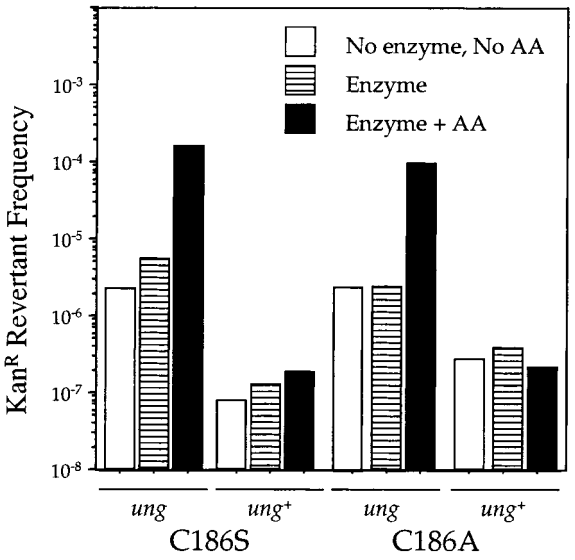

FIGURE 8: Mutations caused by AA and mutants of M.*Eco*RII in *ung*$^+$ and *ung* cells. The reaction conditions used in different experiments are explained in the figure. The M.*Eco*RII mutant used in the reaction and the *E. coli* host into which DNA was electroporated are indicated below the bars.

in the way the catalytic task is shared between the enzyme and the coenzyme. Although this mechanism does ascribe a role for the enzyme in the reaction, it is clear that at least one mutant of the Mtase (C186A) is catalytically inactive in the absence of the analogue. Thus AA can be said to revive this "dead" enzyme for catalysis. While several examples of "chemical rescue" of catalytically inactive enzymes are known (*25*−*28*), in all the reported cases the complementing chemical restores the function missing in the mutant enzyme. AA-promoted cytosine deaminations are unique in that the coenzyme does not substitute for the missing cysteine but promotes instead a reaction pathway that is normally not taken by the enzyme.

Several points regarding the new mechanism remain unclear. For example, we do not know whether AA acts as a general acid or a general base in the reaction (Figure 6). The precise role of the enzyme in the reaction is also unclear. C5 Mtases are known to flip the target cytosine out of the double helix (*21*) and this extrusion of the base makes it accessible for chemistry. Consequently, it is highly likely that the inactive Mtase also assists the direct attack at C4 by flipping the target cytosine. However, no mutants of C5 Mtases are currently available that are defective in this step, and hence the role of base flipping in the reaction cannot be tested. Further, it is likely that the mutant enzyme also promotes this reaction pathway by protonating N3. This can be confirmed by testing whether an E233Q/C186A double mutant of M.*Eco*RII can cause cytosine deaminations in the presence of AA.

We have found that, in addition to AA, several other derivatives of adenosine, including sinefungin, promote cytosine deamination in the presence of WT M.*Eco*RII. While many of these derivatives contain an amine functionality, at least one analogue contains a different functional group (A. Sharath and A. S. Bhagwat, unpublished results). In addition, we have found that one of these other analogues also promotes C to T mutations in the presence of the C186A mutant of the Mtase. This suggests that the reaction mech-

anisms outlined in Figure 6 may also apply to other SAM analogues. This work will be published elsewhere.

Cytosine methyltransferases come in two varieties. One class includes enzymes such as M.*Eco*RII, M.*Hha*I and M.*Hae*III, which methylate the base at position 5, while the other class of enzymes includes M.*Pvu*II and methylates it at N^4. Although the primary structures of these enzymes are very different, the structures of their active sites are similar (*29*). On the basis of this observation, it was suggested that these two types of enzymes may have evolved from a common ancestor. The protonation of N^4 by AA envisioned in the mechanism outlined in Figure 6B is similar to the methyl transfer reaction performed by the cytosine-N^4 Mtases. Consequently, if this version of the direct attack mechanism of cytosine deamination is found to be correct, it would support the hypothesis regarding a common ancestry for the two classes of cytosine Mtases.

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