A clarification of the complex spectrum observed with the ultraviolet circular dichroism of ethidium bromide bound to DNA

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ABSTRACT

Ethidium bromide intercalation strongly affects the circular dichroism spectrum of DNA in the region of 230-300 m μ , in a complex manner. In this report we present a study that quantizes the relationships of the circular dichroism spectrum in the region of 230-300 m μ and the ethidium bromide induced optical activity centered around 308 m μ .

We present evidence of two hidden cooperative bands that are probably the negative counterparts of the 308 m μ band and 330 m μ shoulder positive cooperative bands. The hidden band is quantitatively characterized. We confirm that the direct effect of ethidium bromide on the DNA spectrum is simply linearly proportional to the amount of intercalated dye.

We also observe that the ethidium bromide enters freely when there is a molecule intercalated for every 3 sites, but that the intercalation is more difficult when the molecule intercalates at every second site.

INTRODUCTION

The ethidium bromide interaction with DNA is important for at least two reasons:

- 1) The ethidium bromide molecule can be used as a probe of the DNA conformation in such structures as the closed circular DNA of phages and viruses and the chromatin of eukaryotic cells (1-5).
- 2) The ethidium bromide interaction with DNA, probably an intercalation between two couples of paired bases, can be considered a typical model for a certain class of intercalating agents (6).

The ethidium bromide molecule is an interesting probe also for the structure of RNA molecules (7).

Important steps in the understanding of the ethidium bromide-DNA interaction are the discovery of a metachromatic shift in the absorption spectrum near 500 m μ (8), the observed large increase in the fluorescence quantum efficiency of intercalated ethidium bromide (6), and the observation that the induced optical activity near 308 m μ is dependent on the ratio of the

amount of DNA-bound ethidium bromide to the number of nucleotides (9,10). This has been interpreted as suggesting that the induced circular dichroism of an intercalated ethidium bromide molecule depends upon the presence of a second molecule intercalated in the vicinity of the first.

The ethidium bromide intercalation also strongly affects the circular dichroism spectrum of the DNA in the region of 230-300 m μ in a complex manner. In this report a quantitative analysis is presented of the relationships already suggested by previous authors (11-14) between the effect of ethidium bromide on the DNA circular dichroism spectrum in the region of 230-300 m μ and the ethidium bromide induced optical activity centered around 308 m μ .

EXPERIMENTAL SECTION

Preparation of Solutions

We used liver DNA from male Wistar rats. Purified DNA (Marmur procedure) was a gift of Dr. S. Abanobi (Fels Research Institute, Temple University School of Medicine, Philadelphia, Pa.). The ratios for absorption of this DNA at 260:230:280 m μ were 1.0:0.44:0.52, typical of a purified DNA (15). The molecular weight of this DNA, as measured with sucrose gradient, is approximately 10^7 Daltons.

The DNA was dissolved in SSC 1/20 (NaCl 0.0075 M, Na citrate 0.00075 M, pH 7.0) and dialyzed once against the same buffer. The concentrations of the working solutions were determined spectrophotometrically using an extinction coefficient of 6600 at 260 m μ (16). The ethidium bromide solution in the same buffer was prepared every week and kept in the dark at 4°C until it was used. Ethidium bromide concentrations were determined using a molar extinction coefficient of 5600 at 480 m μ (17).

The DNA-ethidium bromide complex solutions were diluted to the standard volume of 3 ml, always with the SSC 1/20 buffer. The DNA concentration was maintained constant ($0.151 \cdot 10^{-3}$ or $0.121 \cdot 10^{-3}$) and the amount of the dye was varied. Lower DNA concentrations were used only for the binding studies.

Binding Studies

For the study of the equilibrium between free and bound molecules for our DNA and our buffer, we employed the well known equation (Scatchard) (17):

$$\frac{r}{c} = K_n - K_r \quad (1)$$

In our case r is the ratio of bound ethidium bromide per nucleotide, n is the number of binding sites per nucleotide, K is the intrinsic association constant to a site, and c is the free dye concentration. The amount of bound dye was measured spectrofluorimetrically, using for the evaluation the method of J.B. LePecq and C. Paoletti (6). We utilized an Aminco-Bowman spectrofluorimeter with a 1 cm x 1 cm cell. The wavelength of the excitation was 520 m μ and the wavelength of the emission 590 m μ .

The previous method is more sensitive and was used for the low concentration studies needed for the Scatchard plot. For high DNA and ethidium bromide concentrations we measured spectrophotometrically the metachromatic effect in the 400-600 m μ region, following essentially the method of M.J. Waring (8).

Circular Dichroism Measurements

Circular dichroism was measured using a Jasco Model J40 recording spectropolarimeter with CD only. The instrument was standardized as described by Simpson and Sobel (18).

All experiments were carried out at 25°C in nitrogen atmosphere in a fused quartz 1 cm cell. The time constant was 4", the scanning speed 10 nm/min, the dynode voltage was not over 400 volts, and the precision in band wavelength was 0.3 μ m. The degrees of ellipticity, obtained directly from the recorder chart, are designated as ' Ψ '. $[\theta]$, the molar ellipticity is expressed in terms of degrees $\cdot$ cm²/decimole.

RESULTS

Fig. 1 gives the scatchard plot for the DNA-ethidium bromide interaction with rat liver DNA and SSC 1/20 buffer at 26°C. The value for the intrinsic association constant ' K ' is $1.3 \cdot 10^6$ (moles/l).

Figure 1: Scatchard plot for the interaction between DNA and ethidium bromide. Buffer SSC 1/20 (pH 7.0).

In apparent agreement with the Crother's exclusion model (21) it was found that for an ethidium bromide over DNA ratio $> 1/6$, the ethidium bromide seems to intercalate with increasing difficulty (Fig. 2). We are aware of the limitation of Scatchard plot analysis (21) which we used here only to determine the range of dye and DNA concentration where all ethidium bromide is bound ($>95\%$). This condition is, in effect, necessary in our quantitative study of the dye-DNA complex CD band.

For the induced circular dichroism band at 308 and 335 $m\mu$, our findings completely confirm the dependence of these bands on the ratio ethidium bromide/nucleotide observed by Dalglish *et al.* (9), Aktipis and Kindelis (3),

Figure 2: $\square$ — $\square$, $\triangle$ — $\triangle$, are two independent experiments where $[\theta]$ is the molar ellipticity measured per total ethidium bromide. $\circ$ — $\circ$ gives $[\theta]$ per bound ethidium bromide. The bound ethidium bromide was determined spectrophotometrically. The DNA is always $151 \cdot 10^{-6}$ M.

and Cheng, Lee *et al.* (11).

The effect of the ethidium bromide intercalation is apparently rather complex. The 273 $m\mu$ positive peak of the DNA dye complex initially increases in an almost proportional manner to the amount of ethidium bromide intercalated, but then the slope of the increase is progressively reduced and finally the 273 $m\mu$ peak decreases more and more rapidly, as shown in Fig. 3. Dalglish (9) made a similar observation, but no clear explanation was given of this phenomenon. We suggest that by itself the DNA ellipticity θ_{DNA}^{EB} is increased by the ethidium bromide intercalation proportionally to the amount

of the ethidium bromide intercalated

$$\theta_{\text{DNA}}^{\text{EB}}(\lambda) = \theta_{\text{DNA}}^0(\lambda) (1 + K_{\lambda} C_{\text{EB}})$$

where C_{EB} is the concentration of bound ethidium bromide and $\theta_{\text{DNA}}^0(\lambda)$ is the CD spectrum of DNA in the absence of ethidium. The slope K_{λ} is a function of the wavelength and is found from the ethidium bromide effect in the DNA region at low r ($r < 0.03$ when the cooperative effect is negligible); in fact, as shown by Fig. 3, in this region of low r the DNA positive band, centered at 273 $m\mu$, and the negative counterpart, centered at 246 $m\mu$, increased proportionally to the amount of the ethidium bromide intercalated. From the observed CD band, $\theta_{\text{EXP}}^{\text{EB}}(\lambda)$ of the complex at various concentrations of ethidium bromide ($r_1 = 0.065$, $r_2 = 0.130$, $r_3 = 0.176$) between 300 and 230 $m\mu$, we subtract the value of the DNA CD band $\theta_{\text{DNA}}^{\text{ED}}(\lambda)$ extrapolated at the same concentration of ethidium bromide

$$\theta_{\text{HID}}^{\text{EB}}(\lambda) = \theta_{\text{EXP}}^{\text{EB}}(\lambda) - \theta_{\text{DNA}}^{\text{ED}}(\lambda)$$

where $\theta_{\text{HID}}^{\text{EB}}(\lambda)$ is a band, which represents a hidden band continuation of the 300-350 $m\mu$ ethidium bromide positive cooperative band.

Figure 3: Δ --- Δ The peak of the cooperative band at 308 $m\mu$ (' ψ ' proportional to the square of the bound ethidium bromide); $\circ$ — $\circ$ The positive 273 $m\mu$ DNA peak (a positive linear increment for low r followed by a subtraction of the hidden cooperative band for high r); $\square$ $\square$ The negative 246 $m\mu$ DNA peak (a negative linear increment for low r followed by an addition of the hidden cooperative band for high r); x — $\circ$ — x The negative shoulder at 230 $m\mu$ (the negative increment remains linear even for high r , because it is not affected by the hidden band).

Fig. 4 represents a summary showing, for various concentrations of ethidium bromide, the CD spectra for the complex and the components into which we propose to resolve it. Using the above data, we look for the function which best fits the hidden band dependence from the ethidium bromide concentration. A least square fit has been done of the function

$$y = a C_{EB}^b$$

to determine the approximate magnitude of a and b . Using the experimental ellipticity of the hidden band, at 10 different wavelengths between 250 and 300 mμ, the values become

$$b = 2.04 \pm 0.09$$

$$a = \theta_{HID}^1 (\lambda)$$

Figure 4: Summary Figure: A) Observed CD spectra of the complex DNA-ethidium bromide, between 230 and 350 nm, for different ethidium bromide concentrations, at constant DNA concentration of $121 \cdot 10^{-6}$ M.

(----) no ethidium; (○—○) $3.9 \cdot 10^{-6}$ M bound ethidium bromide; (○—○) $7.9 \cdot 10^{-6}$ M; (△—△) $15.8 \cdot 10^{-6}$ M; (□—□) $21.4 \cdot 10^{-6}$ M.

B) Proposed CD spectra for the components (DNA alone and ethidium bromide alone) into which we resolve the observed CD spectra of the complex.

DNA band for different concentrations of bound ethidium bromide: none (---); $3.9 \cdot 10^{-6}$ M (x---x); $7.9 \cdot 10^{-6}$ M (○---○); $15.6 \cdot 10^{-6}$ M (△---△).

Ethidium bromide bands, hidden and known, for different concentrations: (○—○) $3.9 \cdot 10^{-6}$ M; (○—○) $7.9 \cdot 10^{-6}$ M; $15.6 \cdot 10^{-6}$ M (△—△).

$21.4 \cdot 10^{-6}$ M (□—□)

where $\theta_{\text{HID}}^1(\lambda)$ represents the hidden band ellipticity, at given wavelength λ , at the concentration of ethidium used as reference unit. The correlation coefficient was better than 0.996 at all wavelengths, indicating that the function which best fit the hidden band is

$$\theta_{\text{HID}}^{\text{EB}}(\lambda) \approx \theta_{\text{HID}}^1(\lambda) \cdot c_{\text{EB}}^2$$

This would imply that only nearest neighbor interaction contributes to the ethidium exciton band; a "counterprouve" of the existence of such conservative exciton band for the ethidium is given by the fact that the negative and positive sections have opposite and equal rotational strengths (the ratio between the two integrals under the opposite band, is about 1.01).

Figure 5: Comparison of the relationship between the cooperative band and the hidden band (○—○ 308/280-285 ratio; Δ---Δ 308/265 ratio), and the relationship between the cooperative band and the increment of the negative 246 mμ DNA peak (□—□ 308/Δ 246 ratio). The hidden band is cooperative and strictly related to the known cooperative band. On the contrary, Δ 246 is clearly linear and not cooperative.

Furthermore, if the hidden band is related to the 308 mμ ethidium bromide cooperative band, the ratio between the 308 mμ peak and the peak of the hidden band around 285 mμ should remain constant for increasing amounts of ethidium bromide intercalated. Fig. 5 shows this to be the case. The same phenomenon is seen for the ratio of the 330 mμ shoulder over the 265 mμ shoulder (not shown), and of the 308 mμ peak over the 265 mμ shoulder of the hidden band. In contrast, the ratio of the 308 mμ peak over the Δ 246 mμ peak (Δ 246 indicates the increment over the value of this peak for DNA alone) increases pro-

portionally to the dose, in agreement with the fact that the 308 $m\mu$ band is a cooperative one, but $\Delta 246 m\mu$ is simply proportional to the amount of ethidium bromide intercalated. In the negative part of the DNA band a shoulder appears around 230 $m\mu$ with increasing concentrations of bound ethidium bromide (Fig. 6). Fig. 3 shows clearly that the increase in this shoulder has a different slope than the increase in the negative peak at 246 $m\mu$, but it is again rigorously linear and not cooperative. Moreover, because it is completely out of the hidden band region, it increases linearly even in the high r region. This is strongly in favor of our hypothesis of a proportional effect of ethidium bromide on the DNA spectrum.

Figure 6: Evidence of the 230 $m\mu$ shoulder. The observed negative DNA band at different concentrations of bound ethidium bromide: — no ethidium bromide; — ethidium bromide $7.9 \cdot 10^{-6}$ M; - - - - - ethidium bromide $15.8 \cdot 10^{-6}$ M; ○—○—○ ethidium bromide $21.4 \cdot 10^{-6}$ M; ethidium bromide $22.2 \cdot 10^{-6}$ M. The concentration of DNA is $121 \cdot 10^{-6}$ M.

DISCUSSION

Previous authors (10-14) suggested, but never analytically proved, the existence of a single small negative counterpart centered around 290 $m\mu$ for the CD positive band of ethidium bromide at 308 $m\mu$. Our findings, instead, prove analytically the existence of two negative CD bands at about 265 and 285 $m\mu$ of compatible magnitude with respect to the positive counterpart of ethidium bromide CD bands at 308 and 330 $m\mu$. The hypothesis is that the induced CD bands near 308 $m\mu$ and 330 $m\mu$ are the result of direct interaction between two or more regularly ordered ethidium bromide molecules, from which one should expect a conservative doublet characteristic of exciton splitting (19). This expectation is now satisfied. The apparent anomalous effect of

ethidium bromide on the DNA-like doublet centered around 260 m μ disappears. Ethidium bromide, like proflavine, 4-ethyl-9-aminoacridine and 9-aminoacridine (8) increases linearly the DNA spectrum amplitude. Dalglish (8) had inferred from the apparent difference in the behavior between ethidium bromide and these three compounds that major differences exist in the type of intercalation. This is probably not the case.

At 230, 246 and 273 m μ there is a very sensitive effect which is proportional to the amount of ethidium bromide incorporated. Binding studies at low concentrations may become possible with the CD.

Our observations favor the idea that in circular dichroism studies the effects of ethidium bromide on the DNA band and its own induced cooperative band (hidden band + 300-350 m μ region) are specific for a unique site (the intercalation site). In fluorimetry and spectroscopy the secondary sites are only quantitatively but not qualitatively different from the intercalation sites (20). This difference points to a clear advantage of the CD technique for many studies.

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