Conformational changes induced in DNA by *in vitro* reaction with N-hydroxy-N-2-aminofluorene

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ABSTRACT

The conformation of DNA modified *in vitro* by the covalent binding of N-OH-AF was investigated by ultraviolet absorbance, circular dichroism and by radioimmunoassay using specific antibodies against Guo-AAF and nDNA-AAF. The results obtained by both physico-chemical and immunological methods are in agreement with a model involving destabilized regions in the double helical DNA around the carcinogen molecule in which, however, the -AF residues are stacked to the adjacent nucleotides. The RIA results show that the -AF residues are less accessible to antibodies in native than in denatured DNA-AF and thus suggest -AF residues partially buried in the interior of the DNA helix. The present model is compared to the one existing for DNA modified by reaction with N-AcO-AAF (DNA-AAF) (1,2).

INTRODUCTION

It is already well established that ultimate metabolites of chemical carcinogens bind covalently to DNA *in vivo* and, in certain conditions, *in vitro* (3-7). A correlation between the conformational changes induced in DNA and carcinogenesis is supposed (1,8,9). One of the most studied chemical carcinogens is 2-acetylaminofluorene (2-AAF). The conformational changes of DNA due to the binding of the metabolite N-acetoxyacetylaminofluorene (N-AcO-AAF) and several of its halogenic derivatives were already extensively studied (1,2) (8-13). Another metabolite of the same carcinogen, the N-hydroxyaminofluorene (N-OH-AF), was not studied until now from the point of view of conformational changes induced in DNA to which it binds covalently as well, leading to DNA-aminofluorene (DNA-AF) (14). A study of this modification will permit comparison with the ones induced by the other derivatives of the same carcinogen.

Another reason of studying the effect of binding of N-OH-AF to DNA is that after treatment *in vivo* with 2-AAF, around 80 % of DNA bound product is deacetylated (15-19).

On another hand, N-OH-AF belongs to the class of N-hydroxyarylamines proposed as ultimate metabolites of aromatic amines which are urinary bladder carcinogens (3, 20-22).

The conformation of native and denatured DNA modified *in vitro* by N-OH-AF, nDNA-AF and respectively dDNA-AF is investigated in the present study by ultraviolet absorbance, circular dichroism and radioimmunoassay with specific antibodies against Guo-AAF and against nDNA-AAF.

MATERIALS AND METHODS

N-OH-AF was prepared by reduction of nitro-2-fluorene with ammonium sulfide in absolute ethanol and N-N-dimethylformamide (v/v, 1/1) according to a published procedure (23) modified as under. The mixture was stored at -10°C for 24 hours, then added to 3 volumes of ether and washed with water. The ethereal phase was concentrated *in vacuo* until beginning of crystallization of the hydroxylamine. The product is obtained as pale yellow crystals with a melting point of 180°C.

Native DNA is a calf thymus preparation ; denatured DNA was obtained by heating it at 100°C for 15 minutes and rapid cooling in ice. GMP-AAF was prepared by reaction of GMP with N-AcO-AAF (12). GMP-AF was obtained by deacetylation of GMP-AAF in 0.02 M NaOH, at 60°C for 1 hour according to a published procedure (12).

Incubation of DNA with N-OH-AF was performed under nitrogen atmosphere in a thermostated bath at 37°C. N-OH-AF was first solubilized in dimethylsulfoxide and added to a Na-citrate buffered solution of DNA (DMSO : buffer 1/20, v/v) containing 2 mg/ml of sonicated DNA, 10 mM Na citrate, 0.1 mM EDTA at pH 5.0 (the ratio of concentration of carcinogen and that of DNA in moles of phosphate, in the incubation mixture was 2:1).

Samples were incubated for different time intervals in order to get DNA samples with different degrees of carcinogen binding. After incubation for time intervals until 5 hours, the unreacted carcinogen was extracted several times with diethyl ether. The DNA was purified by precipitation in ethanol, then redissolved and applied on Sephadex G-50 column. The same procedure was used for both native and denatured DNA.

Absorption measurements were recorded with a Cary 15 spectrophotometer and the circular dichroism spectra with a Roussel-Jouan dichrograph 3. In the case of CD spectra, $\Delta\epsilon$ values refer to the concentration in -AF residues.

Radioimmunoassay (RIA) were performed using antibodies against Guo-AAF and nDNA-AAF prepared and purified as previously described (24-26). The radioactive antigen used was [^3H] Guo-AF obtained by deacetylation of [^3H] Guo-AAF by the same procedure as the non radioactive GMP-AF. All competition experiments were performed at the same ionic strength (0.25 M NaCl). The radio-

activities were determined in a Beckman scintillation counter.

RESULTS

Determination of percentage of modified bases

The percentage of modified bases over phosphate, (C)/(P), in DNA-AF was determined as follows : (C) was calculated from the U.V. absorbance at 312 nm (this wavelength is isosbestic in the heating of DNA-AF as it will be shown below) using as extinction coefficient the one of GMP-AF, $\epsilon(312) = 25000 \text{ M}^{-1} \text{ cm}^{-1}$. This value was obtained by us when transforming GMP-AAF in GMP-AF by alkali treatment and then passing in neutral pH. The shape of the spectra is similar to that already published for GMP-AF, but the value of ϵ is slightly different from the published one which is 27000 (27). The concentration of nucleotide residues was calculated from the absorbance at 258 nm neglecting the contribution of carcinogen at this wavelength. It was verified that this assumption is valid in our conditions. A good agreement was found for the nucleotide residues concentration deduced from the absorbance at 258 nm and from a radioactive measurement (a *E.coli* [^3H] DNA, 100,000 cpm/mM was modified with N-OH-AF).

A standard curve A_{258}/A_{312} at 20°C as a function of (C)/(P) was established for calf thymus nDNA-AF (Fig. 1).

The reactivity of N-OH-AF was smaller with dDNA than with nDNA. In the same experimental conditions, it was found that 4.5 % bases were modified in nDNA and only 1.5 % in dDNA.

Absorption data. The ultraviolet absorption spectra of both nDNA-AF and dDNA-AF present a band in the region around 320 nm. When heating the DNA solutions a hypochromic effect is observed in this region, as shown in Fig. 2.

Fig. 1 - Standard curve for determination of percentage of modified bases in calf thymus nDNA-AF (buffer : $2 \times 10^{-3} \text{ M}$ Na citrate, 10^{-2} M NaCl, pH 7.4).

Fig. 2 - Ultraviolet absorption spectra of a) native DNA-AF modified at 5 % in 2×10^{-3} M Na citrate, 10^{-2} M NaCl, pH 7.4, $(C) = 6 \times 10^{-6}$ M (AF), b) denatured DNA-AF modified at 1.9 % in the same buffer, $(C) = 2.6 \times 10^{-6}$ M (AF), c) GMP-AF in the same buffer, $(C) = 1.8 \times 10^{-5}$ M (AF), (—) 20°C, (---) 80°C.

The absorbance around 320 nm at high temperatures is similar to that of GMP-AF.

In order to understand the hypochromic effect in the heating of DNA-AF at 320 nm we performed several experiments of interaction of GMP-AF with nucleotides (28). We followed the differential absorbance in the wavelength range between 320 and 400 nm when mixing GMP-AF with AMP or GMP at high concentration (Fig. 3). An increase of absorbance is observed at $\lambda > 320$ nm for the mixture containing AMP and at $\lambda > 330$ nm for that containing GMP. The absorbance difference is larger for higher concentrations of nucleotides.

Melting curves of samples of nDNA-AF containing different percentages of modified bases were determined. The hyperchromicity at 260 nm over the temperature range observed (from 20°C to 75°C) was between 35 and 37 % for samples modified between 0.9 % and 4.5 % of the bases. A control sample of DNA incubated in the same conditions as the mixture of DNA and carcinogen presents a hyperchromicity of 38 %. The $A_{260} = f(T^\circ)$ curves (Fig. 4) show a highly cooperative transition around the T_m . The cooling curve shows a monotonous decrease of absorbance with decreasing temperature. The hypochromicity observed at 330 nm is of 20 %. In contrast to the cooperative curves $A_{260} = f(T^\circ)$, the $A_{330} = f(T^\circ)$ curve presents a monotonous variation of around 6 % before that corresponding to the melting of DNA. This first linear variation is reversible. When cooling down the sample heated until 75°C, half of the hypochromic effect is monotonously disappearing (the cooling curve is paral-

Fig. 3 - Difference ultraviolet spectrum produced by the interaction of GMP-AF and nucleotides : GMP-AF, 2×10^{-5} M and a) GMP (— 10^{-2} M, --- 3×10^{-2} M) ; b) AMP (0.5×10^{-2} M), temperature 20°C .

lel to the first part of the heating curve) (Fig. 4a).

For dDNA-AF, in the same temperature range we observe a monotonous hyperchromic effect of 13 % at 260 nm and a monotonous hypochromic effect of

Fig. 4 - Temperature dependent variation of absorbance for a) nDNA-AF modified at 4.5 % and b) dDNA-AF modified at 1.5 %. $H = A_{T^\circ\text{C}} - A_{20^\circ\text{C}} / A_{20^\circ\text{C}}$ (buffer : 2×10^{-3} M Na citrate, 10^{-2} M NaCl, pH 7.4).

about 10 % at 330 nm, which levels off at about 70°C. This effect is completely reversible (Fig. 4b).

We determined the values of T_m for different samples of nDNA with different degrees of carcinogen binding (Fig. 5). The values of T_m depend linearly on the percentage of modified bases (C)/(P). The value of the slope is - 1.4°C/ percent of modified bases.

In all experiments the heating-cooling cycle was performed in around 3 hours.

Circular dichroism data. Native and denatured DNA-AF which present similar absorption spectra in the wavelength region around 320 nm, present very different circular dichroism spectra in this region. Moreover the CD spectra are different from that of GMP-AF (Fig. 6).

The CD spectra of native DNA-AF depend on the percentage of modified bases (Fig. 7).

The intensity of the CD signal depends on temperature before the T_m of DNA-AF (Fig. 8).

Alkaline deacetylation of DNA-AAF

We treated denatured DNA-AAF by the conditions used for deacetylation of GMP-AAF (60°C, 0.02 M NaOH). We observed the deacetylation by measuring the loss of activity of a sample of DNA-AAF tritiated on the acetyl group. The deacetylation proceeded much slower than in the case of GMP-AAF (after 6 hours we observed 85 % decrease of radioactivity) ; complete stabilization of the absorbance was observed after 24 hours. A sample of DNA-AF was treated in exactly the same conditions and in the same time interval as dDNA-AAF and an evolution of the absorbance was observed. The final UV spectra of the two modified DNA are similar (Fig. 9). We note the corresponding dDNA-AF as dDNA-

Fig. 5 - Dependence of melting temperature of nDNA-AF on the percentage of modified bases (buffer : 2×10^{-3} M Na citrate, pH 7.4).

Fig. 6 - CD spectra of a) GMP-AF, b) dDNA-AF modified at 1.9 %, c) nDNA-AF modified at 1.7 % at 25°C (buffer 2×10^{-3} M Na citrate, 10^{-2} M NaCl, pH 7.4) ; $\Delta\epsilon$ values refer to the concentration in -AF residues.

AF'.

Almost no loss of carcinogen molecule by the alkali-high temperature treatment was observed after exhaustive dialysis (less than 7 %). Moreover, no change in the UV spectrum of this sample was found after precipitation by

Fig. 7 - Dependence of CD spectra on the percentage of modified bases in nDNA-AF (buffer : 2×10^{-3} M Na citrate, 10^{-2} M NaCl, pH 7.4).

Fig. 8 - Variation of CD signal of nDNA-AF (5 % modified bases) with temperature.

alcohol.

Radioimmunoassays

Antibodies anti Guo-AAF. We followed the inhibitory capacity of dDNA-AF and nDNA-AF for the reaction of antibodies against Guo-AAF with [³H]Guo-AF. We

Fig. 9 - Transformation by alkali-high temperature treatment (0.02 M NaCl, 60°C) of a) dDNA-AAF in dDNA-AF' and b) dDNA-AF in dDNA-AF' (spectra at pH 7.0, 20°C).

found that both dDNA-AF and nDNA-AF inhibit the reaction, but dDNA-AF inhibits less than GMP-AF and nDNA-AF inhibits less than dDNA-AF (Fig. 10a). At 50 % inhibition we find $\log C(\text{dDNA-AF}) - \log C(\text{GMP-AF}) = 1.3$ and $\log C(\text{nDNA-AF}) - \log C(\text{GMP-AF}) = 2.3$. In all experiments the ratio $\frac{Ag}{Ab}$ was 1.3 and the salt concentration was kept constant (0.25 M NaCl).

Antibodies anti nDNA-AAF. The same competition experiments were performed with antibodies against nDNA-AF. Also in this case the reaction between the antibodies and $[^3\text{H}]\text{Guo-AF}$ was inhibited by dDNA-AF less than GMP-AF and by nDNA-AF less than dDNA-AF (Fig. 10b). At 50 % inhibition $\log C(\text{dDNA-AF}) - \log C(\text{GMP-AF}) = 1.2$ and $\log C(\text{nDNA-AF}) - \log C(\text{GMP-AF}) = 2.2$. In all experiments the ratio $\frac{Ag}{Ab}$ was 1.5 and the salt concentration was kept constant (0.25 M NaCl).

DISCUSSION

The results presented here are in favour of a conformation of DNA-AF similar, until a certain point, to the one proposed for DNA-AAF. In the case of nDNA-AAF the -AAF moiety is covalently bound in principal (80 %) to the C(8) position of guanine (20,29) (almost 100 % in the dDNA-AAF (29)). This binding destabilizes a region of several base pairs around the carcinogen. The -AAF moiety stacks with the bases of the DNA molecule while guanine is

Fig. 10 - Inhibition of the precipitation reaction between $[^3\text{H}]\text{Guo-AF}$ and a) Ab anti Guo-AAF and b) Ab anti DNA-AAF. The inhibitors : $\square$ GMP-AF, $\times$ dDNA-AF, $\circ$ nDNA-AF.

outside (1,2,8,9). Studies using antibodies as probes have shown that even when bound to nDNA, the -AAF moiety is accessible (24-26).

- a) Results in favor of a destabilized region in nDNA-AF around the -AF residue

The decrease of T_m with increase of percentage of modified bases ($\Delta T_m = -1.4^\circ\text{C}/\text{percent of modified bases}$) shows a destabilizing effect of the -AF residue. In the case of DNA-AAF, ΔT_m is $-1.15^\circ\text{C}/\text{percent of modified bases}$ (8,9). A theoretical treatment of polynucleotides complexes with small internal loops resulting from mismatches predicts that DNA treated with products which interrupt up to 20 % of the nucleotide pairs will show a drop of around $1.2^\circ\text{C}/\text{percent of modified bases}$ (30).

The decrease of intensity of CD signal of nDNA-AF around 300 nm with increase of percentage of modified bases may be interpreted as a denaturing effect of the bound carcinogen (since the CD signal of -AF in dDNA-AF is much smaller than in nDNA-AF). Moreover, the fact that -AF residues are accessible to anti DNA-AAF and anti Guo-AAF antibodies even in nDNA-AF fits to a certain freedom of Guo-AF in DNA.

- b) Results in favor of interactions of -AF residues with the neighboring nucleotides in dDNA-AF and nDNA-AF

At room temperature the UV absorbance and CD spectra above 305 nm (corresponding to the absorbance of aminofluorene residues) of both nDNA-AF and dDNA-AF are different from that of GMP-AF. At high temperature (80°C) the UV absorbance in this region become identical for all. This lets suppose interaction of -AF residues with neighboring bases in DNA at room temperature.

The nucleotides -GMP-AF interaction experiments show that at very high concentration of nucleotides, the -AF residue interacts with nucleotides. These interactions increase the absorption coefficient of -AF in the region around 320 nm as compared to the non interacting state. Thus the unstacking of -AF residues from the neighboring nucleotides in DNA might be the origin of the hypochromicity at $\lambda > 320$ nm observed when increasing temperature of dDNA-AF. The lower association constant of antibodies for -AF in dDNA-AF as compared to GMP-AF is also in agreement with stacking interactions of -AF residues to its neighboring bases. interactions which would render the -AF residues less accessible to antibodies.

The interactions of -AF residues in nDNA-AF are more complex. The CD spectrum in the region corresponding to -AF in nDNA-AF is much different from the one in dDNA-AF. Thus the environment around the bound molecules is different in the two DNAs. This means that the -AF residues, even in a destabili-

zed region of DNA, feel strongly the influence of the double helical structure.

Much below T_m , in nDNA-AF, we observe a temperature dependent reversible variation of A_{330} and of the CD signal which parallels that observed for dDNA-AF. This shows a change in the stacking interactions between -AF residues and neighboring bases in nDNA-AF which implies a movement of the carcinogen. This variation is followed by a cooperative variation of UV absorbance around T_m , due to the disappearance of the helical structure of DNA.

c) The similarity of the spectra of alkali-deacetylated dDNA-AAF and of dDNA-AF treated in the same conditions is in favor of the binding of -AF to DNA in a position similar to that of -AAF, i.e. mostly on the C(8) position of guanine as already suggested by Kriek (20). The study of the deacetylation phenomenon is complicated by the transformation of -AF in -AF'. This transformation was already mentioned to occur in DNA-AF by incubation at pH 7.4 and 37°C (18,31) but -AF' is not yet identified. More work on this subject is in progress in our laboratory.

Until this point the model of DNA-AF seems similar to the one of DNA-AAF : -AF residue covalently bound mostly to C(8) of guanine in dDNA-AF and stacked to its neighboring bases in dDNA-AF and in the destabilized regions of nDNA-AF, but situated under the influence of the double helical structure of DNA.

The difference between the conformation of DNA-AAF and DNA-AF appears in the RIA experiments. In the case of DNA-AAF, the reaction between antibodies against nDNA-AAF and against Guo-AAF and $[^3H]$ Guo-AAF and nDNA- $[^3H]$ AAF is inhibited identically by denatured and native DNA-AAF. This result suggested that the -AAF residues are not completely buried in the interior of the double helical structure (25). Contrary to this case, nDNA-AF has a lower inhibitory capacity on the reaction between anti DNA-AAF and anti Guo-AAF and $[^3H]$ Guo-AF than dDNA-AF. Thus, the conformations of the regions where -AF residues are bound in nDNA-AF and dDNA-AF are different.

This suggests that the -AF residues are situated more in the interior of the double helical structure of DNA-AF, than are the -AAF residues in the nDNA-AAF.

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