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ENZYMATIC METHYLATION OF CHEMICALLY ALKYLATED DNA AND POLY(dG-dC).POLY(dG-dC) IN B AND Z FORMS

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The enzymatic methylation of chemically alkylated DNA and of poly(dG-dC).poly(dG-dC) by beef brain DNA(cytosine-5-)-methyltransferase have been tested. The alkylation by dimethylsulfate, which yields mostly 7 methylguanine (m^7G) and 3 methyladenine (m^3A) do not affect the enzymatic methylation. The dimethylsulfate alkylated poly(dG-dC).poly(dG-dC) converted into the Z-form in the presence of $MgCl_2$, is just as well methylated as the native or the alkylated polynucleotide in the B-form. The alkylation of DNA or of poly(dG-dC).poly(dG-dC) by methylnitrosourea yields, in addition to the above base modifications described for dimethylsulfate, methylphosphotriesters and O^6-methylguanine. The enzymatic methylation of these substrates modified by methylnitrosourea is decreased. This decrease is proportional to the extent of the chemical alkylation of the substrate.

Acetylaminofluorene substituted DNA (DNA-AAF) is less methylated than unmodified DNA by DNA(cytosine-5-)-methyltransferase from purified nuclei (1,2). The inhibition of the methylation is proportional to the modification of the DNA. Moreover, DNA-AAF inhibits irreversibly the methylation of native DNA by blocking the enzyme at the AAF-substituted sites (2). It seemed, therefore, of interest to investigate whether other substitutions in the DNA could also hinder its enzymatic methylation. The chemical methylation of DNA by simple alkylating agents is one of these modifications. It leads, however, to the production of different types of adducts depending on the methylating agent, the most important being 7-methylguanine (m^7G), O^6-methylguanine (m^6G), 3-methyladenine (m^3A) (3,4). The relative proportions of these modifications also vary with the methylating agent, the N^7 position of the guanine being by

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far the main target of the alkylating agents. To distinguish the relative effects of these different alkylations on the enzymatic methylation of DNA, we used both poly(dG-dC).poly(dG-dC) and DNA. The first one is essentially methylated at the N^7 position of guanine by dimethylsulfate, and at the N^7-guanine and O^6-guanine positions by methylnitrosourea, whereas DNA is methylated at the N^7-guanine and N^3-adenine positions by dimethylsulfate and essentially at the N^7-guanine, O^6-guanine and N^3-adenine positions by methylnitrosourea. Furthermore, the phosphates are methylated by methylnitrosourea (5).

When treated under alkaline conditions, m^7G is converted into an open ring form (rom^7G) which blocks DNA replication (6). By heating under neutral conditions, m^7G is excised yielding apurinic sites (3). Apurinic sites also block DNA replication (6-7). We have tested the enzymatic methylation of DNA containing such modifications. Finally, poly(dG-dC).poly(dG-dC) can be converted into a left- handed conformer, similar to the Z form of DNA (8), whose methylation also seemed of interest.

MATERIALS AND METHODS

Materials

Chicken erythrocyte DNA was a gift from Professor Daune and was prepared as described by Kay et al. (9). Poly(dG-dC).poly(dG-dC) was purchased from P.L. Biochemicals. S-Adenosyl-L-(methyl-^{3}H)-methionine (SAM), with a specific radioactivity of 15 Ci/mMole, was from Amersham International (England), non-radioactive SAM was from Boehringer-Mannheim. Dimethylsulfate (DMS) was from Aldrich. Methylnitrosourea (MNU) was a gift from Professor Kleihues, Freiburg (FRG). The scintillator Ready Solv MP was from Beckman Instruments (France). All other reagents were of analytical grade.

Methods

Chemical alkylation

Both poly(dG-dC).poly(dG-dC) and DNA were alkylated with DMS as described for DNA (10) briefly : 1 mg of the polynucleotide or of DNA in 1.2 ml 0.3 M sodium cacodylate pH 7.5, was treated with a 200 mM DMS solution (final concentration 25-100 mM) for 1 h at 37°C.

The solution was desalted by filtration through Sephadex G25. The reaction mixture was filtered through a disposable syringe previously, prepared as follows : swollen Sephadex G25 was poured in a glasswool plugged disposable syringe and centrifuged for 5 min at 1,000 rpm in tubes adapted to both centrifuge and syringe (the outlet should not drop into the filtrate). Only the water in the dead volume remained in the Sephadex G25. The mixture was applied and filtered by centrifugation as above. The poly(dG-dC).poly(dG-dC) or DNA was collected in the centrifuge tube, whereas the salt, DMS, H_2SO_4 and CH_3OH remained in the syringe. The modified polynucleotide was then precipitated with 2 volumes of cold ethanol and redissolved in 2 mM citrate buffer, pH 7.5.

The native and alkylated substrates were hydrolyzed under either neutral or acidic conditions, as described by Lawley (11).

The quantitative analysis of the modified bases was carried out by HPLC, using C18 μ Bondapack column (Waters), as previously described (6,12). Briefly the mobil phase was 50 mM $NH_4H_2PO_4$ (pH 4.0) 5% methanol (v/v), and the elution was performed at a speed of 1.5 ml/ min . The retention times of m^3A,

guanine, adenine, m^7G and m^6G were 4.0, 5.5, 9.0, 11.0 and 23.0 min, respectively. Under the same conditions, two rotamers of rom^7G were observed. They eluted at 4.0 and 5.0 min, respectively. The quantification of the respective adducts was carried out as previously described (6) by measuring of the optical density at 254 nm.

The results are shown in Table I. With poly(dG-dC).poly(dG-dC) the DMS treatment yielded mostly m^7G and barely detectable amounts of m^6G, whereas m^7G and m^3A were mainly formed with DNA.

The poly(dG-dC).poly(dG-dC) and DNA were alkylated with MNU as follows: 1 mg of polynucleotide or DNA in 1.2 ml 0.3 M sodium cacodylate, pH 7.5, was treated with 200 mM MNU solution in acetone (final concentration 25-100 mM) for 16 h at 25°C. The polynucleotide was desalted and precipitated as described above for the alkylation by DMS.

Table I shows that poly(dG-dC).poly(dG-dC), treated with MNU, yielded mostly m^7G and low amounts of m^6G, whereas with DNA m^3A and low amounts of m^6G were found in addition to m^7G.

The alkaline cleavage of the imidazole ring of N^7-methylated guanine, leading to the formation of 7-formamidopyrimidine, was obtained by incubation of DMS modified poly(dG-dC).poly(dG-dC) for 48 h in 50 mM Na_2HPO_4,NaOH (pH 11.4) (6). This treatment converted more than 99% of the m^7G into rom^7G (6).

The excision of m^7G from the methylated poly(dG-dC).poly(dG-dC) was carried out at 100°C for 30 min in 50 mM KPO_4 buffer (pH 7.5) (6).

In order to obtain the Z-form of poly(dG-dC).poly(dG-dC), the polymer was alkylated by DMS in three steps as follows : 500 µg of poly(dG-dC).poly(dG-dC) in 600 µl 0.3 M sodium cacodylate, pH 7.5, were treated with 200 mM DMS for 1 h at 37°C. The poly(dG-dC).poly(dG-dC) was desalted by

Table I. Quantification of base modifications obtained by dimethylsulfate or methylnitrosourea treatment of poly (dG-dC).poly(dG-dC) and DNA

	$\frac{m^7G}{G + m^7G + m^6G}$	$\frac{m^6G}{G + m^7G + m^6G}$	$\frac{m^3A}{A + m^3A}$
poly(dG-dC).(dG-dC) modified by			
0 mM DMS	0	0	0
50 mM DMS	0.15	<0.001	0
100 mM DMS	0.30	<0.001	0
DNA modified by			
0 mM DMS	0	0	0
25 mM DMS	0.07	<0.001	0.01
50 mM DMS	0.20	<0.001	0.05
100 mM DMS	0.40	<0.001	0.10
poly(dG-dC).(dG-dC) modified by MNU			
0 mM MNU	0	0	0
30 mM MNU	0.03	<0.001	0
45 mM MNU	0.04	<0.002	0
DNA modified by			
0 mM MNU	0	0	0
15 mM MNU	0.04	N.M.	N.M.
30 mM MNU	0.09	0.005	0.01
45 mM MNU	0.12	0.005	0.01

The DNA or polynucleotides were alkylated with either DMS or MNU. They were hydrolyzed and the relative amounts of the different bases were determined after separation by HPLC. For details see Materials and Methods.
N.M. : Not measured.

filtration through Sephadex G25, as previously described. The desalted poly(dG-dC).poly(dG-dC) was again alkylated and desalted twice under the same conditions. The polynucleotide was finally precipitated with two volumes of cold ethanol and redissolved in 2 mM citrate buffer pH 7.5. Under these conditions, 95% of the guanine residues were converted into m7G.

Enzymatic methylation and DNA recovery

Beef brain DNA methylase was obtained from nuclei, as described for rat brain DNA methylase (2). The methylation assay was as previously described (2). For kinetic studies of DNA methylation, the DNA was recovered quantitatively as published (13) but instead of collecting DNA on GF/C filters, the precipitated DNA was recovered by centrifugation and further hydrolyzed by 300 µl of 0.5N perchloric acid for 20 min at 95°C. The hydrolysate was quantitatively transferred into 6 ml of scintillator ready Solv MP and its radioactivity was determined.

RESULTS AND DISCUSSION

No difference was found between the *in vitro* enzymatic methylation of poly(dG-dC).poly(dG-dC) untreated or treated with increasing amounts of DMS. Even a 30% modification of G into m^7G did not hinder the enzymatic methylation, which was tested after 90 min, during the linear phase of the reaction, with 4 enzyme concentrations (Fig. 1A). DMS treated poly(dG-dC).poly(dG-dC) was incubated, either under alkaline conditions to generate quantitatively 7-formamidopyrimidine (rom^7G) from m^7G, or heated under neutral conditions to obtain quantitative release of m^7G.Fig. 1B shows that poly(dG-dC).poly(dG-dC) containing 15% or 30% rom^7G was just as well methylated as the untreated control. Similarly, 15% and 30% depurinated poly(dG-dC).poly(dG-dC) were methylated as well as the control (Fig. 1C).

It has been shown that methylation of poly(dG-dC).poly(dG-dC) at the N-7 position of guanine facilitates the transition to the Z-form of the molecule. A polynucleotide containing high amounts of m^7G can easily be converted into a Z-form by NaCl or $MgCl_2$ (8). We used either poly(dG-dC).poly(dG-dC) containing 40% of G modified into m^7G in the presence of 50-200 mM $MgCl_2$, or poly(dG-dC).poly(dG-dC) containing 95% m^7G residues in the presence or absence of 50 mM $MgCl_2$. According to Moller *et al.* (8) the polynucleotide containing 50% m^7G seems to be already partially converted into the Z-form at 50 mM $MgCl_2$. With 95% modified guanines, the polynucleotide is already largely converted into the Z-form in the absence of $MgCl_2$ and completely converted in the presence of 50 mM $MgCl_2$. Table II shows that the polynucleotide in its left handed Z-DNA form is as well methylated enzymatically as in its right-handed B-DNA form.

Since the transformation of G into m^7G did not impair the methylation of the polydeoxynucleotide, we checked the influence of m^3A. This experiment was performed with DMS-treated DNA, which contained m^7G as well as m^3A (Table I). Figure 2A shows that,when up to 10% of adenine are modified into m^3A, the enzymatic methylation did not vary. DNA containing 7-formamidopyrimidine was also methylated just as well as the control (Fig. 2B).

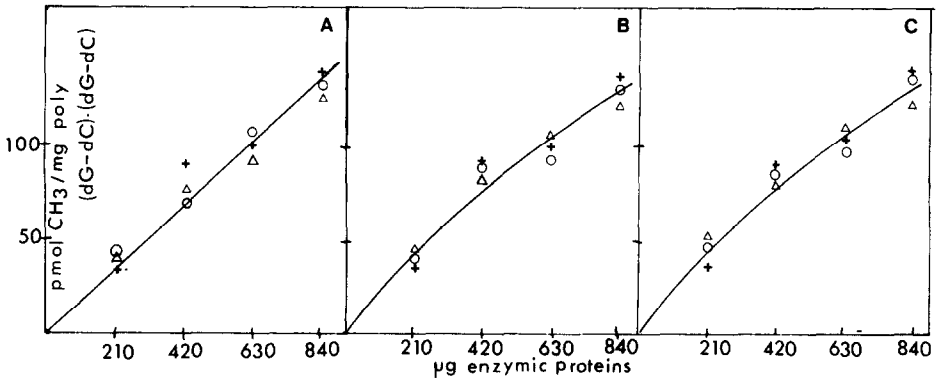

Figure 1. Enzymatic methylation of poly(dG-dC).poly(dG-dC) alkylated by dimethylsulfate and either untreated or further treated at neutral pH (yielding apurinic sites) or alkaline pH (yielding rom^{7}G)

A. The poly(dG-dC).poly(dG-dC) was treated with increasing amounts of DMS. The templates used to measure enzymatic methylation were untreated (+—+), alkylated with 50 mM DMS (○—○), or 100 mM DMS (△—△). See Table I for the quantification of modified base.

B. Poly(dG-dC).poly(dG-dC) treated with DMS and further incubated at alkaline pH. The symbols are as in A .

C. Poly(dG-dC).poly(dG-dC), treated with DMS at neutral pH and heated. The symbols are as in A .

Since m^{7}G and m^{3}A did not influence the enzymatic methylation of DNA, we investigated other lesions. For this purpose, DNA was alkylated with MNU : in addition to m^{7}G and m^{3}A, this drug produces m^{6}G and phosphotriesters in DNA (3-5). Table I shows that MNU-treated DNA or polynucleotides contain

Table II. Enzymatic methylation of poly(dG-dC).(dG-dC) as a function of the conversion of B- into Z-form

	$MgCl_2$ mM	A295/A260nm	Picomoles of CH_3/mg of poly(dG-dC).(dG-dC)
poly(dG-dC).(dG-dC) untreated	0	0.18	62.5
	50	0.17	64.6
	100	0.14	63.1
	200	0.13	60.8
poly(dG-dC).(dG-dC) 40% m^{7}G/G + m^{7}G	0	0.30	59.8
	50	0.38	57
	100	0.42	65
	200	0.46	59
poly(dG-dC).(dG-dC) 95% m^{7}G/G + m^{7}G	0	0.6	65
	50	0.7	61

The ratio A295/A260 increases with the conversion of poly(dG-dC).poly(dG-dC) from B- to the Z-form (14).

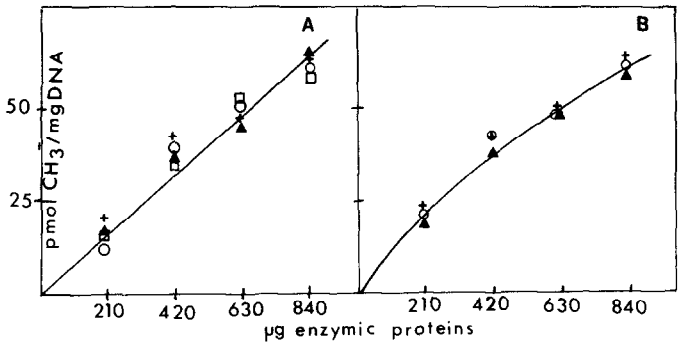

Figure 2. Enzymatic methylation of DNA alkylated by dimethylsulfate and either untreated or further treated at alkaline pH (yielding rom7G).
A. The DNA was alkylated with increasing amounts of DMS. The templates used to measure enzymatic methylation were untreated DNA (+—+), DNA alkylated with 25 mM DMS (O—O),or with 50 mM DMS (▲—▲), or with 100 mM DMS (□—□).
B. The DMS treated DNA was further incubated at alkaline pH. The symbols are as in A . See Table I for the quantification of modified bases.

measurable amounts of m^6G. When MNU-treated DNA was used as substrate for enzymatic methylation, there was inverse relationship between the level of modification and the methylacceptor capacity of the DNA (Fig. 3). A 1% alkylation of the bases inhibited 40% of the enzymatic methylation. When MNU-

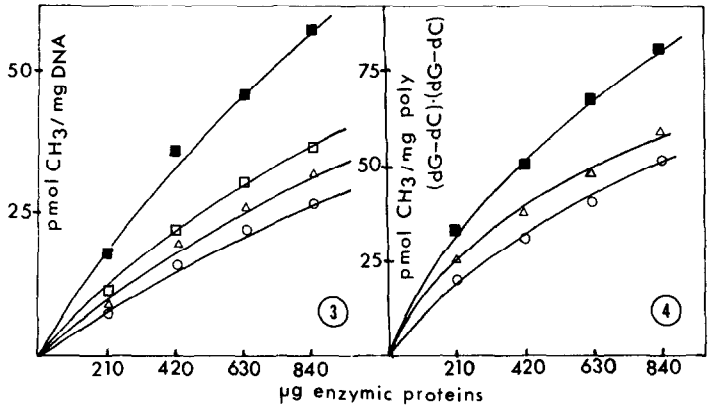

Figure 3. Enzymatic methylation of DNA alkylated with various amounts of MNU.
The DNA was alkylated with increasing amounts of MNU. The templates used to measure enzymatic methylation were untreated DNA (■—■), DNA alkylated with 15 mM MNU (□—□), or 30 mM MNU (△—△), or 45 mM MNU (O—O). See Table I for the quantification of modified bases.

Figure 4. Enzymatic methylation of poly(dG-dC).poly(dG-dC) alkylated with various concentrations of MNU.
Poly(dG-dC).poly(dG-dC) was alkylated with increasing amounts of MNU. The amount of alkylated bases was measured by HPLC as reported in Table I. The templates used to measure enzymatic methylation were untreated (■—■), alkylated with 30 mM MNU (△—△) or with 45 mM MNU (O—O).

treated poly(dG-dC).poly(dG-dC) was used as substrate for methylation (Fig. 4), there was a 38% inhibition of the enzymatic activity for only 1% of alkylated bases. A comparison of the substituted bases in the polynucleotides or DNAs (Table I) used as substrates for DNA(cytosine-5-)methyltransferase does not permit to reach a clear-cut conclusion as to the identity of the real inhibitor (m^6G and/ or phosphotriester). A final conclusion could only be drawn from a modified substrate whose m^6G would have been specifically eliminated.

The methylation of cytosine is the only post-replicational modification so far detected in the DNA of higher eukaryotes and has, therefore, been taken as the basis of several proposed mechanisms of gene activity, cellular differentiation and oncogenesis (for reviews see 15-18). The hypomethylation of DNA caused by its chemical alkylation could be therefore of importance in these biological mechanisms, in addition to its role in premutagenic lesions.

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