In vitro enzymatic methylation of DNA substituted by *N*-2-aminofluorene

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Both the initial velocity and the overall methylation of DNA substituted by aminofluorene, by a rat liver DNA(cytosine-5-)-methyltransferase, are increased as compared to native DNA. The K_m and V_{max} of the modified DNA for the enzyme increase as a function of the extent of modification. The carcinogen may induce a secondary structure favouring the 'walking' of the enzyme along the DNA. The hypermethylation caused by this carcinogen could have a significance in gene activity, cellular differentiation and cancer induction.

DNA-methylase *DNA(cytosine-5-)-methyltransferase* *Acetylaminofluorene* *Aminofluorene* *5-Methylcytosine* *Carcinogenesis*

1. INTRODUCTION

It is already well established that ultimate metabolites of chemical carcinogens bind covalently to DNA in vivo and in vitro. One of the most studied chemical carcinogens is *N*-2-acetylaminofluorene (2-AAF). When administered to rats the following adducts are formed: 70% of non-acetylated C8 guanine adducts [*N*-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF)], 30% of acetylated adducts [24% of *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene (dG-C8-AAF)] and 2% of 3-(deoxyguanosin-N^2-yl)-2-acetylaminofluorene (dG-N^2-AAF) and 4% of non-identified guanine adducts [1–4]. The conformational changes of DNA due to (dG-C8-AAF) were extensively studied [5–8]. DNA modified in vitro by N-OH-AF gives only the deacetylated adduct dG-C8-AF [9,10].

We found that the in vitro enzymatic methylation of DNA-AAF (DNA modified by *N*-AcO-AAF) was decreased as compared to native unmodified DNA [11,12]. However, this modified DNA contained only the acetylated adducts. Therefore, it was interesting to determine whether the deacetylated adduct has an influence on its methylation. Thus, we substituted DNA by aminofluorene (DNA-AF) and utilized it as substrate for enzymatic methylation by a rat liver DNA(cytosine-5-)-methyltransferase.

2. MATERIALS AND METHODS

Chicken erythrocyte DNA was a gift from Professor Daune and prepared as in [13]. Heat denatured DNA in 2×10^{-3} M citrate (pH 7) buffer, was prepared by incubation in a boiling water bath for 3 min followed by rapid chilling in ice. *S*-Adenosyl-L-[*methyl*-^{3}H]methionine (SAM) (spec. act. 15–20 Ci/mmol) was from Amersham (England); non-radioactive SAM was from Boehringer (Mannheim). N-OH-[*ring*-^{3}H]AF was synthesized as in [14].

All other reagents were of analytical grade. The reaction of native DNA with N-OH-AF was done in 2×10^{-3} M Na citrate buffer (pH 5) at 37°C. The DNA-AF was subsequently purified by a phenol Sevag extraction followed by a Sevag extraction and two ethanol precipitation steps. The

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extent of modification was obtained as in [15] and ranged from 0.26 to 1.3% of modified bases. Rat liver methyltransferase was obtained from nuclei as in [12].

The methylation assay was as described [16] and the DNA was recovered quantitatively [16].

3. RESULTS

3.1. *Kinetics of enzymatic methylation of double-stranded DNA-AF*

Fig.1 shows the kinetics of enzymatic methylation of DNA-AF. Both the initial velocity of methylation and the overall methylation plateau are increased.

Fig.2 shows that there is a linear relationship between the degree of modification of the DNA and the initial velocity of the methylation. We determined the K_m and V_{max} of the DNA(cytosine-5)-methyltransferase for DNA-AF plotting $1/S$ *vs* $1/v$. Fig.3 shows that both the apparent K_m of the enzyme for the modified DNA and the V_{max} increase with the extent of modification.

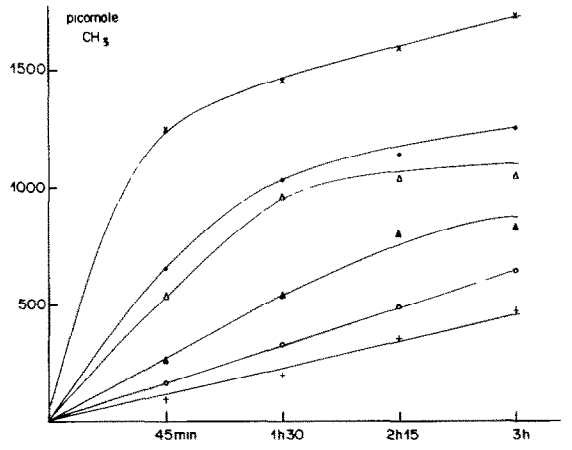

Fig.1. Kinetics of enzymatic methylation of: (+—+) native DNA, (○—○) 0.26% of modified bases, (▲—▲) 0.5% of modified bases, (△—△) 0.63% of modified bases, (●—●) 0.81% of modified bases, (×—×) 1.08% of modified bases. Results are in pmol CH_3 fixed on 20 μg DNA.

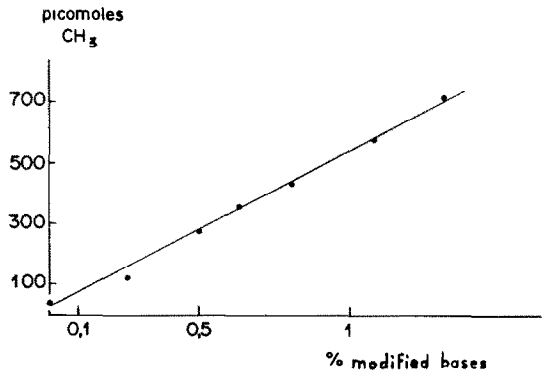

Fig.2. Initial velocity of the enzymatic methylation of 20 μg DNA-AF as a function of modified bases after 30 min incubation.

3.2. *Enzymatic methylation of heat-denatured DNA-AF*

We showed [12] that denatured, non-modified DNA is less methylated than the native form and that its K_m for the enzyme is increased. We also showed that the methylation of denatured DNA-AAF increases as a function of the level of substitution (unpublished) and the K_m is the same for unmodified and modified denatured DNA-AAF [12].

When heat-denatured DNA-AF was used as a substrate for the enzyme, its methylation (fig.4) and its K_m (fig.5) increased with the level of the modification. This hypermethylation is due to an increase of maximal velocity of the enzymatic methylation (fig.5).

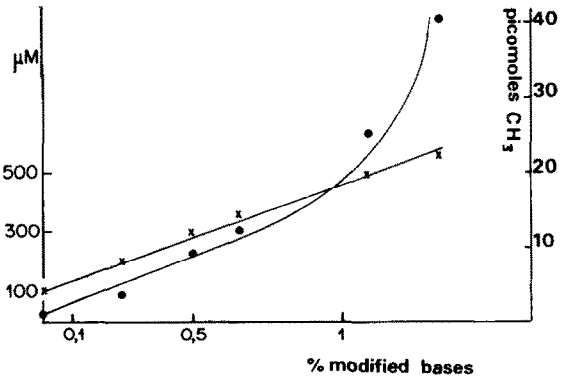

Fig.3. K_m (x) and V_{max} (●—●) of double-stranded DNA-AF as a function of modified bases.

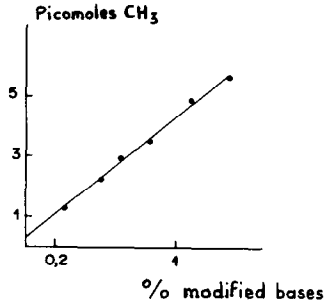

Fig.4. Initial velocity of the enzymatic methylation of 5 μg heat-denatured DNA-AF as a function of modified bases.

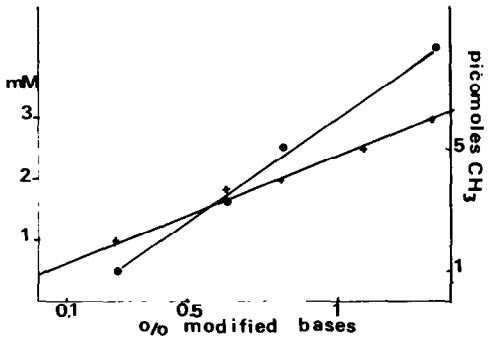

Fig.5. K_m (+) and V_{max} (●—●) of single-stranded DNA-AF as a function of modified bases.

4. DISCUSSION

Here, we analyzed the in vitro enzymatic cytosine methylation of single- and double-stranded DNA substituted by aminofluorene (DNA-AF). We observed a hypermethylation of DNA-AF which contrasts with the hypomethylation observed previously with DNA-AAF [11,12] and DNA treated with methylnitrosourea [17], but which is similar to the data obtained with DNA substituted with 4-aminoquinoline-1-oxide (NQO) [18].

For double-stranded DNA the initial velocity of methylation is a linear function of the extent of modification (fig.2). Both the apparent K_m and the V_{max} increase with the level of modification. Thus, the hypermethylation is probably due to an increase in the maximal velocity of the enzymatic methylation.

In vitro studies showed a major difference in the conformation between DNA-AAF and DNA-AF [8,9]: in the case of double-stranded DNA-AAF, the AAF moiety is essentially bound to the C(8) position of guanine (80%). This binding destabilizes the double helix of the DNA. This phenomenon has been named the insertion-denaturation model [5,6,8]. In this model, the AAF moiety stacks with the bases of the DNA molecule, while guanine is outside [5,6]. In the case of DNA-AF, the AF is also bound to C(8) of guanine, but there is only a minor conformational change and the overall structure is that found in B-DNA with the guanine residue located inside the helix (outside binding model [8]). This conformation seems to be stabilized by the formation of a hydrogen bond between the amino group of AF and the 5′-oxygen of the sugar [9,19]. This interaction also exists in denatured DNA-AF. Furthermore, the -AF adduct, in contrast to the -AAF adduct, does not induce the *anti*⟶*syn* conformational change.

This major conformational difference between -AAF and -AF adducts has an effect on their respective SOS-inducing properties [20]. It has been suggested that -AAF damages block the replication machinery, while -AF adducts have almost no effect on the replication machinery [20,21]. Furthermore, recent in vitro studies have shown that both -AAF- and -AF-modified plasmid DNA are substrate for the *Escherichia coli* uvr ABC endonuclease [21]. However, in vivo the deacetylated adducts are much less toxic than the -AAF adducts, i.e., the survival of the AAF-substituted plasmid DNA is strongly dependent on functional uvr excision-repair pathway [21]. It is proposed that the -AF-substituted plasmid DNA survives equally well in wild type and in a *uvr A* strain because the -AF adduct does not efficiently block the replication machinery [21].

In mammalian genomes, the major methylation of dC occurs in the dinucleotide dCpdG [22]. The formation of adducts on dG has a high probability of altering a potential methylation site. We showed [11,12] that in the case of DNA-AAF, the conformational change of the double helix favors the recognition or binding of the enzyme to the substrate as the affinity of the enzyme for DNA-AAF increases with the extent of the modification, but the AAF residues irreversibly block DNA

methylase. Here, we studied the methylation of DNA-AF and found that it is better methylated than the non-modified DNA, and logically therefore than the DNA-AAF. This comes from an increased V_{max} and not from a lower apparent K_m of DNA-AF for the methylase. This result resembles the one we obtained with DNA substituted with NQO [18]. However, with single-stranded DNA-AF, the V_{max} also increases with the modification.

However, there seems to be a discrepancy between our results of DNA-AF methylation and those observed recently [23] on the methylation of poly(dC-dG)-AF. These authors found that the presence of the -AF adduct severely impairs the methylation of the polynucleotide with a diminished V_{max}. Thus, one cannot compare the results of enzymatic methylation of DNA to that of poly(dC-dG). We already made this observation when we compared the methylation of poly(dC-dG)-NQO to that of DNA-NQO [18].

Authors in [23] suggested that the impairment of methylation is not dependent on helix conformation but on hydrophobic interactions of the fluorene ring with the enzyme. In these conditions one should have similar results with DNA and poly(dC-dG), both substituted with AF, since in both cases the -AF molecule is accessible to the enzyme. As this is not the case we favor the hypothesis that the -AF, and also the -NQO adducts, slightly change the secondary structure of the DNA in a way facilitating the mechanism of action of the enzyme which, as demonstrated [24,25], walks along the double helix. Note that both -AF and -NQO adducts are deacetylated residues.

Our results with DNA-AAF (hypomethylation) and with DNA-AF (hypermethylation) are interesting in the context of recent observations [26] showing that the treatment of murine cells with *N*-acetoxy-*N*-2-acetylaminofluorene, which in situ leads both to AAF and AF adducts in DNA [27,28], yielded sets of cells, one with a higher and another with a lower extent of enzymatic methylation of DNA.

In the field of studies of mechanisms of gene expression, much recent work using the methods of genetic engineering has suggested the hypothesis of a role of DNA methylation in the control of gene activity, with undermethylation being associated with gene expression (reviews [22,29–33]). Recently, authors in [34,35] have shown that some human genes were hypomethylated in carcinomas compared with adjacent normal tissues. Hypomethylation of these genes was even greater in a liver metastasis. In the same way, authors in [36] studying teratocarcinoma and embryonal carcinoma cells, suggested that methylation has a role in controlling gene expression, but that additional mechanisms are necessary to initiate gene expression.

However, not only hypomethylation, leading to gene expression, could be related to the initiation of carcinogenesis. Hypermethylation too, could be a critical event in this initiation as the inactivation of genes can induce retinoblastomas [37–39] or kidney tumors [40–43]. Finally, a hypermethylation can also lead to gene expression, as recently shown [44] for the H-2K gene.

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