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On the mechanism of demethylation of 5-methylcytosine in DNA

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Abstract—DNA methylation is an important biological process that programmes gene expression in vertebrates. The methylation pattern is generated by a combination of methylation and demethylation reactions catalyzed by DNA methyltransferases and putative demethylases. MBD2 binds methylated DNA and possesses DNA demethylase activity. We use here direct analysis of the reaction mixture by GC–MS using a water-tolerant gas chromatographic column to avoid the loss of potential volatile products and identify the leaving residue of the demethylation reaction. We show that the DNA demethylase reaction catalyzed by a recombinant human MBD2 purified from SF9 insect cells releases dideuteroformaldehyde from [Me–$^2H_3$]-5-methylcytosine in DNA. A mechanism of the DNA demethylation reaction is proposed based on this observation.

The pattern of DNA methylation regulating gene expression is generated by methylation and demethylation reactions catalyzed by DNA methyltransferases and putative demethylases. MBD2 is a protein that binds methylated DNA which we reported to have DNA demethylase activity.[1] We have also previously reported[2,3] that DNA demethylase activity extracted from human lung cancer cells mediated demethylation of 5-[Me–^{3}H]-methyl-2′-deoxycytidine to deoxycytidine in 5-methyl-dCpdG-bearing DNA (**1a**, Fig. 1), releasing a tritiated volatile compound. Demethylation of **1b** was shown to be accompanied by production of very small amounts of methanol by GC–MS of the toluene extract of the aqueous reaction mixture.[3]

We report here further experiments demonstrating that the methyl group is initially lost as formaldehyde which would not have been detected in the earlier report due to its inefficient extraction into toluene as retention in the aqueous phase is greatly favoured.

In the present study, dideuteroform-aldehyde released from 5-[Me–$^2H_3$]-methyl-dCpdG-bearing DNA (Fig. 1c) is reduced with sodium borodeuteride (NaB^2H_4) in the aqueous reaction mixture to [Me–$^2H_3$]-methanol which is then directly analyzed by GC–MS using a water-tolerant gas chromatographic column to avoid the loss of volatile products. The trideuteromethylated DNA substrate (**1c**) was synthesized by in vitro methylation of a double stranded $(CpG)_{12}$ substrate (synthesized commercially by Sheldon Biotech, McGill University) with *S*-adenosyl-[Me–$^2H_3$]-methionine (C^2H_3AdoMet) and recombinant His-tagged MBD2/dMtase (His-demethylase) extracted from insect SF9 cells cultured in spinner flasks as previously described.[2] C^2H_3-AdoMet was synthesized enzymatically from commercially available [Me–$^2H_3$]-methionine (Sigma Chemical Co.) and yeast.[4]

Demethylation of [*Me*–$^2H_3$]-*methylated*(CpG)$_{12}$. A reaction mixture (200 μL) consisted of 1 μg of a double stranded [Me–$^2H_3$]-methylated $(CpG)_{12}$ incubated in demethylation buffer (10 mM Tris–HCl, 5 mM $MgCl_2$, pH 7.0) with the purified MBD2/dMTase (5 μL, ~50 ng), for 24 h at 37 °C. Following incubation, a few crystals of NaB^2H_4 (CDN Isotopes, Pointe-Claire, Que., Canada; estimated 50 μg) were added to approximately 30 μL of the reaction mixtures contained in 200 μL conical bottom autoinjector vials. The vials were capped, shaken well to effect dissolution (minute frothing apparent) and held at 30 °C for 10 min. Sodium

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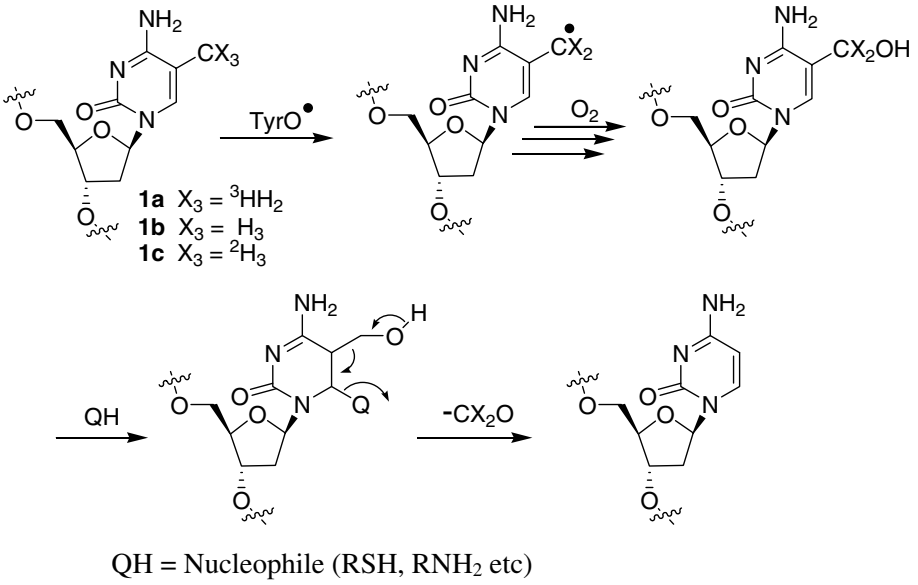

Figure 1. Proposed mechanism for the demethylation of 5-methylcytosine in DNA.

borodeuteride does not reduce CO_2. This reduction converts formaldehyde to methanol avoiding the problem of co-elution of formaldehyde with CO_2 and air components that form the instrumental background and which unavoidably enter the column during injection. Methanol elutes after CO_2 and air component elutions are complete.

Analysis by gas chromatography–mass spectrometry. Aliquots (3–5 μL) of the aqueous reaction mixtures were analyzed with a Hewlett Packard GC–MS model 5988A equipped with a 30 m × 0.25 mm i.d. Phenomenex capillary column coated with a 0.25-μm ZB-Wax (polyethylene glycol) film. The column temperature was programmed from 30 °C after a 0.5-min hold to 100 °C at 15 °C/min, the temperature of the injection port was 200 °C, the GC interface 150 °C and the ion source 200 °C. Electron ionization was used with an emission current of 300 μA and electron energy of 70 eV. Scanning was started immediately following injection and was over the range *m*/*z* 20–80 chosen to avoid recording the intense ion current due to water for the duration of the run. Aqueous samples of authentic methanol, $^2H_1$-, $^2H_2$- and $^2H_3$-methanol, formaldehyde and formic acid were similarly analyzed to establish retention times and to obtain characteristic mass spectra.

Figure 2A illustrates an example of the total ion current chromatogram obtained for a full scan analysis of a borodeuteride reduced sample. The major peak eluting at approximately 1.6 min is due to CO_2 from bicarbonate in the incubation mixture, while the remaining ion current eluting before 2.5 min is due principally to H_2O, N_2 and O_2 introduced at injection, in addition to that present as background in the ion source. Formaldehyde gas co-elutes with CO_2 and cannot be unequivocally detected in these analyses. For this reason we chose to reduce formaldehyde that may be present with NaB^2H_4 and search for deuterium labelling in the methanol produced. Authentic methanol elutes in this chromatographic system at approximately 2.7 min. The same sample analyzed under the same conditions prior to NaB^2H_4 reduction did not contain detectable amounts of labelled or unlabelled methanol.

Figure 2B illustrates the average of five spectra acquired at 2.7 min retention time, the maximum intensity of the ion current for eluting methanol. The spectrum is consistent with the majority of the GC peak being trideuteromethanol (^2H_3COH, fragmentation: Fig. 3). No unambiguous evidence was found for unlabelled or singly- or doubly-labelled methanol.

The third panel (Fig. 2C) is the total ion chromatogram obtained for an enzyme blank incubation mixture similar to that described above but missing the enzyme. There is no chromatographic peak noted at 2.7 min, and five scans centred at 2.7 min showed only ions related to ion source background (data not shown). There was no trace of methanol, either labelled or unlabelled.

Figure 2D is the total ion chromatogram obtained for another enzyme blank to which 50 ng of ^2H_3COH (1 μL of a solution of 1.2 μL ^2H_3COH in 20 mL H_2O) was added. The average of five scans collected at 2.7 min under the same conditions as the reduced complete incubation sample is shown in Figure 2E.

The principal difference between mass spectra B and E is the relative intensity of the major ions due to ion source background (*m*/*z* 28 and 32, N_2 and O_2, respectively). Ions due to trideuteromethanol in Figure 2E are found in the same relative ratios in B (*m*/*z* 35, 33 and 30). The integrated areas of the ^2H_3COH peaks in 2A relative to 2D show that the yield of ^2H_3COH is approximately 3 ng, or 50% theoretical, based upon 1 μg of labelled oligomer substrate. Due to difficulties associated with the scale of these experiments, reaction kinetics were not feasible. In earlier work with a radio-

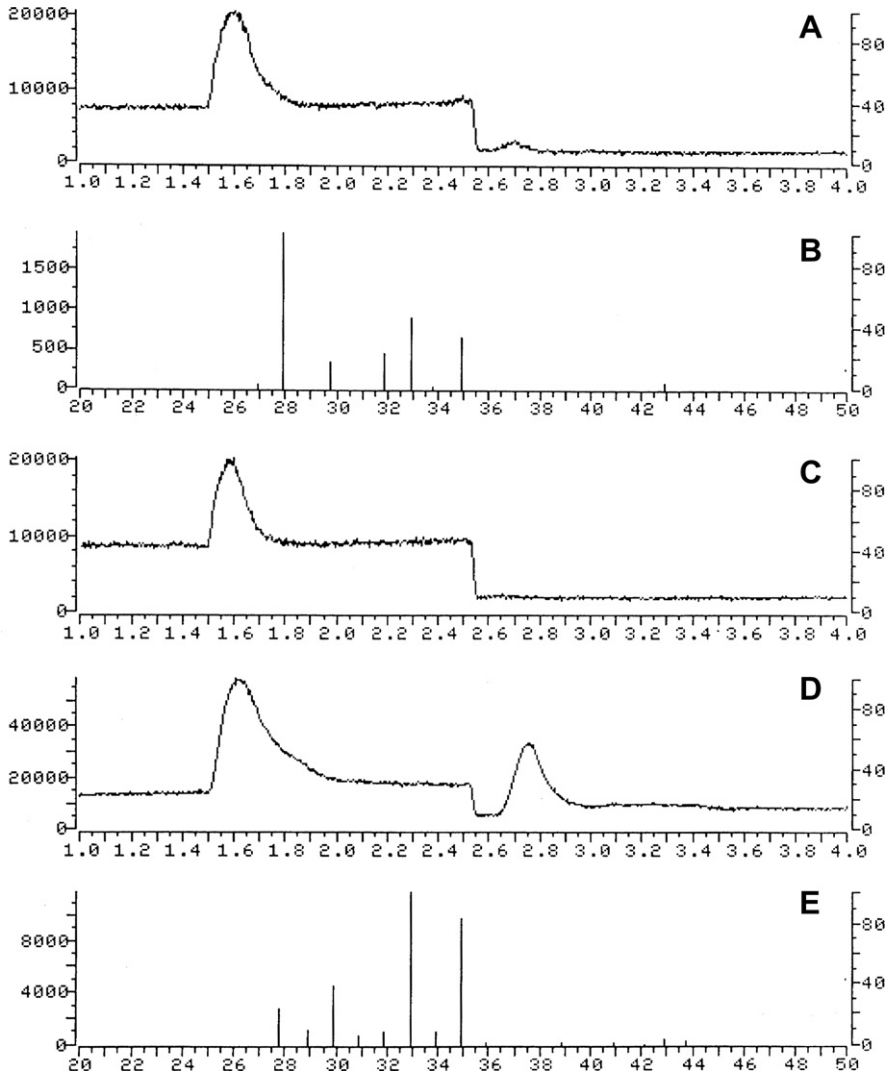

Figure 2. (A) Total ion-gas chromatogram obtained for the complete aqueous reaction mixture. (B) Average of five electron ionization mass spectra centred on the peak at 2.7 min in Figure 1A. (C) Total ion-gas chromatogram obtained for the blank aqueous reaction mixture missing only the enzyme. (D) Total ion-gas chromatogram obtained for another blank mixture as in (C) to which a few nanograms of authentic $^{2}H_3COH$ was added. (E) Average of five electron ionization mass spectra centred on the peak at 2.7 min in (D).

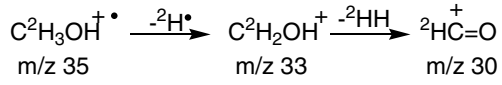

Figure 3. Fragmentation of trideuteromethanol in electron ionization mass spectrometry.

labelled substrate we showed that demethylation was essentially complete after 200 min.[3]

We propose an oxidative pathway, (Fig. 1) supported by these findings.

Homology modelling. In this report, computational approaches compensate for the lack of structural information for MBD2/dMTase. The first goal was to generate a computer model of the MBD2 protein.[5] Fortunately, a structure of MBD1 bound to methylated DNA was available[6] which has 50% identity with MBD2 in the methylated DNA binding domain (MBD) and no residue differences in the binding site. We are therefore reasonably confident in using MBD1 as a template for MBD2. This construction was followed by optimization of the side chain conformations leading to eight similar models which were smoothly relaxed in explicit water using a combination of Molecular Dynamics simulations and energy minimization with the CHARMM22 force field. A close look at the eight similar models exemplified by the model shown in Figure 4 revealed that the Tyr178 side chain was perfectly positioned to be involved in the process of demethylation. Tyrosine side chains are known to form radicals as in the cyclooxygenase reaction[7] and to be good nucleophiles and these two factors can be considered relevant in proposing a mechanism.

Our earlier work[3] reporting methanol detection with unlabelled substrates included an extraction step into toluene for GC–MS analysis. We can only speculate that in that first study, formaldehyde liberated enzy-

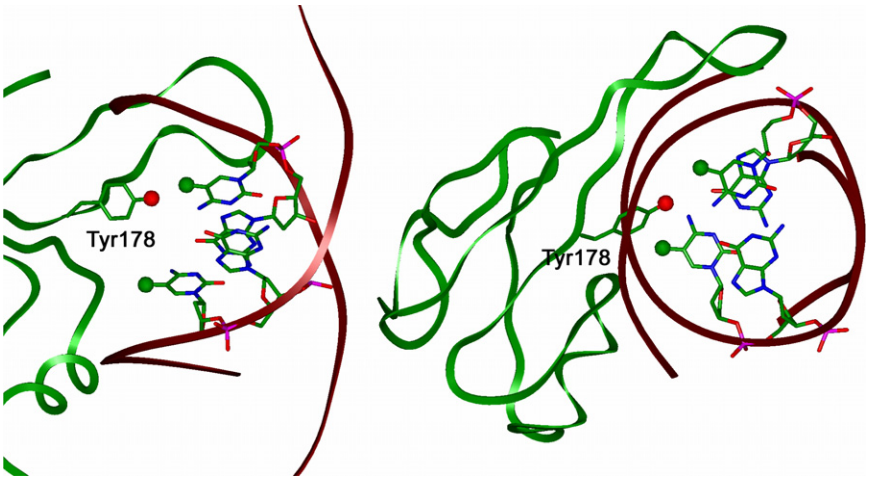

Figure 4. Model of the MBD2/methylated DNA complex. Two points of view are given. The protein is represented as a green ribbon showing the Tyr178 side chain. The DNA is represented by a red ribbon, four bases of which are shown. The methyl groups are represented by green balls and the tyrosyl hydroxyl group as a red ball.

matically by MBD2/dMTase in the aqueous incubation medium may have been converted into methanol by an unknown dehydrogenase contaminant of the crude demethylase preparation in use at that time. Under those circumstances, the inefficiency of extraction of methanol from the aqueous phase into toluene led to the very small quantity of methanol detected in the first report. In the present work however, the extraction step was omitted, and the crude aqueous reaction mixture was reduced with NaB^2H_4 and subjected to GC–MS analysis on a water-tolerant capillary column. Only by NaB^2H_4 reduction of product dideuteroformaldehyde was methanol made detectable.

From these results it is clear that the loss of the methyl group is due to an oxidative demethylation perhaps similar to that proposed in Figure 1. Although the first single electron transfer is most likely attributed to the presence of a tyrosyl radical, the second transfer can be achieved by a hydroxyl radical or another radical species. Formation and regeneration of the tyrosyl radical, for instance, remains obscure. While confirmation will emerge once the X-ray or NMR structure of MBD2 is known, further experiments will be required to fully understand DNA demethylation. The present work demonstrates that the leaving moiety in this reaction is formaldehyde, and this finding will be central to further elucidation of the mechanism of this important reaction.

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