Enzymatic methylation of DNA and poly(dG–dC)·poly(dG–dC) modified by 4-acetoxyaminoquinoline-1-oxide, the ultimate carcinogen of 4-nitroquinoline-1-oxide

A. Pfohl-Leszkowicz, S. Galiegue-Zouitina+, B. Bailleul+, M.H. Loucheux-Lefebvre+ and G. Dirheimer

Institut de Biologie Moléculaire et Cellulaire du CNRS et Faculté de Pharmacie, Université Louis Pasteur, 15 rue René Descartes, 67084 Strasbourg Cedex and *+Institut de Recherches sur le Cancer de Lille et U124 INSERM BP 311, 59020 Lille Cedex, France*

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Both the initial velocity and the overall methylation of Ac-4HAQO modified DNA by a calf brain DNA (cytosine-5-)-methyltransferase are increased as compared to native DNA. The affinity of the modified DNA for the enzyme decreases as a function of the extent of the modification. Heat-denatured, single-stranded DNA shows exactly the opposite results: the more it is modified, the less it is methylated. The poly(dG–dC)·poly(dG–dC) modified by 4NQO is as well methylated as the non-modified one. The carcinogen may induce a tertiary structure favouring the 'walking' of the enzyme along the DNA. The hypermethylation caused by this carcinogen could have a significance in gene activity and cellular differentiation.

DNA methylation *4-Nitroquinoline-1-oxide* *DNA(cytosine-5-)-methyltransferase* *Brain*
Poly(dG–dC)·poly(dG–dC) *Chemical carcinogen*

1. INTRODUCTION

4-Nitroquinoline-1-oxide (4NQO) is a potent carcinogen (reviews [1,2]). The *0,0'*-diacetyl-4-hydroxyaminoquinoline-1-oxide (diAc-4HAQO) reacts covalently with DNA [3,4]. The monoacetyl derivative, 4-acetoxyaminoquinoline-1-oxide (Ac-4HAQO), constitutes a very attractive model of ultimate carcinogen for the in vitro study of carcinogenesis by 4NQO [5]. The reaction between the purine nucleosides and Ac-4HAQO yields 5 adducts, of which only one is well characterized: *N*-(deoxyguanosine-C^8-yl) 4-aminoquinoline-1-oxide [6]. This adduct is recovered from the substituted DNA in vivo [6] and in vitro [5]. The covalent binding of the quinoline moiety to DNA induces a destabilization of the double helix [4,5]. A destabilization is also observed for AAAF-modified DNA [7]. We found that the enzymatic methylation of this DNA–AAF was decreased [8] as compared to native unmodified DNA. The same is true for DNA alkylated by methylnitrosourea [9–11]. Therefore, it was interesting to determine whether the modification of DNA by Ac-4HAQO has an influence on its enzymatic methylation.

Thus, we modified DNA and poly(dG–dC)·po-

Abbreviations: 4NQO, 4-nitroquinoline-1-oxide; 4HAQO, 4-hydroxyaminoquinoline-1-oxide; SAM, *S*-adenosyl-L-methionine; Ac-4HAQO, 4-acetoxyaminoquinoline-1-oxide; diAc-4HAQO; *0,0'*-diacetyl-4-hydroxyaminoquinoline-1-oxide; AAF, 2-acetylaminofluorene; AAAF, *N*-acetoxyacetylaminofluorene; DNA–AAF, DNA modified by AAAF; DNA–NQO, DNA modified by Ac-4HAQO; poly(dG–dC)–NQO, poly(dG–dC)·poly(dG–dC) modified by Ac-4HAQO

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ly(dG–dC) by Ac-4HAQO and utilized these modified products as substrates for enzymatic methylation by a calf brain DNA(cytosine-5-) methyltransferase.

2. MATERIALS AND METHODS

Chicken erythrocyte DNA was a gift from Professor Daune and prepared as in [12]. Poly(dG–dC)·poly(dG–dC) was purchased from PL Biochemicals. Heat-denatured DNA 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. 20 Ci/mmol) was from the Commissariat à l'Energie Atomique (CEA, Saclay); non-radioactive SAM was from Boehringer (Mannheim); 4NQO was obtained from Fluka (Buchs); Ac-4HAQO was prepared as in [6]. All other reagents were of analytical grade.

The reaction of native and denatured DNA with Ac-4HAQO and subsequent purification of modified DNA were described in [5]; 0.3–2% of modified bases were present. The reaction of poly(dG–dC)·poly(dG–dC) with Ac-4HAQO was done in the same way as for DNA. The two samples prepared correspond to 2.7% and 3.7% of modified bases.

2.1. *Methylation of chicken erythrocyte DNA by calf brain DNA(cytosine-5-)-methyltransferase*

The standard assay mixture contained in 80 μl 50 mM Tris–HCl (pH 7.6) and 0.5 mM DTE, 16 μg DNA, 1.75 μg pancreatic RNase, 50 μg enzyme preparation, 1 μCi [^{3}H]SAM and 0.5 nmol SAM. The mixture was incubated at 37°C for various times after which 20 μl proteinase K (1 mg/ml) in a buffer containing: Tris–HCl 10 mM, NaCl 10 mM, SDS 0.5% and EDTA 10 mM (pH 8) were added and the tubes were incubated for 10 min at 60°C. The DNA was collected as in [13]. This method permits a quantitative recovery of the methylated DNA [13]. Calf brain DNA methylase was obtained from nuclei as in [8].

3. RESULTS

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

Fig.1. shows the kinetics of enzymatic methylation of DNA. The reaction reaches a plateau after about 6 h (not shown). For DNA–NQO both the initial velocity of methylation and the overall methylation plateau are increased.

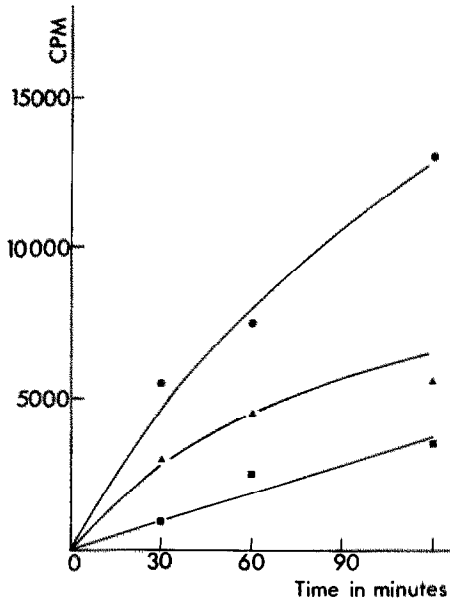

Fig.1. Kinetics of enzymatic methylation of DNA–NQO: (■—■) native DNA; (▲—▲) 0.5% of modified bases; (●—●) 2% of modified bases.

We calculated the pmol CH_3 incorporated into 1 mg DNA for 30 min. Fig.2 shows a linear variation between the degree of modification and the initial velocity of the methylation.

3.2. *Lineweaver–Burk plot*

The initial methylation reaction velocities of variable amounts of modified DNA were measured. Plotting $1/v$ *vs* $1/S$ gave the following K_m expressed in phosphate residues: 150 μM, 400 μM, 770 μM, 1430 μM and 2500 μM, respectively for 0%, 0.3%, 0.5%, 1.5% and 2% of modified bases. Thus, the affinity of the enzyme for the modified DNA decreases with the extent of modification. This decrease is linear.

3.3. *Enzymatic methylation of heat-denatured DNA–NQO*

We had shown that denatured, unmodified DNA was less methylated than the native form and that its K_m for the enzyme was increased [8]. When heat-denatured DNA–NQO was used as a

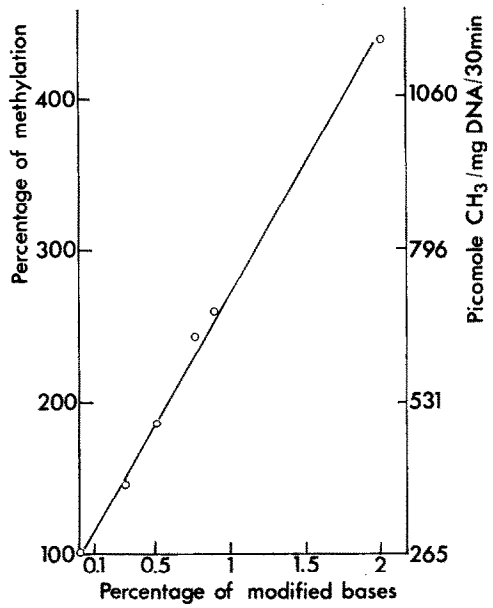

Fig.2. Initial velocity of the enzymatic methylation of DNA–NQO as a function of modified bases.

substrate for the enzyme, this methylation further decreased as a function of the level of substitution (table 1). Thus, we obtained the inverse result of that with double-stranded DNA–NQO. On the other hand, the K_m is the same for unmodified and modified, denatured DNA (400 μM).

3.4. *Enzymatic methylation of poly(dG–dC) –NQO*

Finally, we tested the methylation of modified poly(dG–dC)·poly(dG–dC). Table 2 shows that its modification does not affect the enzymatic methylation.

4. DISCUSSION

Here, we have analyzed the in vitro enzymatic methylation of DNA and poly(dG–dC)·poly(dG–dC) modified by the ultimate carcinogen of 4-nitroquinoline-1-oxide.

We have observed a hypermethylation of DNA–NQO which contrasts with the hypomethylation observed with the DNA–AAF [8] and DNA alkylated with methylnitrosourea [9,10]. For double-stranded DNA the initial velocity of methylation is a linear function of the extent of modification (fig.2). However, the affinity of the DNA–NQO is decreased, the K_m increasing with the level of modification. The hypermethylation is in fact due to an increase in the maximal velocity of the enzymatic methylation, which is more rapid than the decrease in affinity of the modified DNA for the DNA methylase.

No difference was found between the methylation of poly(dG–dC)·poly(dG–dC) treated or not with Ac-4HAQO. One could assume that the substitution modifies the tertiary structure in a way favouring the 'walking' of the methylase along the DNA strands [14]. Against this hypothesis is the result obtained with poly(dG–dC)·poly(dG–dC). However, as the structure of this synthetic polynucleotide is very tight, the substitution of only 3.7% of the bases, which is the highest modification performed, might have a lower effect on it than on DNA. Concerning the methylation, the results obtained with DNA–NQO are just the opposite of those obtained with DNA–AAF [8]. Since it could be expected that a relationship exists between the type of structural modification observed on the substituted DNA by these two carcinogens and their methylation, let us establish a comparison between DNA–NQO and DNA–AAF from a conformational viewpoint. For both modified DNA, a thermal destabilization was

Table 1

Enzymatic methylation of heat- denatured DNA modified by 4NQO after 1 h of incubation

	Non-modified control	Percentage of modified bases		
		0.55	1.5	2.0
cpm	1845	1420	1300	1000
% Methylation	100	77	70	54

Table 2

Enzymatic methylation of 4NQO modified poly(dG–dC)·poly(dG–dC)

Time (h)	Percentages of modified bases		
	0	2.7	3.7
1	3730[a]	3970	3740
2	7590	7250	–
3	13650	13150	13365

[a]Values are expressed in cpm

observed and the melting temperature depression had about the same value for the two samples: −1.2°C [5] and −1.13°C [7] per 1% of modified bases for DNA–NQO and DNA–AAF, respectively. As expected these two modified DNA are sensitive to digestion by S_1 endonuclease which is specific of single-stranded DNA, but the enzyme differently recognizes the lesions by NQO and AAF [15]. In addition it was established that poly(dG–dC)–AAF is almost completely resistant to S_1 endonuclease digestion [17] and this result provides evidence for the lack of denatured regions and for a particular restructuring (Z-form) of the modified polymer [16] essentially due to the guanyl C-8 arylamination of poly(dG–dC). Poly(dG–dC) which in this case represents 80% of the total modification. On the contrary, poly(dG–dC)–NQO is sensitive to the S_1 endonuclease and this result has to be related to the circular dichroism study which shows that poly(dG–dC)–NQO does not exhibit an induction of the Z-form [18]. Since in this case the C-8 guanine adduct represents only about 30% of the total polymer modification [19] we can think that the role played by the adducts other than the C-8 guanine adduct in conformational modification is more important in the case of 4NQO. The characterization of the other NQO adducts is in progress in our laboratory as well as their influence on the conformation of DNA–NQO. Finally, with single-stranded DNA, the substitution with 4NQO inhibits methylation as was already found with AAF [8]. The result showing that the K_m of the enzyme is the same for single stranded DNA, modified or not, must confirm the conclusion that the enzyme is essentially sensitive to the type of the double helix.

Methylation of cytosine is the only replicational modification detected so far in DNA of higher eucaryotes. The degree of methylation of cytosine has been shown to be important in the control of gene activity, hypomethylation being associated with gene expression (reviews [20–24]). If the hypermethylation we observed in vitro also occurs in vivo, this could play an inhibitory role in gene expression.

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