A CHROMATIN FACTOR IN RAT LIVER WHICH DESTROYS O^6-ETHYLGUANINE IN DNA

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1. Introduction

Adapted *Escherichia coli* cells (i.e. pretreated with a low concentration of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) contain a factor able to promote the disappearance of O^6-methylguanine from DNA [1,2]. The O^6-methylguanine is not released as a free base and the transformation product remains in DNA [2]; the factor seems to be active at 0°C and to disappear during the reaction [1].

Renard et al. [3] observed the disappearance of O^6-ethylguanine from the DNA of isolated rat liver nuclei treated in vitro with ethylnitrosourea and Pegg [4] has found, in the total proteins of a rat liver extract, an activity which induces the disappearance of O^6-methylguanine from DNA. As in *E. coli*, the glycosylic bond is not hydrolyzed to release free O^6-methylguanine.

In this communication, we show that, in rat liver, chromatin has the highest concentration of the factor capable to decrease the O^6-ethylguanine content of an added ethylated DNA. Some properties of the chromatin factor are also presented.

2. Materials and methods

2.1. *Rat liver preparations*

2.1.1. Total proteins

Liver is homogenized in 5 vol. of buffer A (50 mM Tris · HCl, 0.1 mM EDTA, 1 mM 2-mercaptoethanol, pH 7.8); the suspension is centrifuged at 5000 × *g* and the proteins are precipitated by addition of solid ammonium sulfate up to 85% saturation at 0°C. The precipitate, collected by centrifugation, is dissolved in buffer A and dialyzed overnight against the same buffer. Cloudiness which appears on dialysis is eliminated by centrifugation.

2.1.2 Nuclei proteins

The nuclei, isolated following the method of Thibodeau and Verly [5], are suspended in buffer A and solid ammonium sulfate is directly added up to 85% saturation at 0°C. The precipitate, collected by centrifugation, is treated as above. The final solution is free of DNA.

2.1.3. Chromatin proteins

Chromatin is prepared from isolated nuclei following the method of Thibodeau and Verly [5]. Chromatin proteins are then prepared in either two ways.

Chromatin is dissociated in 10 vol. of 1 M KCl in buffer A at 0°C during 30 min, then the suspension is centrifuged 18 h at 300 000 × *g*. The supernatant, which is free of DNA, is dialyzed against buffer A. The small precipitate which forms during dialysis is eliminated by centrifugation. This preparation is called 'chromatin 1 M KCl proteins'.

Chromatin is sheared in a Potter–Elvehjem apparatus of 10–20 μm clearance in buffer B (10 mM K phosphate, 10 mM Tris · HCl, pH 8.0) to have about 300 μg DNA per ml. Heparin-Sepharose (Pharmacia) in buffer B (2.5 mg heparin per ml) is then added to have the same weight of heparin and chromatin DNA. The mixture is stirred for 30 min at 0°C leading to a complete dissociation of chromatin. The heparin-Sepharose-DNA-protein complex is collected by centrifugation; the supernatant is practically devoid of DNA and protein. The pellet is washed with buffer B, then extracted twice with 0.5 M KCl in buffer B. The pooled KCl extracts are dialyzed against buffer A; no precipitate appears

during dialysis. This preparation is called 'chromatin heparin-Sepharose proteins'.

2.2. *Substrates*

Rat liver DNA prepared from isolated nuclei or calf thymus DNA (Sigma) is alkylated with [^{3}H]-ethylnitrosourea (3 or 5 Ci/mmol; IRE Belgium). The ethylated DNA is used as such or after partial depurination according to [2]. The number of O^6-ethylguanines per 10^9 guanines is indicated for each preparation. Before the experiment, the substrate is dialyzed against buffer C (10 mM Tris · HCl, 1 mM EDTA, pH 7.8).

2.3. *The assay*

1 ml of substrate solution is mixed with 1 ml of protein solution or buffer A, and the mixture is incubated at 37°C for 1 h. DNA is then precipitated by addition of 0.25 ml of 5 M $NaClO_4$ and 2.25 ml of ethoxyethanol. After collection by centrifugation, it is submitted to depurination for purine analysis or to enzyme hydrolysis for nucleoside analysis.

In some experiments, DNA and proteins are precipitated by addition of 2 vol. of 95% ethanol and discarded by centrifugation. The pooled supernatants of several identical assays are divided in two parts. One is directly analyzed for free purines; the other is submitted to depurination before performing the same analysis.

2.4. *Purine analysis*

The pellet of DNA or the lyophilized supernatant is taken in 1 ml of 0.1 M HCl containing 100 μg of unlabeled O^6-ethylguanine and warmed at 70°C during 50 min. We checked that, in these conditions, the purines are completely released as free bases and that the O^6-ethylguanine is not significantly destroyed. After neutralization with 1 M KOH, 2 vol. of 95% ethanol are added and, after 30 min in ice, the precipitate is discarded by centrifugation and the supernatant lyophilized. The residue is dissolved in 1.1 ml of buffer D (50 mM ammonium formate: formic acid, pH 5.0/absolute ethanol; 90/10 by vol.) and the solution is passed through a 0.45 μm Millipore filter.

The solution is placed onto a 30 × 1 cm column of Sephasorb HP (Pharmacia); the elution is carried out with buffer D at a flow rate of 0.9 ml/min; 41 fractions of 3.2 ml are collected; absorbance at 260 nm and radioactivity are measured on each fraction.

The adenine absorbance peak is always clearly separated. The amount of adenine in the sample is estimated knowing the extinction coefficient in buffer D at 260 nm; the guanine content is then calculated using a guanine/adenine ratio of 0.76 for calf thymus DNA or 0.75 for rat liver DNA.

The total radioactivity in the O^6-ethylguanine peak is measured. Knowledge of the specific radioactivity of the [^{3}H]ethylnitrosourea used to alkylate the substrate enables to calculate the amount of O^6-ethylguanine in the sample. The result is given as such or as the ratio of O^6-ethylguanines per 10^9 guanines.

2.5. *Nucleoside analysis*

The DNA precipitate is dissolved in 100 μl of 10 mM Mg acetate, pH 6.5. 30 μl of 50 mM Tris · HCl, 5 mM Mg acetate, pH 7.5, containing 0.5% bovine serum albumin and 60 units of DNase I (3.1.4.5; Boehringer), are added and the solution is incubated for 30 min at 37°C. The solution is then completed with 18 μl of 1 M Tris · HCl, pH 8.5, 0.8 unit of *E. coli* alkaline phosphatase (3.1.3.1; Boehringer) and 0.4 unit of snake venom phosphodiesterase (3.1.4.1; Boehringer) before a further incubation of 18 h at 37°C. The pH is then lowered to 7.0 with 1 M HCl before heating 5 min at 100°C; the protein precipitate is discarded by centrifugation.

The supernatant is applied on a Whatman 3MM paper which is eluted during 15 h with a mixture of butanol/ethanol/water (80/10/25 by vol.) [6]. The paper is dried, then cut into 1 cm strips which are eluted with buffer D for radioactivity measurements. The O^6-ethyldeoxyguanosine spot also contains the O^2-ethyl- and O^4-ethyl-derivatives of deoxythymidine [6].

3. Results

3.1. *The chromatin repair factor*

Chromatin heparin-Sepharose proteins in buffer A are mixed with ethylated DNA in buffer C and the mixture is incubated at 37°C for 1 h. After precipitation, the DNA is submitted to depurination and purine analysis. Fig.1(a,b) shows an example in which 93% of the O^6-ethylguanine has disappeared from the DNA. No new radioactivity peak is observed.

In these experiments, the supernatants obtained after ethanol precipitation of DNA and proteins do not contain free O^6-ethylguanine and the ratio of

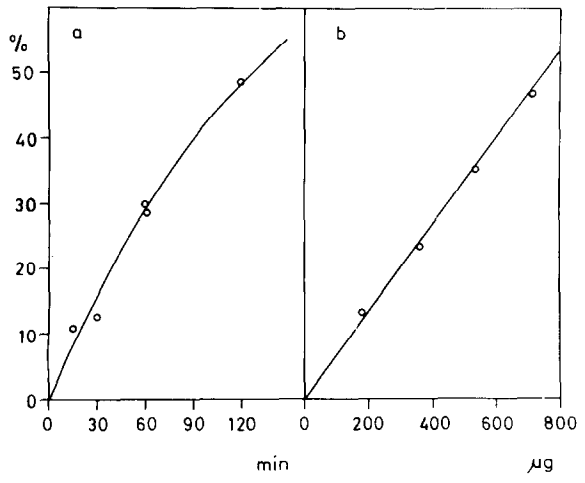

Fig.1. Disappearance of O^6-ethylguanine from DNA exposed to chromatin proteins: purine and nucleoside analyses. Chromatin heparin-Sepharose proteins (300 μg) in 1 ml of buffer A are incubated for 1 h at 37°C together with 1 ml of buffer C containing 27 μg of ethylated calf thymus DNA partially depurinated (128 fmol of O^6-ethylguanine; 7820 O^6-ethylguanines/10^9 guanines) and 250 μg of not treated DNA. Two such assays are run in parallel as well as two controls without proteins. (1) The DNAs of one control (a), and one assay (b) are depurinated and the purines analyzed by chromatography on Sephasorb HP. Absorbance at 260 nm (A; dotted line) and radioactivity (dpm; continuous line) are measured on the controlled fractions. The graphs show the adenine absorbance peaks and the O^6-ethylguanine radioactivity peaks. N = fraction number. (2) The DNAs of one control (c) and one assay (d) are enzymatically hydrolyzed to nucleosides which are separated on Whatman 3MM paper. The graphs give the percents of total radioactivity in the 43 fractions as a function of the migrated distance (cm). The O^6-ethyldeoxyguanosine is located in the last peak.

the number of O^6-ethylguanines/10^9 guanines is the same in the oligonucleotides as in the precipitated DNA.

The conclusion is that chromatin contains a protein factor which causes the disappearance of O^6-ethylguanine from ethylated DNA. The O^6-ethylguanine is not released as a free base by a DNA glycosylase or in an oligonucleotide by the conjugated action of a specific endonuclease and an exonuclease. The O^6-ethylguanine thus seems to be converted into another compound (compound X) which is not released by the depurination procedure or which is sensitive to HCl hydrolysis. These results on purine analysis do not exclude that the factor might be a dealkylase.

3.2. *Further analysis of the reaction product*

In an experiment using ethylated DNA partially depurinated and chromatin heparin-Sepharose proteins, in which 50% of the O^6-ethylguanine has disappeared, the DNA is submitted to nucleoside analysis. The peak containing the O^6-ethyldeoxyguanosine, obtained by chromatography on Whatman 3MM paper, represents 29% of the total radioactivity as well before as after the 1-h incubation at 37°C with the chromatin proteins. It seems that compound X still has the ethyl group (the repair factor is thus not a dealkylase), that it remains bound to the sugar-phosphate backbone of DNA, and that the deoxyriboside of X comigrate with the O^6-ethyldeoxyguanosine in the chromatographic system.

The phosphatase used in the preceding experiment is still active, but outdated. When the experiment is repeated with a fresh preparation of phosphatase, the peak containing the O^6-ethyldeoxyguanosine of the control represents 30% of the total radioactivity, but it has decreased to 16% after the incubation with the chromatin proteins [fig.1(c,d)]. The difference is the value expected from the disappearance of O^6-ethylguanine measured by purine analysis (93% in this experiment). No new radioactivity peak is observed in the chromatogram. The conclusion might be that X is destroyed, likely with the formation of a radioactive volatile compound (ethanol?) by an impurity present in fresh phosphatase preparation and which has vanished from the outdated preparation; this impurity might be adenosine deaminase.

3.3. *A few properties of the chromatin repair factor*

The chromatin factor is active in absence of divalent cations in 0.55 mM EDTA. Introduction of Mg^{2+} rather decreases the activity. The factor is inhibited by 0.1 M KCl.

Fig. 2(a) shows, as a function of time, the disappearance of O^6-ethylguanine from ethylated DNA incubated at 37°C with chromatin heparin-Sepharose proteins. The process is still going on after 120 min. On the other hand, the factor is inactive at 0°C.

The same amount of substrate is incubated with increasing amounts of chromatin heparin-Sepharose proteins for 1 h at 37°C. Fig.2(b) shows that the number of fmol of destroyed O^6-ethylguanine is proportional to the amount of protein (the largest quantity of protein used removes less than 50% of the O^6-ethylguanine from the DNA). As shown in table 1 (experiment 1), the number of fmol of

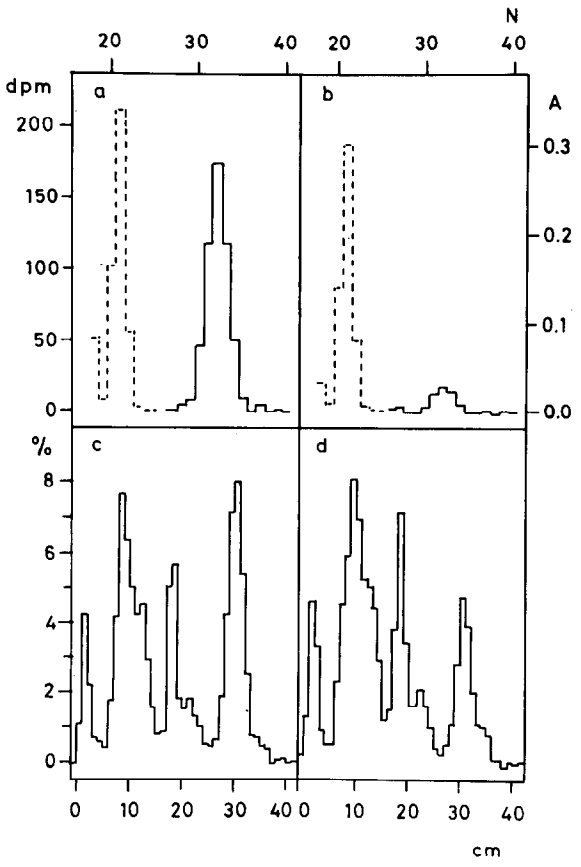

Fig.2. O^6-ethylguanine lost by DNA as a function of time of incubation or as a function of amount of chromatin proteins. Samples of 1.4 mg calf thymus DNA ethylated and partially depurinated (78 fmol of O^6-ethylguanine; 92 O^6-ethylguanines/10^9 guanines) are incubated at 37°C with chromatin heparin-Sepharose proteins in a total volume of 2 ml. The DNA is depurinated and the purines analyzed on Sephasorb HP; the O^6-ethylguanine lost by DNA is given as percent of the initial O^6-ethylguanine content. In (a), 260 μg of protein are used and the incubation time varies from 0 to 120 min. In (b), the incubation time is 1 h and the amount of protein varies from 0 to 700 μg.

O^6-ethylguanine that disappears depends on the concentration of O^6-ethylguanine in the incubation medium; there is also an effect of O^6-ethylguanine frequency (number of O^6-ethylguanines/10^9 guanines).

3.4. *Cellular localization of the repair factor (table 1)*

The specific activity of nuclear proteins (number of fmol of O^6-ethylguanine destroyed/h/mg protein for a given amount of substrate) is 25 times higher than the specific activity of total cellular proteins and 3/4 of the cellular activity is found in the nucleus (experiment 2). The specific activity of chromatin

Table 1

Exp.	Substrate DNA			Proteins		Activity	
	Nature	O^6-etG/10^9 G	O^6-etG (pM)	Origin	mg/ml	fmol/h/mg protein	fmol/h/g liver
1	calf thymus alk-dep	92	43	chromatin hep-Seph	0.13	81	
	calf thymus alk-dep	7820	64	chromatin hep-Seph	0.13	274	
	calf thymus alk-dep	7820	640	chromatin hep-Seph	0.13	1320	
2	rat liver alk	120	6	total	10	0.4	32
	rat liver alk	120	6	nuclear	0.4	10	25
3	rat liver alk-dep	120	6	nuclear	0.20	10	
	rat liver alk-dep	120	6	chromatin 1 M KCl	0.22	19	
4	calf thymus alk-dep	92	43	chromatin 1 M KCl	0.08	71	
	calf thymus alk-dep	92	43	chromatin hep-Seph	0.05	93	

The substrate is rat liver or calf thymus DNA alkylated with [^{3}H]ethylnitrosourea (alk) and sometimes partially depurinated (alk-dep). The number of O^6-ethylguanine (O^6-etG)/10^9 guanines (G) is given as well as the O^6-ethylguanine concentration (pM) in the incubation medium. The total cellular proteins (total), the nuclear proteins (nuclear) or the chromatin proteins (1 M KCl or heparin-Sepharose) are used and their concentration in the incubation medium is indicated (mg/ml). The specific activity of the proteins (fmoles of O^6-ethylguanine destroyed/h/mg protein) is recorded; some results have also been calculated per g of liver (assuming the usual 60% yield in the preparation of the nuclei). The difference between the results obtained with the chromatin 1 M KCl proteins in experiments 3 and 4 is mostly due to a higher concentration of O^6-ethylguanine in experiment 4

proteins is even greater than that of the nuclear proteins (experiment 3). There is no important difference between the specific activities of chromatin 1 M KCl and heparin-Sepharose proteins (experiment 4).

4. Discussion

Rat liver possesses a factor which destroys O^6-ethylguanine in DNA. This factor is mostly localized in chromatin. It exists constitutively in contrast with the *E. coli* factor which is absent in non-adapted bacteria [2]. We however have data showing that the chromatin activity can be increased by treatment of the rats with low doses of diethylnitrosamine.

The *E. coli* factor seems to react stoichiometrically with O^6-methylguanine, even at 0°C, and to disappear during the reaction [1]. The data presented here suggest that the rat liver chromatin factor is behaving more like an enzyme: it is not active at 0°C and, in the presence of an excess substrate, it is still working after 120 min. Kinetic analysis are being done to decide if the factor is a stoichiometric reagent or an enzyme.

Our results are in agreement with the hypothesis that the chromatin repair factor modifies O^6-ethylguanine into compound X which stays in DNA. Compound X is probably destroyed in the conditions of acid depurination; it also appears to be sensitive to an impurity of the *E. coli* alkaline phosphatase preparation when the DNA is enzymatically hydrolyzed to nucleosides.

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