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PAPER

Substrate promiscuity in DNA methyltransferase M.*Pvu*II. A mechanistic insight†

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M.*Pvu*II is a DNA methyltransferase from the bacterium *Proteus vulgaris* that catalyzes methylation of cytosine at the N4 position. This enzyme also displays promiscuous activity catalyzing methylation of adenine at the N6 position. In this work we use QM/MM methods to investigate the reaction mechanism of this promiscuous activity. We found that N6 methylation in M.*Pvu*II takes place by means of a stepwise mechanism in which deprotonation of the exocyclic amino group is followed by the methyl transfer. Deprotonation involves two residues of the active site, Ser53 and Asp96, while methylation takes place directly from the AdoMet cofactor to the target nitrogen atom. The same reaction mechanism was described for cytosine methylation in the same enzyme, while the reversal timing, that is methylation followed by deprotonation, has been described in M.*Taq*I, an enzyme that catalyzes the N6-adenine DNA methylation from *Thermus aquaticus*. These mechanistic findings can be useful to understand the evolutionary paths followed by *N*-methyltransferases.

Introduction

DNA methyltransferases are a family of enzymes that transfer the methyl group from cofactor S-adenosyl-L-methionine (AdoMet) to cytosine or adenine bases in DNA, converting AdoMet into S-adenosyl-L-homocysteine (AdoHcy).[1] Enzymatic DNA methylation is an important biochemical process that provides DNA with new information, which is not encoded in the nucleotide sequence. These enzymes are found both in prokaryotes and in eukaryotes. In the former, DNA methyltransferases are mostly components of restriction-modification systems whose central function is protection of the host cell from foreign DNA.[2,3] Moreover, these enzymes coordinate DNA replication and cell division and direct postreplicative mismatch repair. In eukaryotes, DNA methylation contributes to the regulation of gene expression, protection of the genome against selfish DNA, maintenance of genome integrity, parental imprinting, inactivation of the X-chromosome, carcinogenesis, *etc.*[1,4–7]

DNA methylation can occur at the exocyclic amino groups of adenine (N6-adenine DNA methyltransferase) and cytosine (N4-cytosine DNA methyltransferase) or the 5 position of cytosine (C5-cytosine DNA methyltransferase) yielding 6-methyladenine, 4-methylcytosine or 5-methylcytosine respectively. Structural and biochemical studies have shown that all DNA methyltransferases share the mechanistic feature that methylation is preceded by flipping the target nucleotide out of the DNA helix,[8] and then it is inserted into the binding pocket.[9] DNA methyltransferases have similar catalytic domains, one large domain containing the binding sites for the cofactor and the flipped base and one smaller domain that participates in DNA binding and recognition.[10–15] The two types of amino methyltransferases are likely to be more closely related between them than with the C5-cytosine DNA methyltransferase. Not only do they have a common target, the exocyclic amino group of cytosine or adenine, but they also contain several conserved residues in their major structural and functional motifs.[16] In addition, it has been demonstrated that amino methyltransferases not only show structural but also functional similarity, they can methylate both target bases, adenine and cytosine.[17] That is, N6-adenine DNA methyltransferases M.*Eco*RV, M.*Eco*RI, *E. coli* Dam and M.*Fok*I also modify cytosine residues at the N4 position[18,19] and N4-cytosine DNA methyltransferase from the bacterium *Proteus vulgaris* (M.*Pvu*II) also methylates adenine residues.[17] Therefore, some amino methyltransferases show substrate promiscuity. Understanding this overlap of substrates between N4 and N6 methyltransferases can be important to unravel the molecular evolution of these enzymes.

The reaction mechanism for methylation of cytosine catalyzed by M.*Pvu*II has been previously explored by us using hybrid quantum mechanics/molecular mechanics (QM/MM) methods.[20] The reaction mechanism involves a methyl transfer from AdoMet to the exocyclic nitrogen atom of the base and a proton transfer from this atom to Ser53, which in turn transfers a proton to Asp96. Different timings for the proton transfer and

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methylation steps have been explored at the AM1/MM and B3LYP/MM levels including localization and characterization of stationary structures. It was found that the deprotonation of the exocyclic amino group (from NH_2 to NH^-) occurs first through a proton relay mechanism that involves Ser53 and Asp96. The amino group of the deprotonated base is then more nucleophilic, facilitating the subsequent methyl transfer step from AdoMet.[20] Interestingly, a previous theoretical analysis showed that the reaction mechanism for N6-adenine DNA methylation from *Thermus aquaticus* (M.*Taq*I) takes place with a reverse ordering of the two chemical events, first methyl transfer followed by proton transfer.[21] As said before, and in spite of the reported mechanistic differences, it has been experimentally shown that N4-cytosine DNA methyltransferase M.*Pvu*II also methylates adenine residues.[17]

We herein present a theoretical study of the reaction mechanism of the adenine methylation catalyzed by M.*Pvu*II by means of hybrid QM/MM methods. Our results point out that the reaction mechanism for adenine methylation in this enzyme is the same as that for cytosine methylation but with a slightly higher energy barrier. The nature of the residues of the active site determines the type of reaction mechanism while the optimization of the barrier depends on the optimization of the interactions established with the substrate. These findings could help to trace the evolutionary path of the different kinds of DNA *N*-methyltransferases.

Methods

A QM/MM approach is here used to study substrate promiscuity of DNA methyltransferase M.*Pvu*II following the same computational procedure employed to analyze the primary activity in our previous study.[20] The initial coordinates of the protein were taken from the X-ray crystal structure of *Pvu*II DNA-(cytosine N4)-methyltransferase complexed with AdoMet, PDB code 1BOO.[13] Since there is no X-ray structure crystallized for the enzyme with DNA, we then built a small model of the reacting system that consists of inserting an adenine base into the active site. This model was introduced inside a cubic box of water molecules of side 79.5 Å. We performed several optimization cycles followed by QM/MM molecular dynamics (MD) simulations using the DYNAMO program.[22,23] The QM subsystem, which includes the base, part of the AdoMet cofactor and side chains of residues Ser53 and Asp96 (see Fig. 1), was described by the semiempirical method AM1[24] while the classical atoms were described by means of the OPLS-AA[25,26] and TIP3P[27] force fields as implemented in the DYNAMO program. To saturate the valence of the QM/MM frontier, we used the link atoms procedure.[28,29] To treat the nonbonding interactions, a switch function with a cutoff distance in the range 14–18 Å was used. The NVT ensemble, with a reference temperature of 300 K, and periodic boundary conditions were employed in the simulations. The time step was 1 fs.

The final structure obtained after 200 ps of MD simulation was used as the starting point for the exploration of the potential energy surface (PES). Stationary structures localization and characterization and intrinsic reaction coordinates (IRCs) were performed using the micro/macroiteration optimization algorithm.[30–32] This method consists of dividing the coordinate space into two subsets: the control space (that usually matches up with the QM region) and the complementary space (the rest of the system). Optimization on the control space (microiterations) makes use of a Hessian-based algorithm and at each step in the control space the complementary space is fully minimized using gradient vectors (macroiterations). This exploration was carried out at the B3LYP/MM level, using a combination of DYNAMO/GAUSSIAN03 programs.[33] The 6-31G* basis set was selected for the exploration of the PES and single-point energy calculations of the stationary structures (reactants, TSs, intermediates and products) were afterwards obtained using a larger 6-311+G** basis set.

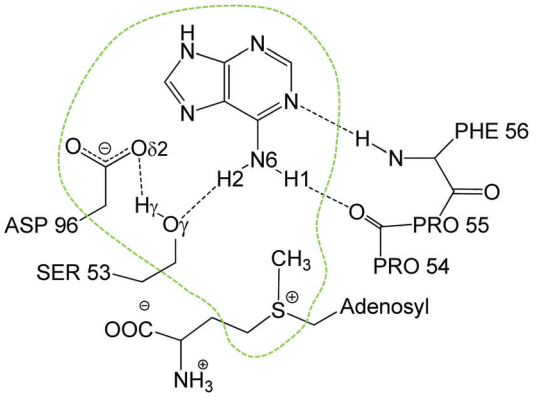

Fig. 1 Active site of M.*Pvu*II containing the adenine base. The region surrounded by a dashed line is the quantum region defined in our calculations (37 QM atoms).

Results

Reaction mechanism of adenine methylation in M.*Pvu*II

A preliminary exploration of the PES was carried out using as distinguished coordinates the antisymmetric combinations of the distances defining the methyl transfer from S(AdoMet) to N6(Ade), the proton transfer from N6(Ade) to Oγ(Ser53) and the proton transfer from Oγ(Ser53) to Oδ2(Asp96).

From these explorations the possibility of a methyl transfer preceding the proton transfer was excluded and the only possible reaction mechanism is described as a stepwise one in which deprotonation of the exocyclic amino group of adenine is followed by a direct methyl transfer from AdoMet to the deprotonated amino group (see Fig. 2). This reaction mechanism is the one found as the most favorable (lower barrier) in the case of N4-cytosine methylation.[20]

Potential energy calculations with the methyl transfer to N6(Ade) preceding the deprotonation of the exocyclic amino group systematically resulted in spontaneous proton transfer. We then explored the methyl transfer by applying a constraint to the antisymmetric combinations of the distances defining the proton transfer from N6(Ade) to Oγ(Ser53) and the proton transfer from Oγ(Ser53) to Oδ2(Asp96), and the resulting potential energy barrier was much higher than for the case in which proton transfer precedes methyl transfer (see Fig. S1 in ESI†).

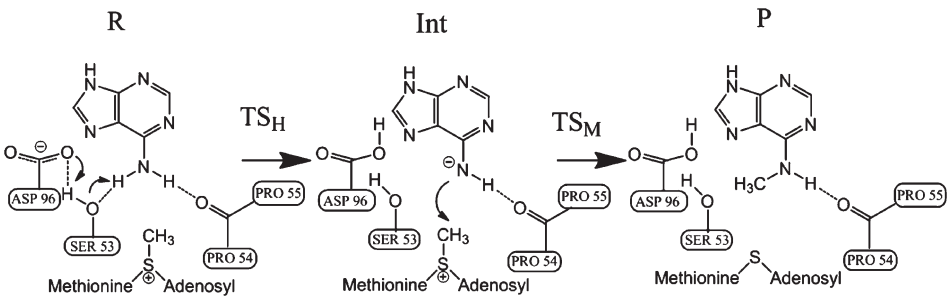

Fig. 2 Reaction mechanism found in this work for adenine in M.*Pvu*II, in which proton transfer is followed by methylation. R, TS_H, Int, TS_M and P designate reactant, transition structure of proton transfer, intermediate, transition structure of methylation and product respectively.

Table 1 Relevant geometrical parameters (distances in Å, angles in °) found for the stationary structures of the reaction mechanism explored at the B3LYP/6-31G*/MM level for the adenine methylation in M.*Pvu*II[a]

	R	TSH	Int	TSM	P
d(S(AdoMet)-CH_3(AdoMet))	1.831	1.838	1.848	2.166	3.888
d(CH_3(AdoMet)-N6(Ade))	3.688	3.366	3.202	2.458	1.463
d(N6(Ade)-H2(N6)(Ade))	1.024	1.236	1.862	2.091	3.894
d(Oγ(Ser53)-H2(N6)(Ade))	1.981	1.307	1.001	0.985	0.979
d(Oγ(Ser53)-Hγ(Ser53))	1.028	1.633	1.838	1.954	1.816
d(Oδ2(Asp96)-Hγ(Ser53))	1.571	1.026	0.989	0.983	0.985
d(S(AdoMet)-N6(Ade))	5.466	5.185	5.027	4.616	4.788
d(O(Pro54)-H1(N6)(Ade))	1.697	1.855	1.927	1.975	1.860
a(S(AdoMet)-CH_3-N6(Ade))	163.13	169.87	168.71	173.2	119.8
v		961.2i		342.1i	
Δ*E*	0	14.3	6.0	10.7	−42.3

[a] Imaginary frequencies of transition structures are given in cm^{-1}, and relative energies (obtained from single point calculations using the 6-311+G** basis set) in kcal mol^{-1}.

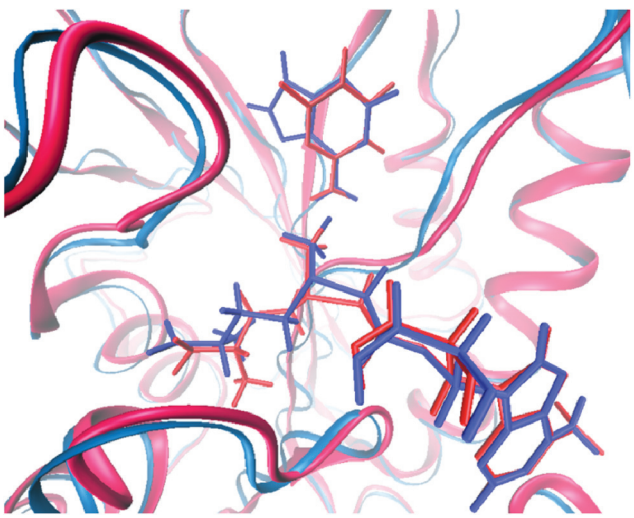

Fig. 3 Overlap of the reactant structures of adenine (blue) and cytosine (red) methylation in the M.*Pvu*II active site. The base and the cofactor (AdoMet) are drawn using stick representation.

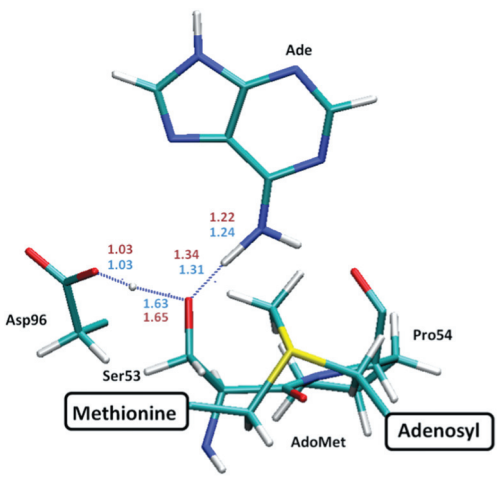

Fig. 4 Representation of the transition structure found for deprotonation of the exocyclic amino group of adenine in the M.*Pvu*II active site. Relevant distances for adenine (blue) and cytosine (red) are given in Å.

The most relevant geometrical parameters and the relative internal energies of the stationary structures found in the exploration of the PES are given in Table 1. Representations of the reactant structure (R) and the two transition structures (TS_H and TS_M) are shown in Fig. 3–5, respectively. Finally the energy profile, together with that found in the case of N4-cytosine methylation, is shown in Fig. 6.

Adenine is accommodated in the active site of M.*Pvu*II using the same set of interactions as in the case of cytosine. As can be seen in Fig. 3, the positions of these bases in the active site

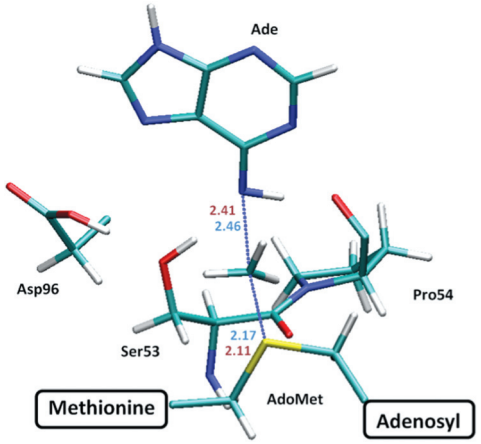

Fig. 5 Representation of the transition structure found for the methylation step of adenine in the M.*Pvu*II active site. Relevant distances for adenine (blue) and cytosine (red) are given in Å.

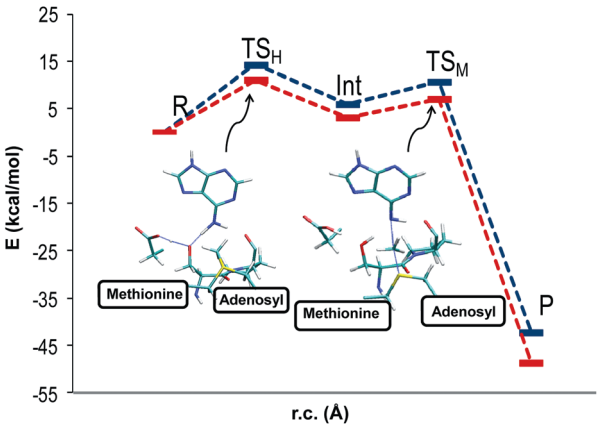

Fig. 6 Potential energy profiles obtained for the reaction mechanism where proton transfer precedes methylation, for both the M.*Pvu*II–cytosine system (red) and the M.*Pvu*II–adenine system (blue).

overlap quite nicely. The exocyclic amino group of adenine establishes two hydrogen bond interactions: one with the carbonyl oxygen of Pro54 (1.697 Å; see Table 1) and another with the hydroxylic oxygen atom of Ser53 (1.981 Å). This residue in turn donates a hydrogen bond to one of the oxygen atoms of the carboxylate group of Asp96 (1.571 Å). The same hydrogen bond interactions but with slightly larger hydrogen bond distances were reported in the case of cytosine.[20] In addition the positively charged methyl group of AdoMet is oriented towards the lone pair of the nitrogen atom of the exocyclic amino group of adenine. The distance between the methyl donor (S(AdoMet)) and the acceptor atom is larger for adenine (N6) than for cytosine (N4), 5.466 and 4.985 Å respectively. These differences found between the reactants structure of cytosine and adenine could then explain why the mechanism with methyl transfer preceding proton transfer is not observed in the case of adenine and that the only available mechanism is the one in which the proton transfer takes place first, increasing then the nucleophilic character of the exocyclic amino group.

After exploration of the PES we located a transition structure (TS_H), shown in Fig. 4. Diagonalization of the Hessian matrix corresponding to this structure results in a unique negative eigenvalue from which an imaginary frequency of 961.2i cm^{-1} was obtained (see Table 1). The transition vector corresponds to the proton transfer from the N6 atom of adenine to Oγ of Ser53 and the proton transfer from this last atom to Oδ2 of Asp96. In the transition structure the latter proton transfer is almost completed while the proton transfer from the exocyclic amino group of adenine to Ser53 is halfway, as reflected in the distances given in Table 1 and in Fig. 4. So, deprotonation of the adenine amino group is initiated by the proton transfer from the hydroxyl group of Ser53 to Asp96 and then the activated Ser53 abstracts one of the protons of the amino group. In this transition structure the distance between the methyl donor and acceptor (the S(AdoMet) and N6(Ade) atoms) is reduced up to 5.185 Å.

From this transition structure we located an intermediate structure (Int) that presents a protonated Asp96, a neutral Ser53, and a formally negatively charged N6 atom of adenine. The deprotonated base is stabilized by means of hydrogen bond interactions with Ser53 (1.862 Å) and Pro54 (1.927 Å). In this intermediate the distance from the N6 of adenine to the sulfur atom of AdoMet is already significantly reduced (5.027 Å) but still longer than the distance shown by the intermediate corresponding to cytosine methylation (4.738 Å).[20]

From the intermediate structure we located a new transition structure (TS_M) corresponding to the direct methyl transfer from the sulfur atom of the cofactor (S(AdoMet) in Table 1) to the target nitrogen atom of the base (N6(Ade)). This is an early transition state where the distance from the carbon atom of the methyl group to the N6 position of the aromatic ring (2.458 Å) is longer than the distance from the carbon atom of the methyl group to the sulfur atom of the cofactor (2.166 Å), as seen in Fig. 5 and in Table 1. This process is accompanied by a slight lengthening of the hydrogen bond interactions established between the exocyclic amino group and Ser53 and Pro54 residues (see Table 1).

Tracing the IRC from TS_M directly leads to the final product. In this structure the exocyclic amino group of adenine obviously has a unique hydrogen bond interaction with the protein. The distance from the hydrogen atom to the carbonyl oxygen atom of Pro54 is 1.860 Å. In this product structure Asp96 is protonated and has a hydrogen bond interaction with Ser53. The latter residue in turn acts as a hydrogen bond donor to the carbonyl oxygen of Asp96 (1.910 Å).

The energy profile obtained by means of single-point calculations at the B3LYP/MM level using the 6-311+G** basis set is depicted in Fig. 6, together with that obtained for cytosine methylation at the same computational level. Both bases follow the same reaction mechanism in the active site of M.*Pvu*II. For both cytosine and adenine bases the highest energy transition structure is the first one, which corresponds to the proton transfer from Ser53 to Asp96 and from the N6 nitrogen atom of adenine to Ser53. The energy barriers, as determined from the potential energy difference between TS_H and R structures, are 11.1 and 14.3 kcal mol^{-1}, for cytosine and adenine, respectively. The larger energy barrier obtained for adenine reflects the fact that the enzyme M.*Pvu*II is specialized in N4-cytosine methylation and thus it has been optimized by evolution to catalyze the

reaction on this substrate. We confirmed this observation by calculating the averaged energy barriers for the rate-determining step in the case of adenine and cytosine, starting from four different reactant structures selected from the MD simulation and spaced by at least 10 ps. The averaged barriers were 11.2 ± 0.8 and 14.9 ± 0.5 kcal mol^{-1} for cytosine and adenine, respectively. Thus the averaged difference in the barrier is 3.7 ± 0.9 kcal mol^{-1}.

The preference of the enzyme for its natural substrate is reflected in the fact that the interactions established between the exocyclic amino group and the protein are better designed to speed up the process in the case of cytosine. First, the distance from the methyl group to be transferred to the target nitrogen atom is systematically larger in the case of adenine than for cytosine. Second, the hydrogen bond interactions established between the amino group and the protein (Ser53 and Pro54 residues) in the intermediate structure (Int) are shorter in the case of cytosine. It is interesting to point out that these distances are shorter for adenine in the reactant state, but then these distances are lengthened in the rest of the stationary structures. The opposite trend is found in the case of cytosine,[20] reflecting the optimization of the hydrogen bond interactions not with the sole purpose of stabilizing the reactant state but to catalyze the reaction; this is to diminish the energy barrier.

A general mechanistic view of *N*-methyltransferases

N-Methyltransferases catalyze methylation of the exocyclic nitrogen of adenine (N6) and cytosine (N4). This reaction necessarily involves two chemical steps: deprotonation of the amino group and methyl transfer from AdoMet to this group. According to our studies it seems that two mechanistic routes are available for *N*-methyltransferases: (i) a reaction mechanism in which deprotonation occurs first, by means of a proton transfer to a protein residue, followed by methyl transfer to an activated amino group and (ii) a mechanism in which the methyl transfer from AdoMet takes place first, producing a positively charged amino group, followed by a proton transfer from the acidified exocyclic amino group. These two mechanistic routes can be optimized using different strategies. The first route would require the presence of a proton acceptor in the active site (typically a glutamate or an aspartate).[20] The second one could be favoured by an active site designed to assist electrostatically the transfer of a positive charge from the AdoMet cofactor to the target atom.[34–36] This classification can be useful to unravel the path followed by evolution in these enzymes. M.*Pvu*II (an N4-methyltransferase) is an example of the first case while M.*Taq*I (an N6-methyltransferase) belongs to the second category. In this work we showed that the same substrate (an adenine base) can be methylated following one or another mechanism depending on the environment, and this environment is dependent on the nature of the active site.

Conclusions

In this work we have analyzed the promiscuous activity of an N4-cytosine methyltransferase (M.*Pvu*II) as an N6-adenine methyltransferase. The reaction mechanism for this promiscuous activity implies a stepwise process in which a proton transfer from the exocyclic amino group to the protein (Ser53 and Asp96 residues) is followed by the direct methyl transfer from AdoMet to the target nitrogen atom. This mechanism is the same as that found when this enzyme methylates its natural substrate, cytosine. Substrate specificity is reflected in the smaller energy barrier obtained for cytosine. This specificity seems to be the consequence of the subtle optimization of the interactions established with the target amino group with the positively charged methyl group of AdoMet and the hydrogen bonds established with Ser53 and Pro54.

When these results are related to other theoretical analysis of *N*-methyltransferases reactivity,[20,21] we found that two different mechanistic routes can be followed to methylate the target base, irrespective of its nature. Depending on the presence or not of a basic residue in the proximity of the substrate, deprotonation of the amino group precedes methylation or methylation precedes deprotonation. This finding could be useful to investigate the evolutionary paths followed by these enzymes, because different strategies are required to optimize the rate of the chemical step depending on the actual mechanism.

Acknowledgements

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