ISSN 0006-2979, Biochemistry (Moscow), 2009, Vol. 74, No. 6, pp. 613-619. © Pleiades Publishing, Ltd., 2009.
Published in Russian in Biokhimiya, 2009, Vol. 74, No. 6, pp. 753-760.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM08-242, March 15, 2009.

Epigenetic DNA-(Cytosine-5-Carbon) Modifications: 5-Aza-2′-deoxycytidine and DNA-Demethylation

S. K. Patra[1,2]* and S. Bettuzzi[1,2]

[1]*Division of Biochemistry, Department of Experimental Medicine, University of Parma, Parma, Italy; E-mail: skpatra_99@yahoo.com*
[2]*Istituto Nazionale Biostrutture e Biosistemi (I.N.B.B.), Roma, Italy*

Received July 29, 2008
Revision received September 16, 2008

Abstract—DNA (cytosine-5-carbon) methylation is one of the hallmarks of mammalian chromatin modifications. Distinct methylation pattern can generate synergistic or antagonistic interaction affinities for CpG-islands associated with methylated or unmethylated cytosine binding proteins, which also may dictate histone modifications and dynamic transition between transcriptionally silent or transcriptionally active chromatin states. The enzymes and cofactors associated with DNA-methylation reactions are convincing in terms of chemistry and chemical thermodynamics. The mechanism of demethylation, the candidate enzyme(s) exhibiting direct demethylase activity, and associated cofactors are not firmly established. Use of azanucleosides, such as 5-azacytidine and 5-aza-2′-deoxycytidine (AzadC), in cell culture produces re-expression of certain genes, which otherwise were repressed in association with hypermethylated CpG-rich promoters. Hence the notion developed that AzadC is a demethylating agent. Here we discuss the broad global pictures with the following points: first, chemical definition and recent advances regarding the mechanism of DNA (cytosine-5-carbon) methylation (^{Me}CpG-DNA or ^{Me}CpNpG-DNA formation) and ^{Me}CpG/^{Me}CpNpG-DNA-demethylation, and then with the mechanistic basis of inactivation of DNA-methyltransferase 1 by AzadC. This will clarify that: (i) AzadC has nothing to do with DNA-demethylation; (ii) it cannot prevent even *de novo* methylation in non-replicating cells; (iii) it can only prevent replication coupled maintenance as well as *de novo* methylations. Finally, we would like to suggest that terming/designating AzadC as DNA-demethylating agent is a serious misuse of chemistry and chemical terminology.

DOI: 10.1134/S0006297909060042

Key words: epigenetics, DNA-methylation, DNA-demethylation, DNA-methyltransferase, 5-aza-2′-deoxycytidine

Methylated cytosine at the 5-carbon position (^{Me}C) was discovered serendipitously by R. D. Hotchkiss [1] while separating and quantifying DNA bases from nuclear preparations. Subsequently it was revealed that DNA-methylation at cytosine-5-carbon position is the post-replicative addition of a methyl (-CH_3) group to cytosine at CpG-DNA, CpNpG-DNA, or some specific sequences of DNA by DNA-methyltransferases (DNMTs), where S-adenosylmethionine (SAM) is the donor of the -CH_3 group (Fig. 1). Thus ^{Me}CpG-DNA and ^{Me}CpNpG-DNA are formed. The DNMTs (DNMT1, DNMT3A, and DNMT3B) catalytically remove the -CH_3 group from SAM and put it onto the 5-carbon (connecting through C–C covalent bonding) of cytosine, and SAM changes to S-adenosylhomocysteine (SAH) [2-8]. Existence of genome wide hypomethylation and regional hypermethylation remains a paradox in carcinogenesis. In zygotic development just after fertilization, paternal DNA experiences drastic loss of DNA-methylation before the zygote starts dividing (before its DNA is replicated). The enzymes and cofactors involved are still unknown. However, the egg-derived DNA remains mostly methylated [7, 8]. Concomitant with differentiation, high levels and cell-type-specific patterns of DNA methylation and histone modifications are acquired for various physiological functions, including memory formation ([2-5], for recent reviews see [7, 8]). Such epigenetic momentum may be difficult to reverse during cloning, since the signals and mechanisms for gene-specific regional hypermethylation and global demethylation patterns are not yet completely elucidated.

Abbreviations: AzadC, 5-aza-2′-deoxycytidine; BER, base excision repair; DNMTs, DNA methyltransferases; NER, nucleotide excision repair; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; TRD, transcriptional repression domain.

* To whom correspondence should be addressed.

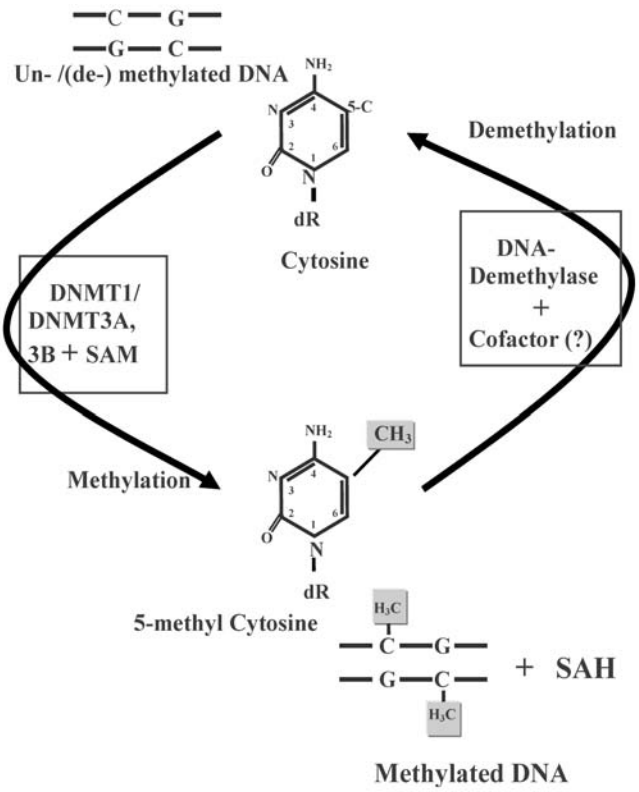

Fig. 1. Depiction of reversible methylation modification of DNA at the 5-carbon position of cytosine in CpG-dinucleotide sequences. Chemical/biochemical definition of DNA methylation is the covalent attachment of methyl (-CH_3) group (for examples, guanine methylation at O6, adenine methylation at N6, cytosine methylation at C6 and N4 (of C4-NH_2 of cytosine)). DNA methyltransferases (DNMT1, DNMT3A, and DNMT3B) catalytically put the -CH_3 group on 5-C of cytosine, where SAM donates the -CH_3 group and changes to SAH. The enzyme remains intact after the complete reaction and can be available for the next catalytic cycle (further details with reaction mechanism are presented in Fig. 2a; see also [1-4, 6, 7, 9-19]). The exact chemical definition of DNA-demethylation is the removal of the sole -CH_3 group from methyl-cytosine (^{Me}C) of DNA [13, 27-30, 51-53, 57-59, 64]. The chemical reaction based mechanism of demethylation is not firmly established, since the enzymes and cofactors involved are not precisely and accurately known (for outstanding discussions, see Patra et al. [7]).

DNA-(cytosine-5-carbon) methylation mechanism. The mechanisms for bacterial and mammalian DNMT-mediated catalysis have been proposed elsewhere [9-18] and reviewed in the recent past [6, 7, 19]. In brief, the catalytic mechanism of DNMT as presented in Fig. 2a involves the formation of a covalent bond between the thiol of a cysteine residue in the active site of the enzyme and carbon 6 (^{6}C) of cytosine DNA (Fig. 2a, Step I). This event increases the electron flow to carbon 5 (^{5}C), with subsequent attack on the methyl group of SAM. Abstraction of the proton from ^{5}C followed by β-elimination allows reformation of the ^{5}C=^{6}C double bond and release of the enzyme and substrate DNA with a methylated cytosine [6, 7, 9, 13-15, 17-19]. Following the cytosine ^{5}C exchange reaction with DNMT1, the preference of DNMT1 for different DNA substrates, the allosteric regulation, and also a basis for comparison with the bacterial enzymes were recently characterized [17]. It was determined that the methyl transfer is rate-limiting (Fig. 2a, Step II) and steps up to and including the cysteine–cytosine covalent intermediate (cytosine ^{6}C-S(Cys)-DNMT) are in rapid equilibrium [15, 16, 18]. Changes in these rapid equilibrium steps account for many of the previously described features of DNMT1 catalysis and specificity including faster reactions with pre-/hemi-methylated DNA versus unmethylated DNA [20], faster reactions with DNA in which guanine is replaced with inosine (poly(dG-dC) versus poly(dI-dC)) [21], and 10-100-fold slower catalytic rates with DNMT1 relative to the bacterial enzyme M.HhaI ([14, 17] and references therein). Interactions of DNMT1 with the guanine (G) within the CpG-recognition site can prevent the premature release of the target base and solvent access to the active site that could lead to mutagenic deamination. The results of Svedruzic and Reich [17] suggest that the β-elimination (Fig. 2a, Step III) following methyl transfer is not mediated by free solvent. DNMT1 shows a kinetic lag in product formation and allosteric inhibition with unmethylated DNA, which is not observed with pre/hemi-methylated DNA. Thus, they suggested that the enzyme undergoes a slow relief from allosteric inhibition upon initiation of catalysis on unmethylated DNA. Notably, this relief from allosteric inhibition is not caused by self-activation through the initial methylation reaction, as the same effect is observed during the cytosine ^{5}C exchange reaction in the absence of SAM [17]. An elaborated mechanism involving DNA substrate, the enzyme, and SAM was described recently [15]. On the basis of amino acid sequence alignments and structural data of related enzymes, Gowher et al. performed a mutational analysis of 14 amino acid residues in the catalytic domain of murine Dnmt3a [15]. The targeted residues are located within the ten conserved amino acid sequence motifs characteristic for DNMTs [19] and in the putative DNA recognition domain of the enzyme (transcriptional repression domain, TRD). Testing the catalytic properties of those purified mutant proteins and their abilities to bind DNA and SAM, a structural model of Dnmt3a was proposed [15]. It was suggested that Phe50 (at motif I) and Glu74 (at motif II) are important for SAM-binding and catalysis. D96A (at motif III) showed reduced SAM binding but increased activity under conditions of saturation with SAM, indicating that the contact of Asp96 to SAM is not required for catalysis. R130A (following motif IV), R241A and R246A in the TRD, and R292A and R297A (both located in front of motif X) showed reduced DNA binding. R130A displayed a strong reduction in catalytic activity and a complete change in flanking sequence preferences, indicating that Arg130 has an important role in the DNA interaction of Dnmt3a.

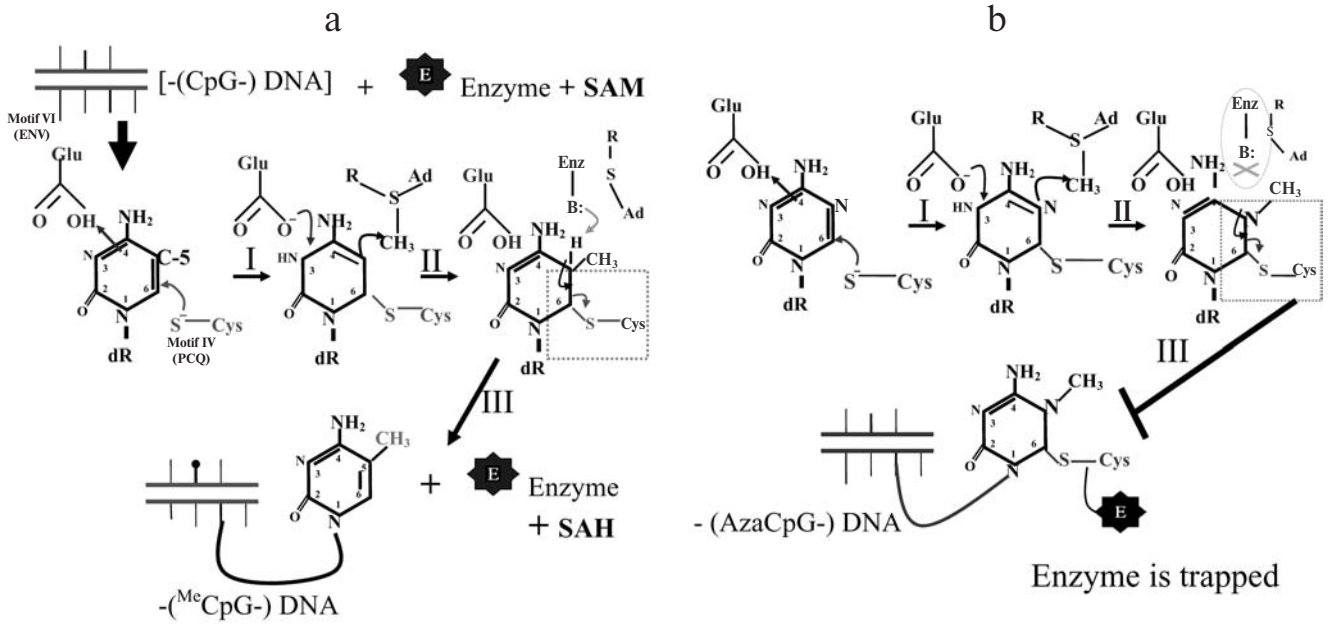

Fig. 2. a) Major steps for mammalian DNMT3a-mediated methylation of cytosine ^{5}C. Step I: from motif IV of the enzyme active site a Cys thiol attack at ^{6}C of cytosine, which results in electron cloud in ^{5}C, and from motif VI of the enzyme Glu -COOH proton (H^+) donation to ^{3}N, apparently stabilize the transition state within the mentioned cytosine complexed with the enzyme through cytosine ^{6}C-S(Cys)-DNMT. Step II: ^{5}C-carbanion attack at -CH_3 of SAM (bound to enzyme) and abstraction of the proton from H-^{3}N by Glu -COO^- (of motif VI) result in the formation of ^{5}C(H)-CH_3, retaining the ^{6}C-S(Cys)-DNMT linkage. Step III: abstraction of the proton from ^{5}C by a base of the DNMT followed by β-elimination allows re-formation of the ^{5}C=^{6}C double bond (marked within dotted lined square), and release of the enzyme and substrate DNA with a methylated cytosine (the "lollypop"). (See also [6, 7, 9-21]; after [89]). b) Possible mechanism for inactivation/trapping of mammalian DNMTs during the course of methylation of 5-aza-2′-deoxycytosine (AzadC) incorporated DNA at ^{5}N position of AzadC. This apparently mimics the Steps I and II involved in the reactions shown in Fig. 2a. Step I: from motif IV of the enzyme active site a Cys thiol attack at ^{6}C of AzadC, which results in electron cloud in ^{5}N and from motif VI of the enzyme a Glu -COOH proton (H^+) donation to ^{3}N, apparently stabilize the transition state within the AzadC complexed with the enzyme through AzadC ^{6}C-S(Cys)-DNMT. Step II: ^{5}N(−) anion attack at -CH_3 of SAM (bound to enzyme) and abstraction of the proton from H-^{3}N by (motif VI) Glu -COO^- resulting in the formation of ^{5}N-CH_3, retaining the ^{6}C-S(Cys)-DNMT linkage. Step III: since there is no question of abstraction of a proton from ^{5}N (by a base of the DNMT, shown within the ellipsoid), β-elimination and re-formation of the ^{5}N=^{6}C double bond (marked within dotted lined square) is prevented; and thus, instead of release, the DNMT is trapped/inactivated and not available for the next catalytic cycle.

R292A also displayed reduced activity and changes in the flanking sequence preferences, indicating a potential role in DNA contacts farther away from the CG target site. N167A (motif VI) and R202A (motif VIII) have normal SAM and DNA binding but reduced catalytic activity. While Asn167 might contribute to the positioning of residues from motif VI, according to structural data Arg202 has a role in catalysis of DNMTs [15, 19].

(5-Carbon methylated cytosine, ^{Me}C) DNA-demethylation chemistry. ^{Me}C-DNA demethylation (Fig. 1), as such, is the removal of methyl groups from DNA [7]. Replication-independent DNA demethylation would imply the existence of a mammalian DNA demethylase enzyme that can either actively remove the methyl group from 5-methylcytosine or can remove the entire methylated base or nucleotide, perhaps in base/nucleotide excision repair (BER/NER)-like pathways [7, 22-35]. Although direct breakage of a carbon–carbon bond seems energetically unfavorable [6, 7, 19, 22], enzymatic removal of a methyl group (demethylation) is essential for transcription of many genes. Demethylation could be obtained by the following possible paths: (i) direct active process—demethylation [27-30]; (ii) indirect active process—nucleotide/base excision repair [31-36]; (iii) passive loss of the methyl group due to failure of maintenance of methylation after replication (for reviews see [7, 22, 32]); or (iv) mechanical inhibition/DNMT-trapping by AzadC incorporated into DNA in cultured cells. The nature of the mammalian DNA demethylase has remained obscure. Earlier, the recombinant MBD2b protein was reported to possess DNA demethylase activity *in vitro* [27-29], although this finding has so far not been repeated in other laboratories [7, 30]. Current trends of DNA-demethylation research implicate the role of various transcription factors in the local activation of nucleosomes by BER/NER pathways [22-26], where an association of DNMT1 is also indicated [25, 26], but the precise role of DNMT1 in this scenario remains to be investigated further. In this context, an interesting hypothesis that DNA demethylation mechanism may follow "Michael retrogression" was proposed earlier by Patra et al. [7]. Recently, repair-mediated DNA demethylation by

Gadd45a has been shown to promote epigenetic *Oct4* gene activation [24], but this finding has been questioned very recently in another laboratory [36]. Moreover, if not strand-specifically coordinated, the BER pathway would put the genome at risk for DNA double strand breakage [7, 23, 36]. The mechanistic steps and proteins with appreciable rate of activity involving DNA demethylation in mammals do need exhaustive investigations. In plants, a demethylase pathway involving a DNA glycosylase activity has been identified [37-39], but these proteins do not appear to have mammalian homologs. The mammalian cytidine deaminases AID and APOBEC1 have ^{Me}C deaminase activity *in vitro* [40] and the deaminated base, thymine, can be removed by BER pathways [7, 31-35].

5-Aza-2′-deoxycytidine cannot demethylate DNA and should not be identified as a DNA-demethylating agent. As discussed above (see also Fig. 1), DNA-methylation and DNA-demethylation are two distinct, exactly opposite conceptually defined chemical reactions (similar to other types; like, acetylation and deacetylation, phosphorylation and dephosphorylation, which are frequently used in studying the dynamic epigenome). Inhibition of methylation, by any means, should not be interpreted as demethylation. AzadC has long been suggested for clinical use [10]. Trials for assessment of safety/toxicity and efficacy for treatment of various tumors had been done and are still ongoing [41-46]. Molecular analyses of gene expression patterns at the levels of mRNAs and proteins after AzadC treatment of cells in culture confirmed re-expression of many genes, which were under the repression condition in association with higher CpG-island methylation [47-56]. But nowhere the mechanism is clearly focussed. As a possibility, it has been suggested that AzadC inhibits DNA methylation only when incorporated into DNA [9, 10, 55]. We propose that the formation of a covalent bond between the Cys-thiol in the active site of the enzyme and C^6 of AzadC allows the formation of the DNMT–AzadC adduct, which can be considered stable since the presence of nitrogen at 5 position (of the AzadC ring) will not allow electron reshuffling for β-elimination (compare between Figs. 2a and 2b, Step III). This reasoning clarifies that trapping of DNMTs would prevent maintenance methylation and may keep the daughter strand in sub-normal/hypo-methylation state. The absence of $-CH_3$ groups reduces the affinity of methyl-CpG-sequence binding domain (MBDs and MeCP2) proteins to the respective promoters [7, 8, 56]. This event may cause reduction of repression potential of the respective gene promoters and enhancement of nucleosome activation for transcription. DNMT–AzadC complexes may be tethered to distinct regulatory sites via modified (AzadC in places of C) DNA sequences that drive the interactions with the nucleosome itself. Hence, sections of DNA incorporating AzadC will not be properly packaged to chromatin, even when histone deacetylases are hyperactive, unless repair enzymes operated [4-7, 55]. This assumption requires that the DNMT itself avidly binds to unusual structures formed in AzadC damaged genes that were to be packed into heterochromatin. In this model, transition state analogs formed in these unusual structures would prevent the enzyme DNMT from completing its catalytic cycle (Fig. 2b). Thus, once trapped, the DNMTs must not be able to progress further and surely target appropriate repair, recombination, or condensation complexes to the CpG-sites in the DNA [6, 7]. Transcriptional activation within a permissive domain frequently correlates with additional, targeted demethylation of DNA at promoter nucleosomes [22-26]; however, there are notable exceptions [57-59]. The most intriguing question is how transcription machinery binds and rolls if AzadC entraps DNMTs. An attractive solution may be following the path similar to NER/BER [7]. Whereas tethering of DNMTs to defined sites in the DNA via AzadC explains local non-methylation, it is less obvious how the methylation of large domains is prevented during replication or demethylation of large domains is achieved. Possible mechanisms include the recruitment of histone acetyltransferases (HATs) to distinct "entry sites" from which they "spread" throughout a domain [7, 29, 58-61], possibly by attachment to a tracking protein associated with RNA polymerase II transcription complex [7, 13, 62]. Alternatively, the residence of a particular chromosomal domain within a demethylation-competent nuclear compartment may ensure relatively uniform modification. If transcription activation complex itself were to generate high-affinity binding sites for demethylase, schemes for explaining propagation could be envisaged.

Mammalian DNMTs in cell cycle and other biological functions. DNA-Methylation within the CpG-dinucleotide by DNMTs is implicated in regulating transcription, maintaining genome stability, imprinting, and X-chromosome inactivation [7, 13, 19, 63]. In mammalian cells, DNA-methylation is catalyzed by two important classes of DNMTs. DNMT1 (EC 2.1.1.37) resides at the replication fork and methylates CpG-dinucleotides in the newly synthesized strand [15, 62]. DNMT1 is essential for maintaining DNA-methylation patterns in proliferating cells [19, 34, 64]. DNMT1 binds proliferating cell nuclear antigen (PCNA; 176740), an auxiliary factor for DNA-replication and repair [65]. DNMT1 binds p53, and the two proteins colocalize in the nucleus of human colon carcinoma (CCR) cell lines [66]. AzadC activates the p53/p21(Waf1)/Cip1 pathway to inhibit cell proliferation [67]. The polycomb group (PcG) protein EZH2 (601573) interacts, within the context of the polycomb repressive complexes 2 and 3 (PRC2/3), with DNMT1, DNMT3A, and DNMT3B and associates with DNMTase activity *in vivo* [68]. Binding of DNMTs to several enhancers of zeste homolog 2 (EZH2)-repressed genes depends on the presence of EZH2. EZH2 recruitment of DNMTs highlights a direct connection between

the two key epigenetic repression systems [68]. The level of mRNA encoding EZH2 significantly increased in malignant versus benign human prostate tumors [69]. Upregulation of EZH2 in prostate cancer leads to transcriptional repression of many genes, including cell-cycle-control and apoptotic genes. The HP1 proteins are sufficient to target DNMT1 activity *in vivo*, and HP1-dependent repressions require DNMT1 [70]. DNMT1 and DNMT3a expressed in transgenic *Drosophila melanogaster* cooperate to establish and maintain methylation patterns. Genomic DNA methylation impairs the viability of transgenic flies, suggesting that cytosine methylation has functional consequences for *Drosophila* development. The expression of DNMT3a but not DNMT1 caused developmental defects in *Drosophila*, with the majority dying in the pupal stage. Tissue-specific expression of DNMT3a in the *Drosophila* eye resulted in small or absent eyes [71].

Two members of the second class of DNMTs (DNMT3A and DNMT3B) are required for *de novo* methylation during embryonic development (for recent reviews see [7, 19]). DNMT3L cooperates with the DNMT3 family to establish maternal imprints in mice [19]. For *de novo* methylation of nucleosomal DNA, the mammalian DNMT1 cooperates with DNMT3A [16]. Overexpression of DNMTs and hypermethylation of CpG-islands at the regulatory regions of certain genes in cells, including cancers, are maintained by coordinate action of DNMT1 and DNMT3B [14, 72], DNMT1, and histone deacetylase activity [60, 73]. Global hypomethylation [5, 74-77], as well as site selective demethylation [7, 19, 24, 48-50, 59], are now well known to participate in regulation of transcription in close association with crucial cellular events [7, 25, 26, 59, 64, 78-80]. Mutation in *DNMT3B* gene is associated with immunodeficiency-chromosome instability-facial anomalies (ICF-syndrome) [19, 81]. The *ATRX* gene, which encodes a chromatin-remodeling protein of the SNF2 family and contains a characteristic ATPase/helicase domain, also contains a cysteine-rich region similar to domains present in the DNMT3B family. Mutations in this X-linked gene cause mental retardation, urogenital abnormalities, and α-thalassemia caused by down regulation of the α-globin genes [22, 82].

Conclusion and perspective. Here we have described the mechanisms of methylation of DNA that are well established [19, 64, 83, 84]. However, the enzymes either for direct demethylation of intact DNA or the glycosylase meditated repair proteins and transcription factors induced BER mechanisms need more experimental support. DNA-methylation imposes a rigid structure to form heterochromatin, and DNA-demethylation generates a relaxed euchromatin, allowing it to participate in gene transcription.

The active mechanisms for ^{Me}C DNA demethylation, other than the excision of the -CH_3 group, actually damage the DNA. Removal the total ^{Me}C-base, total ^{Me}C-nucleotide, or the ^{Me}CpG dinucleotide is obviously active, but it must not be considered as truly chemically defined DNA-demethylation. Moreover, BER/NER reactions, in terms of cellular economy, may not account for genome-wide loss of methyl groups. If the cellular chemistry and physiology normally permits deamination of ^{Me}C-base and removal by BER/NER pathway, then why does the genome remain methylated? This rationale suggests that other factors are certainly responsible for reversible regulation of DNA methylation. New functions of DNMT1 are emerging, as mentioned elsewhere [7, 22-26], for demethylation of DNA. Understanding the functions of DNMTs in more detail will shed new light on the molecular biology of reversible DNA modifications and on a variety of pathologic states, including cancer.

5-Aza-2′-deoxycytidine, an analog of cytosine base [6], has the potential to kill tumor cells. The genotoxic stress imposed to the surrounding normal cells has questioned its therapeutic use [10, 13, 41-46]. Potent inhibitors of DNA methylation with fewer side effects and less toxicity originating from bioflavonoids, including tea catechins are emerging [85-88]. AzadC may be applied for studying the expression profile of genes that were under repression status; but, certainly, AzadC is neither a demethylating agent nor can be considered as a classical enzyme inhibitor for the DNMTs. Inhibition of methylation by any means should not be misinterpreted as demethylation. However, and certainly, AzadC is a potential destroyer/killer of the enzymes DNMTs; since, for effective recognition of the promoters by an RNA-polymerase and for proper rolling of RNA-polymerase, a cell needs to pay a price for recruiting DNA-repair teams and protein degradation machines to remove the AzadC (in DNA)–DNMT adducts (Fig. 2b). Finally, we would like to suggest that designating AzadC as a DNA-demethylating agent in molecular medicine is certainly a potential misinterpretation of chemistry and chemical terminology.

S. K. P. is supported by a fellowship from the Istituto Nazionale Biostrutture e Biosistemi (INBB), Rome, Italy. Grant sponsor: FIL 2007, University of Parma, Italy; Abo Project 2006, Venice, Italy; AICR (UK) Grant No. 06-711.

Note added in proof

Since this article was accepted for publication, the following studies have appeared: Wang, J., et al. (2009) The lysine demethylase LSD1 (KDM1) is required for maintenance of global DNA-methylation, *Nat. Genet.*, **41**, 125-129; Rai, K., et al. (2008) DNA demethylation in zebrafish involves the coupling of a deaminase, a glycosylase, and Gadd45, *Cell*, **135**, 1201-1212; Ma, D. K., et al. (2009) Neuronal activity-induced Gadd45b promotes

epigenetic DNA demethylation and adult neurogenesis, *Science*, **323**, 1074-1077; Engel, N., et al. (2009) Conserved DNA methylation in Gadd45a(−/−) mice, *Epigenetics*, **4**, in press.

Wang et al. have shown that the lysine specific demethylase LSD1, previously known to demethylate H3K4 to repress active genes, also demethylates and stabilize DNMT1 in mouse, and thus proving a mechanistic link between the histone and DNA-methylation systems. Notably, DNMT1 methylation at K1096 makes the enzyme inactive and unstable [90]. Rai et al. have provided evidence for a role of Gadd45 in base excision repair mediated demethylation of ^{Me}CpG-DNA [91]. Ma et al. have suggested that Gadd45b links neuronal circuit activity to epigenetic DNA modification, demethylation, and expression of secreted factors in mature neurons for extrinsic modulation of neurogenesis in the adult brain [92]. Engel et al. reported that neither global nor locus-specific methylation is increased in Gadd45a(−/−) mice. Thus debates on active mechanisms of ^{Me}CpG-DNA-demethylation are continuing [93].

REFERENCES

1. Hotchkiss, R. D. (1948) *J. Biol. Chem.*, **175**, 315-332.
2. Razin, A., Cedar, H., and Riggs, A. D. (1984) *DNA Methylation, Biochemistry, and Biological Significance*, Springer-Verlag, New York.
3. Adams, R. L. P., and Burdon, R H. (1985) *Molecular Biology of DNA Methylation*, Springer-Verlag, New York.
4. Jost, J.-P., and Saluz, H. P. (1993) *DNA Methylation: Molecular Biology and Biological Significance*, Birkhauser-Verlag, Berlin.
5. Jones, P. A., and Baylin, S. B. (2007) *Cell*, **128**, 683-692.
6. Christman, J. K. (2002) *Oncogene*, **21**, 5483-5491.
7. Patra, S. K., Patra, A., Rizzi, F., Ghosh, T. C., and Bettuzzi, S. (2008) *Cancer Metast. Rev.*, **27**, 315-334.
8. Klose, R. J., and Bird, A. P. (2006) *Trends Biochem. Sci.*, **31**, 89-97.
9. Santi, D. V., Garrett, C. E., and Barr, P. J. (1983) *Cell*, **33**, 9-10.
10. Wilson, V. L., and Jones, P. A. (1983) *Cell*, **32**, 239-246.
11. Klimasauskas, S., Kumar, S., Robert, R. J., and Cheng, X. (1994) *Cell*, **76**, 357-369.
12. Erlanson, D., Dhen, L., and Verdin, G. L. (1993) *J. Am. Chem. Soc.*, **115**, 12583-12584.
13. Patra, S. K., Patra, A., Zhao, H., and Dahiya, R. (2002) *Mol. Carcinog.*, **33**, 163-167.
14. Reither, S., Li, F., Gowher, H., and Jeltsch, A. (2003) *J. Mol. Biol.*, **329**, 675-684.
15. Gowher, H., Loutchanwoot, P., Vorobjeva, O., Handa, V., Jurkowska, R. Z., Jurkowski, T. P., and Jeltsch, A. (2006) *J. Mol. Biol.*, **357**, 928-941.
16. Gowher, H., Stockdale, C. J., Goyal, R., Ferreira, H., Owen-Hughes, T., and Jeltsch, A. (2005) *Biochemistry*, **44**, 9899-9904.
17. Svedruzic, Z. M., and Reich, N. O. (2005) *Biochemistry*, **44**, 9472-9485.
18. Daujotyte, D., Liutkeviciute, Z., Tamulaitis, G., and Klimasauskas, S. (2008) *Nucleic Acids Res.*, **36**, E57.
19. Goll, M. G., and Bestor, T. H. (2005) *Annu. Rev. Biochem.*, **74**, 481-514.
20. Bacolla, A., Pradhan, S., Roberts, R. J., and Wells, R. D. (1999) *J. Biol. Chem.*, **274**, 33011-33019.
21. Pfeifer, G. P., and Drahovsky, D. (1986) *FEBS Lett.*, **207**, 75-78.
22. Kress, C., Thomassin, H., and Grange, T. (2001) *FEBS Lett.*, **494**, 135-140.
23. Kress, C., Thomassin, H., and Grange, T. (2006) *Proc. Natl. Acad. Sci. USA*, **103**, 11112-11117.
24. Barreto, G., Schafer, A., Marhold, J., Stach, D., Swaminathan, S. K., Handa, V., Doderlein, G., Maltry, N., Wu, W., Lyko, F., and Niehrs, C. (2007) *Nature*, **445**, 671-675.
25. Metivier, R., Gallais, R., Tiffoche, C., le Peron, C., Jurkowska, R. Z., Carmouche, R. P., Ibberson, D., Barath, P., Demay, F., Reid, G., Benes, V., Jeltsch, A., Gannon, F., and Salbert, G. (2008) *Nature*, **452**, 45-50.
26. Kangaspeska, S., Stride, B., Metivier, R., Polycarpou-Schwarz, M., Ibberson, D., Carmouche, R. P., Benes, V., Gannon, F., and Reid, G. (2008) *Nature*, **452**, 112-115.
27. Bhattacharya, S. K., Ramchandani, S., Cervoni, N., and Szyf, M. (1999) *Nature*, **397**, 579-783.
28. Cedar, H., and Verdine, G. L. (1999) *Nature*, **397**, 568-569.
29. Cervoni, N., and Szyf, M. (2001) *J. Biol. Chem.*, **276**, 40778-40787.
30. Ng, H. H., Zhang, Y., Hendrich, B., Johnson, C. A., Turner, B. M., Erdjument-Bromage, H., Tempst, P., Reinberg, D., and Bird, A. (1999) *Nat. Genet.*, **23**, 58-61.
31. Jost, J.-P., Thiry, S., and Sigman, M. (2002) *FEBS Lett.*, **519**, 128-134.
32. Kapoor, A., Agius, F., and Zhu, J.-K. (2005) *FEBS Lett.*, **579**, 5889-5898.
33. Swisher, J. F. A., Rand, E., Cedar, H., and Pyle, A. M. (1998) *Nucleic Acids Res.*, **26**, 5573-5580.
34. Vairapandi, M., Liebermann, D. A., Hoffman, B., and Duker, N. J. (2000) *J. Cell. Biochem.*, **79**, 249-260.
35. Wu, P., Qiu, C., Sohail, A., Zhang, X., Bhagwat, A. S., and Cheng, X. (2003) *J. Biol. Chem.*, **278**, 5285-5291.
36. Jin, S.-G., Guo, C., and Pfeifer, G. P. (2008) *PLoS Genet.*, **4**, e1000013.
37. Agius, F., Kapoor, A., and Zhu, J. K. (2006) *Proc. Natl. Acad. Sci. USA*, **103**, 11796-11801.
38. Gehring, M., Huh, J. H., Hsieh, T. F., Penterman, J., Choi, Y., et al. (2006) *Cell*, **124**, 495-506.
39. Arnaud, P., and Feil, R. (2006) *Cell*, **124**, 468-470.
40. Morgan, H. D., Dean, W., Coker, H. A., Reik, W., and Petersen-Mahrt, S. K. (2004) *J. Biol. Chem.*, **279**, 52353-52360.
41. Claus, R., Almstedt, M., and Lubbert, M. (2005) *Semin. Oncol.*, **32**, 511-520.
42. Wijermans, P., Lubbert, M., Verhoef, G., Bosly, A., Ravoet, C., Andre, M., and Ferrant, A. (2000) *J. Clin. Oncol.*, **18**, 956-965.
43. Yoo, C. B., and Jones, P. A. (2006) *Nat. Rev. Drug Discov.*, **5**, 37-50.
44. Lavelle, D., Vaitkus, K., Hankewych, M., Singh, M., and DeSimone, J. (2006) *Exp. Hematol.*, **34**, 339-347.
45. Lavelle, D., Saunthararajah, Y., and Desimone, J. (2008) *Blood*, **111**, 2485.

46. DeSimone, J. (2004) *Semin. Hematol.*, **41**, 1-3.
47. Hellebrekers, D. M., Melotte, V., Vire, E., Langenkamp, E., Molema, G., Fuks, F., Herman, J. G., van Criekinge, W., Griffioen, A. W., and van Engeland, M. (2007) *Cancer Res.*, **67**, 4138-4148.
48. Sato, N., Maehara, N., Su, G. H., and Goggins, M. (2003) *J. Natl. Cancer Inst.*, **95**, 327-330.
49. Pulukuri, S. M., Estes, N., Patel, J., and Rao, J. S. (2007) *Cancer Res.*, **67**, 930-939.
50. Li, L. C., Okino, S. T., and Dahiya, R. (2004) *Biochim. Biophys. Acta*, **1704**, 87-102.
51. Lucarelli, M., Fuso, A., Strom, R., and Scarpa, S. (2001) *J. Biol. Chem.*, **276**, 7500-7506.
52. El Kharroubi, A., Piras, G., and Stewart, C. L. (2001) *J. Biol. Chem.*, **276**, 8674-8680.
53. Zhu, X., Leav, I., Leung, Y. K., Wu, M., Liu, Q., Gao, Y., McNeal, J. E., and Ho, S. M. (2004) *Am. J. Pathol.*, **164**, 2003-2012.
54. Santoso, B., Ortiz, B. D., and Winoto, A. (2000) *J. Biol. Chem.*, **275**, 1952-1858.
55. Ferguson, A. T., Vertino, P. M., Spitzner, J. R., Baylin, S. B., Muller, M. T., and Davidson, N. E. (1997) *J. Biol. Chem.*, **272**, 32260-32266.
56. Patra, S. K., Patra, A., Zhao, H., Carroll, P., and Dahiya, R. (2003) *Biochem. Biophys. Res. Commun.*, **302**, 759-766.
57. Santos, F., Hendrich, B., Reik, W., and Dean, W. (2002) *Dev. Biol.*, **241**, 172-182.
58. Rodriguez, J., Frigola, J., Vendrell, E., Risques, R. A., Fraga, M. F., Morales, C., Moreno, V., Esteller, M., Capella, G., Ribas, M., and Peinado, M. A. (2006) *Cancer Res.*, **66**, 8462-9468.
59. Bruniquel, D., and Schwartz, R. H. (2003) *Nat. Immunol.*, **4**, 235-240.
60. Patra, S. K., Patra, A., and Dahiya, R. (2001) *Biochem. Biophys. Res. Commun.*, **287**, 705-713.
61. Dong, E., Guidotti, A., Grayson, D. R., and Costa, E. (2007) *Proc. Natl. Acad. Sci. USA*, **104**, 4676-4681.
62. Wittschieben, B. O., Otero, G., de Bizemont, T., Fellows, J., Erdjument-Bromage, H., Ohba, R., Li, Y., Allis, C. D., Tempst, P., and Svejstrup, J. Q. (1999) *Mol. Cell*, **4**, 123-128.
63. Pfeifer, G. P., Steigerwald, S. D., Hansen, R. S., Gartler, S. M., and Riggs, A. D. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 8252-8256.
64. Patra, S. K. (2008) *Exp. Cell. Res.*, **314**, 1193-1201.
65. Chuang, L. S.-H., Ian, H.-I., Koh, T.-W., Ng, H.-H., Xu, G., and Li, B. F. L. (1997) *Science*, **277**, 1996-2000.
66. Esteve, P.-O., Chin, H. G., and Pradhan, S. (2005) *Proc. Natl. Acad. Sci. USA*, **102**, 1000-1005.
67. Zhu, W. G., Hileman, T., Ke, Y., Wang, P., Lu, S., Duan, W., Dai, Z., Tong, T., Villalona-Calero, M. A., Plass, C., and Otterson, G. A. (2004) *J. Biol. Chem.*, **279**, 15161-15166.
68. Vire, E., Brenner, C., Deplus, R., Blanchon, L., Fraga, M., Didelot, C., Morey, L., Eynde, V. A., Bernard, D., Vanderwinden, J.-M., Bollen, M., Esteller, M., di Croce, L., de Launoit, Y., and Fuks, F. (2006) *Nature*, **439**, 871-874.
69. Varambally, S., Dhanasekaran, S. M., Zhou, M., Barrette, T. R., Kumar-Sinha, C., Sanda, M. G., Ghosh, D., Pienta, K. J., Sewalt, R. G., Otte, A. P., Rubin, M. A., and Chinnaiyan, A. M. (2002) *Nature*, **419**, 624-629.
70. Smallwood, A., Esteve, P.-O., Pradhan, S., and Carey, M. (2007) *Genes Dev.*, **21**, 1169-1178.
71. Lyko, F., Ramsahoye, B. H., Kashevsky, H., Tudor, M., Mastrangelo, M.-A., Orr-Weaver, T. L., and Jaenisch, R. (1999) *Nat. Genet.*, **23**, 363-366.
72. Rhee, I., Bachman, K. E., Park, B. H., Jair, K.-W., Yen, R.-W. C., et al. (2002) *Nature*, **416**, 552-556.
73. Fuks, F., Burgers, W. A., Brehm, A., Hughes-Davies, L., and Kouzarides, T. (2000) *Nat. Genet.*, **24**, 88-91.
74. Ehrlich, M. **(**2006) *Curr. Top. Microbiol. Immunol.*, **310**, 251-274.
75. Feinberg, A. P., and Vogelstein, B. (1983) *Biochem. Biophys. Res. Commun.*, **111**, 47-54.
76. Bedford, M. K., and van Helden, P. D. (1987) *Cancer Res.*, **47**, 5274-5276.
77. Goelz, S. E., Vogelstein, B., Hamilton, S. R., and Feinberg, A. P. (1985) *Science*, **228**, 187-190.
78. Lopez-Serra, L., Ballestar, E., Fraga, M. F., Alaminos, M., Setien, F., and Esteller, M. (2006) *Cancer Res.*, **66**, 8342-8346.
79. Jin, S. G., Jiang, C. L., Rauch, T., Li, H., and Pfeifer, G. P. (2005) *J. Biol. Chem.*, **280**, 12700-12709.
80. Zhang, J., Liu, L., and Pfeifer, G. P. (2004) *Oncogene*, **23**, 2241-2249.
81. Xu, G. L., Bestor, T. H., Bourc'his, D., Hiseh, C. L., Tommerup, N., Bugge, M., Hulten, M., Qu, X., Russo, J. J., and Viegas-Pequignot, E. (1999) *Nature*, **402**, 187-191.
82. Gibbons, R. J., McDwell, T. L., Raman, S., O'Rourke, D. M., Garrick, D., Ayyub, H., and Higgs, D. R. (2000) *Nat. Genet.*, **24**, 368-371.
83. Patra, S. K., and Bettuzzi, S. (2007) *Oncol. Rep.*, **17**, 1279-1290.
84. Patra, S. K. (2008) *Biochim. Biophys. Acta*, **1785**, 182-206.
85. Fang, M. Z., Wang, Y., Ai, N., Hou, Z., Sun, Y., Lu, H., Welsh, W., and Yang, C. S. (2003) *Cancer Res.*, **63**, 7563-7570.
86. Lee, W. J., Shim, J.-Y., and Zhu, B. T. (2005) *Mol. Pharmacol.*, **68**, 1018-1030.
87. Patra, S. K., and Szyf, M. (2008) *FEBS J.*, **275**, 5217-5235.
88. Fang, M., Chen, D., and Yang, C. S. (2007) *J. Nutr.*, **137**, 223S-228S.
89. Reither, S., Li, F., Gowher, H., and Jeltsch, A. (2003) *J. Mol. Biol.*, **329**, 675-684.
90. Wang, J., Hevi, S., Kurash, J. K., Lei, H., Gay, F., Bajko, J., Su, H., Sun, W., Chang, H., Xu, G., Gaudet, F., Li, E., and Chen, T. (2009) *Nat. Genet.*, **41**, 125-129.
91. Rai, K., Huggins, I. J., James, S. R., Karpf, A. R., Jones, D. A., and Cairns, B. R. (2008) *Cell*, **135**, 1201-1212.
92. Ma, D. K., Jang, M. H., Guo, J. U., Kitabatake, Y., Chang, M. L., Pow-Anpongkul, N., Flavell, R. A., Lu, B., Ming, G. L., and Song, H. (2009) *Science*, **323**, 1074-1077.
93. Engel, N., Tront, J. S., Erinle, T., Nguyen, N., Latham, K. E., Sapienza, C., Hoffman, B., and Liebermann, D. A. (2009) *Epigenetics*, **4**, in press.