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STRUCTURAL EVIDENCE ON DNA CARCINOGEN INTERACTIONS. N-ACETOXY-N-2ACETYLAMINOFLUORENE BINDING TO DNA

Bengt NORDÉN

Department of Inorganic Chemistry, University of Lund, Chemical Center, P.O. Box 740, S-220 07 Lund, Sweden

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Linear dichroism (LD) gives useful information on the interaction between DNA and the directly acting carcinogen N-acetoxy-N-2-acetylaminofluorene (AAAF). In 50% methanol solvent with low ionic strength only a weak complex (van der Waals) appears. However, above 40° C strand separation takes place and a covalent aminofluorene complex forms. After renaturation a characteristic positive LD band is observed at 306 nm. The average angular orientation of the long-axis of the fluorene moiety (47° to the local helix axis) is inconsistent with intercalation. It can be explained for instance by a free rotation around a C(DNA)-N(aminofluorene) bond or by a major groove site. The occupation density was 1-2 aminofluorene residues per 100 bases. With native DNA, AAAF slowly forms a covalent complex which has a negative LD at 307 nm. The orientation (70–90°) is consistent with steric direction by the strand.

1. Introduction

An important unifying concept for chemical carcinogenesis has been the recognition that many carcinogens interact with DNA and often are active only after metabolism by the host. In almost all cases this involves generation of some electrophilic species [1–3]. Microsomal oxidative enzymes can be responsible for a number of metabolic activations [4], e.g. the formation of reactive epoxides of benzpyrene and other aryl-hydrocarbons. This has led to numerous efforts to synthesize polycyclic hydrocarbon epoxides and to test their carcinogenic activity: benzopyrene 7,8-diol-9,10-epoxides for instance have been found to be among the most potent mutagens ever tested [6].

The identification, due to the work of James and Elisabeth Miller, of N-hydroxy-acetylaminofluorene as the electrophilic metabolite of the carcinogen 2-acetylaminofluorene [7,8] in conjunction with the demonstration by Kriek et al. [9] that the C-8 position of guanine is a principal site of reaction with DNA, has stimulated several studies of interactions between different N-hydroxy esters of aromatic amine carcinogens and DNA [10–12].

At this stage structural studies on the nature of carcinogen-DNA complexes can be particularly valuable. Linear dichroism offers a method of deciding relative orientations of chromophoric ligands to DNA. This report shows that linear dichroism provides unique structural information on the interaction between N-acetoxy-2-acetylaminofluorene and DNA.

2. Experimental

Linear dichroism measuring techniques and a suitable flow cell have been described elsewhere [13]. Calf thymus DNA (type I) was obtained from Sigma Chemical Co; a salt-free 2.30 mM (phosphate) aqueous stock solution was used. The standard buffer "10^{-1} SSC" (= 0.015 M NaCl, 0.0015 M Na_3citrate) was used. The carcinogen N-acetoxy-N-2-acetylaminofluorene (AAAF) was a gift from Dr. Ronald Pero, Wallenberg-laboratoriet, Lund. AAAF was added as a 3.30 mM stock solution with either methanol or dimethylsulfoxide (DMSO) as a solvent. Due to the high toxicity only a very small amount of AAAF (15 mg) was used. Ten AAAF-DNA solutions, compositions according to table 1, were examined.

Table 1
Spectroscopic observations on different AAAF-DNA solutions

Sample	Conditions [a]	LD_{257}/cm	A_{257}/cm	$LD_{301} \times 10^2$/cm	A_{301}/cm
I. 3 ml 2.3 mM DNA + 0.5 ml 10^{-1} SSC	2 h (22°) [a],	−1.9	10	−6.8	2.4
+ 3 ml 0.33 mM AAAF (methanol)	3 h (22°)	−2.0	–	−6.9	
	22° immediately after treating to 60°	−0.2	10	+0.8 (0.97 [b])	2.5 (1.4 [b])
II. 3 ml 2.3 mM DNA + 0.5 ml 10^{-1} SSC	3 h (22°)	−2.2	> 30	−8.8	2.4
+ 3 ml 3.3 mM AAAF (ethanol)	6.5 h (22°)	−0.8		−4.7	–
	37° (after 0.1 h)	−0.25		+1.0	2.2
	22° (8 h)	−0.44		−1.4	–
	37°	−0.2		+1.0	
	loose ligands extracted [c]	−0.9	8.4	+0.4 [b]	0.5 [b]
III. 3 mg DNA + 0.1 ml H_2O (to give a gel) + 0.3 ml 10^{-1} SSC + 3 ml 3.3 mM AAAF (ethanol) + (after 2h) 4 ml 0.25 M NaCl	30 h (22°)		13		
	loose ligands extracted [c]	−0.94	6	−2.4 [b]	0.1–0.3 [b]
IV. 3 ml 2.3 mM DNA + 0.7 ml 1 M NaCl	6 h (22°)	−1.2	9	−3.6	1.2
+ 3 ml methanol + 0.3 ml 3.3 mM AAAF (DMSO)	57°	−0.9	10	−1.9	0.8
V. 3 ml 2.3 mM DNA + 0.7 ml H_2O +	6 h (22°)	−1.7	9	−5.4	
3 ml methanol + 0.3 ml 3.3 mM	45°	−0.2	13	−1.5	–
AAAF (DMSO)	loose ligand extracted [c]	−0.1	4.8	+0.1	0.5
VI. 3 ml 2.3 mM DNA + 0.5 ml 10^{-1} SSC	1 h (22°)	−1.5	8.2	−4.8	0.25 (0.1 [b])
+ 2 ml methanol = *reference sample*	60°	−0.03	11	−0.6	–
VII. 3 ml 2.3 mM DNA + 0.5 ml 10^{-1} SSC	2 h (22°)	−1.4	10.5	−4.1	2.6
+ 2 ml methanol + 0.3 ml 3.3 mM	12 h (22°)	−1.4	11	−5.0	2.1
AAAF (DMSO)	loose ligands extracted	−1.4	–	−4.8	0.3
VIII. 3 ml 2.3 mM DNA + 0.5 ml 10^{-1} SSC	0 h, 22°	−1.4	> 35	−4.8	9.3
+ 2 ml methanol + a ml 3.3 mM	12 h (22°)	−1.4		−4.8	
AAAF (DMSO)					
IX. 3 ml 2.3 mM DNA + 0.5 ml 10^{-1} SSC	1 h (22°)	−0.6	ca 30	−2.2	3.2
+ 2 ml methanol, heating to 60°C	12 h (22°)	−0.8	ca 30	−2.7	2.5
(1 h), cooling to −70°C, + 0.3 ml 3.3 mM AAAF (DMSO)	loose ligands extracted	−0.26	9.5	−1.0 (−0.4 [b]) [s]	0.5
X. 3 ml 2.3 mM DNA + 0.5 ml 10^{-1} SSC	12 h (22°)	−1.2		−4.6	11
+ 2 ml methanol + 2 ml 3.3 mM	50° (1 h)	−1.0			
AAAF (DMSO)	loose ligands extracted	−0.25	7.5	+0.6 [b]	0.6 [b]
XI. 2 ml 2.3 mM DNA + 0.5 ml 10^{-1} SSC	12 h (22°)	−0.6	> 30	−2.1	11
+ 2 ml methanol + 0.2 ml 1 M NaCl	50° (1 h)	−0.3		−1.5	
+ 2 ml AAAF (DMSO)	loose ligands extracted	−0.50	7.9	−0.2 [b]	0.2 [b]

[a] E.g., sample II: stored at 22°C for 3 h, then measured (at 22°), then stored for further 3.5 h and measured, then heated to 37°C and measured after 0.1 h at this temperature, thereafter stored for 8 h at 22°C and measured, then measured at 37°C, then the DNA was precipitated and washed with ethanol and redissolved in distilled water (measurement at 22°)
[b] At 306 nm. [c] Concentration according to A_{257}. [s] Shoulder in spectrum.

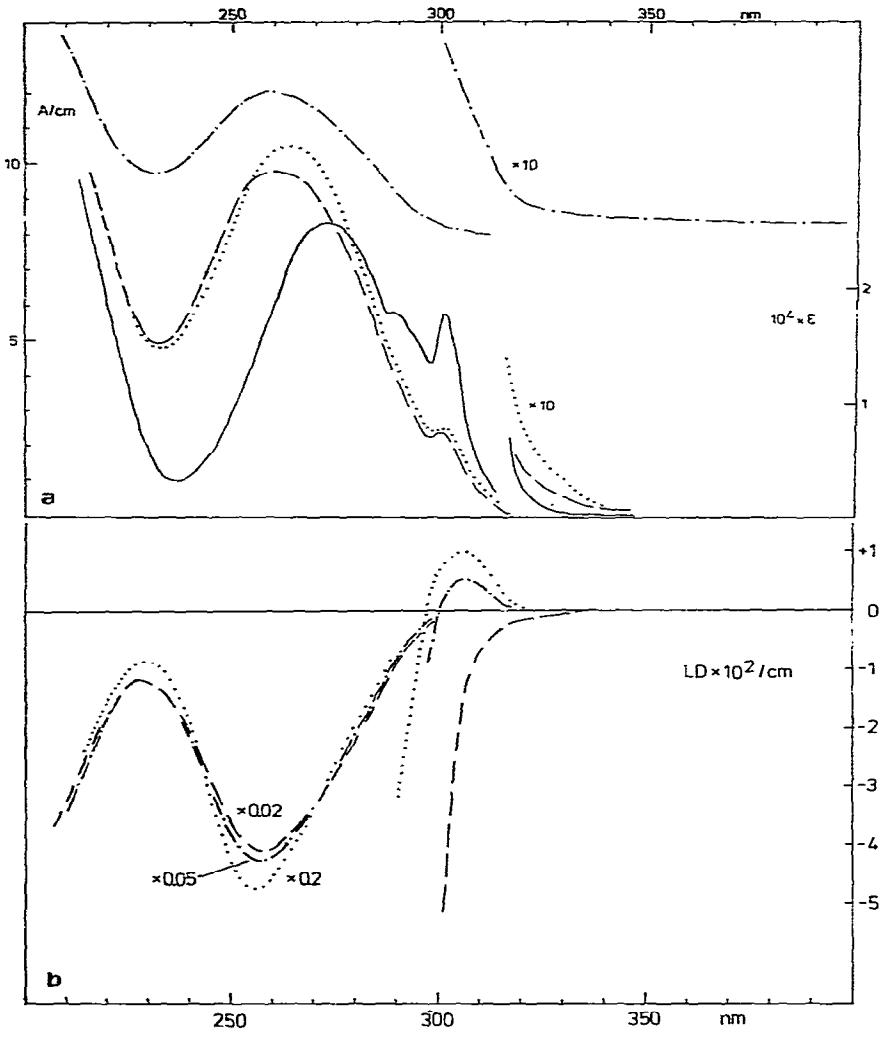


Fig. 1. Absorption (a) and linear dichroism (b) spectra of AAAF in ethanol (——) and of sample I, before (– – –) and after (· · ·) denaturation; sample II after extracting non-covalently bound ligands with ethanol (– · –, absorption curve is vertically offset).

3. Results

The behaviour of the samples is shown in table 1 and in figs. 1–2. Neither in sample I (low AAAF) nor in II (high AAAF) is there any marked separate LD due to the fluorene chromophore even after several hours at room temperature. Closer study reveals a very weak negative contribution in the region 270–300 nm (cf. the absorption spectrum of AAAF, fig. 1). This LD can be explained by a fraction of AAAF solubilized as a complex bound at hydrophobic DNA sites.

A remarkable change, characterized by the development of a positive band at 307 nm in the LD spectrum, is observed when a "sodium-free" sample is heated and

thereafter cooled to room-temperature. The wavelength of this band coincides with an absorption band observed by Miller et al. [7] in denatured DNA treated with AAAF. The LD band corresponds to a weak shoulder in the absorption spectrum (fig. 1). The positive band is also observed in sample II after raising the temperature.

The temperature effect is clearly related to denaturation or partial denaturation: heating to 60° (sample I) and to 37° (sample II) respectively, results in 87 and 80% decreases in the LD signal at 257 nm. The LD at 257 nm is almost entirely due to absorption from bases located in (oriented) double-stranded regions, and can thus be taken as an index of the amount of ordered double-helix structure. The positive LD bands at 306 nm are of the same order of magnitude in II and I indicating that the formation of the AAAF-DNA complex reaches saturation in I.

When sample II is brought from 37°C to room-temperature, some reassociation occurs (according to the LD_{257}). The effect of the reassociation appears to be a weakening of the positive band at 306 nm and this

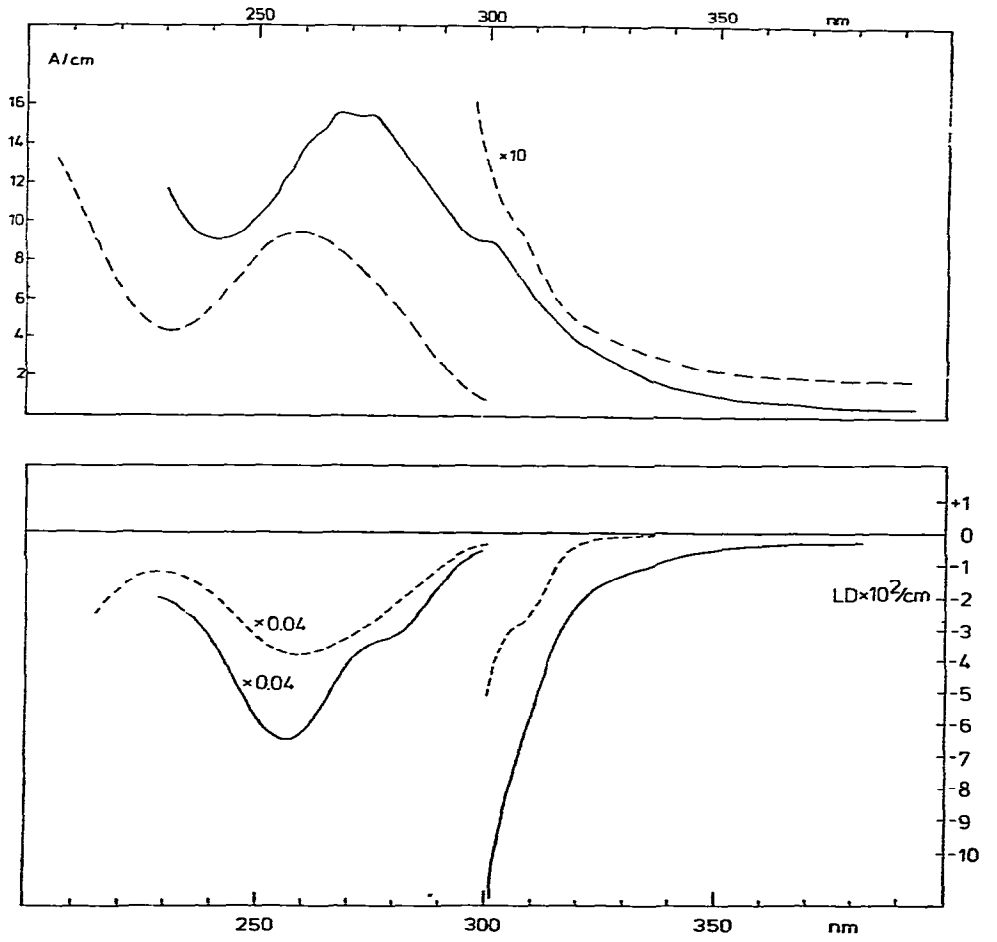


Fig. 2 (a).

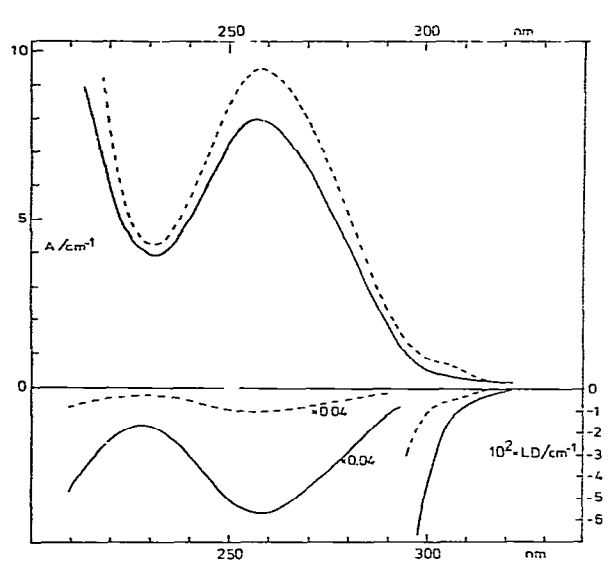


Fig. 2 (b)

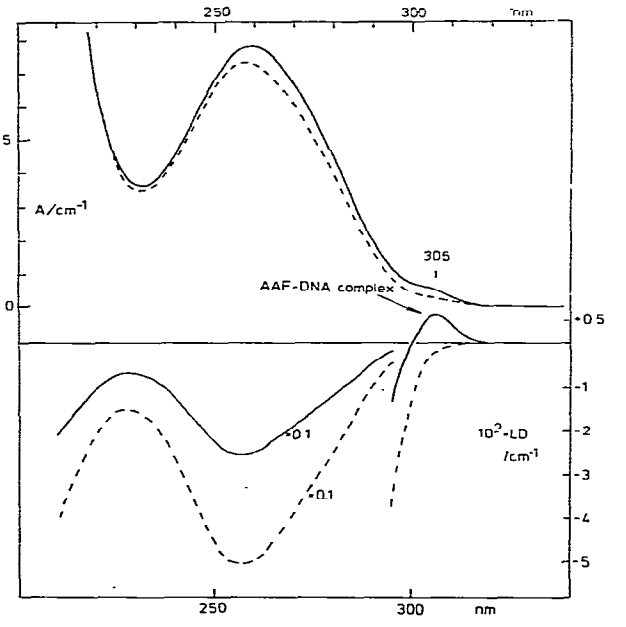


Fig. 2 (c)

Fig. 2. Absorption and linear dichroism spectra of: (a) sample III 30 hours after mixing (——) and after extracting weakly bound ligands (– – –), (b) sample VII (——) and sample IX (– – –) after extraction with ethanol (c) sample X (——) and sample XI (– – –) after extraction with ethanol.

band is finally swamped in a negative LD slope. The positive band appears again on heating the solution to 37°C. The negative LD seems to be due to a weaker (van der Waals) aminofluorene complex which dissociates when the strands are beginning to separate. The negative LD is qualitatively consistent with intercalation. That the positive LD is due to the covalent complex, is shown by the LD spectrum observed after precipitating and washing the DNA with ethanol (removing the weaker complex) and then redissolving it in water, see fig. 1.

As can be seen in fig. 2 the native DNA in sample III also reacts with AAAF but more slowly. The long-wavelength tail in the absorption suggests the formation of species (hydrolysis products) other than those observed in samples I and II. That the interaction is different in this experiment is indicated by the LD spectrum which has a negative shoulder at around 310 nm, and two additional (negative) shoulders at 280 nm and 330 nm. A decrease in LD at 257 nm indicates 10–30% strand separation.

Part of the negative fluorene LD is due to "intercalated", weakly bound AAAF since the 280 nm shoulder disappears when the DNA is precipitated and washed with ethanol and redissolved in water. However, the negative shoulder at longer wavelength (now at 306 nm) remains, demonstrating that in this covalent complex the fluorene has a different orientation from that in the complex formed with denatured DNA.

Reassociated DNA reacts more readily with AAAF than native DNA (cf. sample VII and sample IX). The LD spectrum of this complex shows evidence of a positive contribution at 300 nm (see fig. 2b).

The effect of denaturation on the reaction with AAAF is definitely established by the appearance of a positive LD band at 306 nm, and a corresponding shoulder in the absorption, of sample X, but not of XI, after heating to 50°C and extraction with ethanol. Sample XI, with a higher salt content, is not denatured at 50° and as is seen from fig. 2c the amount of reacted AAAF is practically negligible.

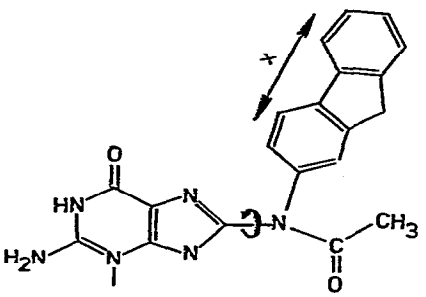
4. Discussion

The average angular orientation of a separate absorbing transition dipole (at wavelength λ) in a DNA complex can be related to the LD observed for the $\pi \rightarrow \pi^*$ transitions of the base chromophores at 257 nm:

$$\text{“}\alpha\text{”} = \arccos\{[1-(LD/A_r)_\lambda/(LD/A_r)_{257}]/3\}^{1/2}, \quad (1)$$

where A_r is the absorbance of the sample at random orientation. Eq. (1) presumes 90° between the base-planes and the effective orientation axis (the local axis) of each segment. α is the angle the dipole makes with the local axis [14] (“α” denotes that the value is the formal angle corresponding to an orientation average $\langle\cos^2\alpha\rangle$). The LD/A_r value +0.01 observed for the 306 nm band in the covalent aminofluorene complex formed under conditions giving strand separation is consistent with “α” = 47°. Quantum mechanical [15] and linear dichroism [16] results show that this transition is polarized parallel to the x-axis in the fluorene nucleus.

The negative LD observed in the complex with native DNA suggests a slightly different orientation: “α” = 67–90° (90° if the absorbance of the background is subtracted from the shoulder). This difference indicates that in the first case fluorene is oriented with its long axis more parallel to the reassociated helix; this may be a consequence of steric direction by the strands or it may be an effect of the orientation of a free loop. All evidence indicates that the single-stranded regions of reassociated DNA give no LD [14] so that the observed fluorene LD is likely to derive from material located within, or close to, double-stranded regions. It is noteworthy however that if the C_8 (guanine)-N aminofluorene) bond has a radial direction with respect to the local helix axis, as it would in standard DNA double helix, and the x-axis in fluorene is at the angle 109° to this bond and is freely rotating around the bond, an “α” of 53° would be expected (this is most easily shown by means of the “statistical ensemble method” [17]). In any case it can be concluded that this result is inconsistent with binding according to the intercalation model. In the case of the native complex intercalation is feasible, but models suggest a major groove site extending across the phospho ribose chain as more probable.



A pertinent question is the number of covalently bound fluorene residues. Since it is reasonable to assign the 306 nm band in the complex to the transition responsible for the 302 nm band in the free parent acetoxy compound a rough estimate can be made by employing the same extinction coefficient (1.8×10^4 $M^{-1}cm^{-1}$). Depending on how the contribution to the 306 nm shoulder of the redissolved DNA sample is evaluated, this gives in the present samples 1–2 aminofluorene residues per 100 bases.

5. Concluding remarks

The observations described here show that LD techniques give useful information on ligand binding to DNA: they demonstrate unequivocally the formation of a complex (since unbound ligand would give no LD), they give some idea of the orientation of the ligand and in the present case they suggest that there are several types of binding.

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