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THE USE OF FLUORESCENCE ANISOTROPY DECAY OF POLY d(A–T) ETHIDIUM BROMIDE COMPLEX TO ESTIMATE THE UNWINDING ANGLE OF THE DOUBLE HELIX[1]

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We measured the fluorescence decay under polarized light, of ethidium bromide bound to the poly d(A–T) isolated from Cancer Pagurus. The decay of the whole fluorescence is a single exponential function revealing a good homogeneity of the binding sites. The anisotropy decay due to energy transfers between the ethidium bromide molecules bound to a same poly d(A–T) molecule has been analysed, with a Monte Carlo calculation. We found that the dye unwinds the poly d(A–T) duplex by an angle of 17° ± 2°. This result is in agreement with the value previously found in the case of calf thymus DNA–ethidium bromide complex, although the base compositions of the two nucleic acids are different.

1. Introduction

In previous work [1, 10] we found that ethidium bromide (EB), inserted between two DNA base pairs, leads to a local unwinding of the DNA double helix of about 16°. This result had been obtained by studying the fluorescence anisotropy decay of EB bound to DNA.

It was shown that the decay increases when P/D (ratio of molar concentrations of nucleotide and dye) decreases. This phenomenon was attributed to energy migration [2] from chromophore to chromophore bound to a same macromolecule.

Experimental results were compared with calculated curves obtained by simulation on a computer. The rate of the elementary transfer was given by Förster's formula [3].

DNA was isolated from calf thymus and contained 42% G–C base pairs. One can therefore think that the measured angle is only a mean value among the unwinding angles associated to the different possible fixation sites of EB on DNA.

It was then interesting to study a simpler nucleic acid by the same method. We chose the alternating poly d(A–T) from Cancer Pagurus.

2. Materials and methods

2.1. Biochemicals

Poly d(A–T) isolated from Cancer Pagurus is a DNA rich in A–T base pairs. It is composed of 90% of alternating A–T, 7% of non-alternating A–T, and 3% of G–C [4].

The extraction of total DNA has been realized by a modified Baranovska method [5]. From total DNA, we separated the constituent which is rich in A–T, by elution with a linear gradient of potassium phosphate buffer in a hydroxyapatite column at 70°C [6]. This method is based on the lower melting temperature of DNA rich in A–T.

The average molar weight of the sample used was about 900 000 and was deduced from its sedimentation coefficient in SSC medium (0.15 M NaCl,

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0.015 M Na Citrat) $S_{20w} = 10.23$ [7].

Solutions of poly d(A–T)–EB complex were studied in 0.15 M NaCl. The concentrations of poly d(A–T) are of the order of 10^{-3} M/ℓ in nucleotides. These concentrations as well as the concentrations of ethidium bromide were measured with a Cary 14 Spectrophotometer. The molar extinction coefficients were $\epsilon_{d(A-T)} = 6\,000\ M^{-1} \cdot cm^{-1}$ at 260 nm for poly d(A–T) and $\epsilon_{EB} = 5600\ M^{-1} \cdot cm^{-1}$ at 480 nm for free EB.

2.2. Fluorescence measurements

The fluorescence decays in polarized light were measured by a method already described [8]. Samples were excited by a vertically polarized pulse and the decays of the principal polarized components of the fluorescence, $i_V(t)$ and $i_H(t)$ were recorded at 90° of the exciting beam. The following functions are then deduced:

$$s(t) = i_V(t) + 2i_H(t), \qquad d(t) = i_V(t) - i_H(t).$$

These curves depend on the response function $g(t)$ of the apparatus in the following way:

$$s(t) = \int_0^t g(T)\,S(t-T)\,dT,$$

$$d(t) = \int_0^t g(T)\,D(t-T)\,dT,$$

where $S(t)$ and $D(t)$ are the decays corresponding to the ideal case of an infinitely short excitation associated with a detection system of infinite resolution. $g(t)$ is determined with a reference solution [9] of DODCI (3–3′ diethyloxadicarbocyanine iodide) in ethanol, whose fluorescence lifetime has been found equal to 0.93 ns.

The fluorescence anisotropy decay is defined by

$$r(t) = D(t)/S(t).$$

2.3. Analysis of results

The principle of the analysis of the results has already been described in detail [1,10]. $S(t)$ is determined by a deconvolution [11] of experimental curves $s(t)$. $r(t)$ is assumed to be equal to

$$r(t) = r_0 r_B(t) r_T(t),$$

where r_0 is the anisotropy at time $t = 0$, $r_B(t)$ the decay anisotropy due to Brownian motion and $r_T(t)$ the decay anisotropy due to energy migration. $r_0 r_B(t)$ is the anisotropy of a solution of the complex having a high P/D ratio. Under these conditions, energy transfers are negligible.

$r_T(t)$ is obtained by a Monte Carlo simulation performed with a computer. The poly d(A–T)–EB complex structure is taken into account. We assume that poly d(A–T) is in the B form of DNA. In this form the angles of twist and of tilt, and the distances between the bases and the helix axis are very small so that we can adopt a simplified model in which these parameters are taken equal to zero.

EB is inserted between two base pairs. All the sites are equally distributed, with the restriction that two successive sites cannot be occupied [12]. Two chromophores are then separated by at least two base pairs. The normal distance and the normal angle between two base pairs are respectively 3.4 Å and 36°. When a dye is intercalated, this distance and this angle increase respectively by 3.4 Å and by δ. The elementary transfer rate between two EB molecules is given by Förster's formula [3]:

$$V = k_T K^2 / R^6.$$

The value of k_T has been obtained in our preceding work [1] ($k_T = 0.27 \times 10^7$ Å^6/ns). This constant depends on the following parameters: the overlap integral determined by measurements of the absorption and emission spectra, the refractive index of the medium and the natural lifetime of the excited state.

In the present case we have

$$K^2 = \cos^2 \theta,$$

where θ is the angle made by the dipolar moments of transition of two molecules separated by a distance R. θ depends on the unwiding angle δ. We then compute

$$D'(t) = r_0 r_B(t) r_T(t) S(t)$$

and the convolution of $D'(t)$ with $g(t)$ is calculated. One obtains a computed curve $d'(t)$ which is compared to the experimental curve $d(t)$. The average weighted residue F is calculated by the following formula [14]:

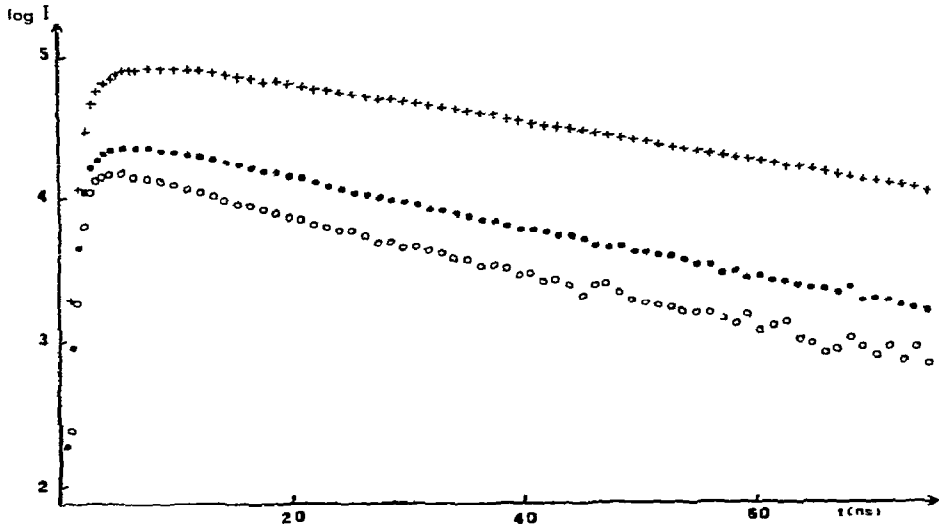

Fig. 1. Experimental curves $s(t)$ (+) and $d(t)$ (•: P/D = 100; ○: P/D = 7.35).

$$F = n^{-1} \sum_{i=1}^{n} (d_i - d'_i)^2 / d_i,$$

where n is the total number of the experimental points, d_i and d'_i the values of $d(t)$ and $d'(t)$ at time t_i. The best value of δ is the value which corresponds to the curve $d'(t)$ with the smallest value of F.

3. Results

We studied two solutions, 1 and 2, in which the value of P/D was respectively equal to 100 and 7.35. Fig. 1 shows the experimental curves $s(t)$ and $d(t)$. One finds that the $S(t)$ curves are practically the same for the two samples. They correspond to a single exponential decay:

$$S(t) = e^{-t/\tau},$$

with $\tau = 24.27$ ns for sample 1 and $\tau = 24.50$ ns for sample 2. r_0 and $r_B(t)$ are determined by analysis of the $d(t)$ curve of sample 1. We obtained $r_0 = 0.32$ and $r_B(t) = 0.6\, e^{-t/\theta} + 0.4$ with $\theta = 20$ ns.

In the analysis of sample 2(P/D=7.35), we used the $r_T(t)$ functions computed in our preceding work [1]. We varied δ from $-45°$ to $+20°$.

On fig. 2, the mean weighted residues F have been plotted as function of δ. One can see that the best fit is obtained for $\delta = -17°$. The errors on this determination lead to an uncertainty of 2°.

Fig. 3 shows a comparison between experimental

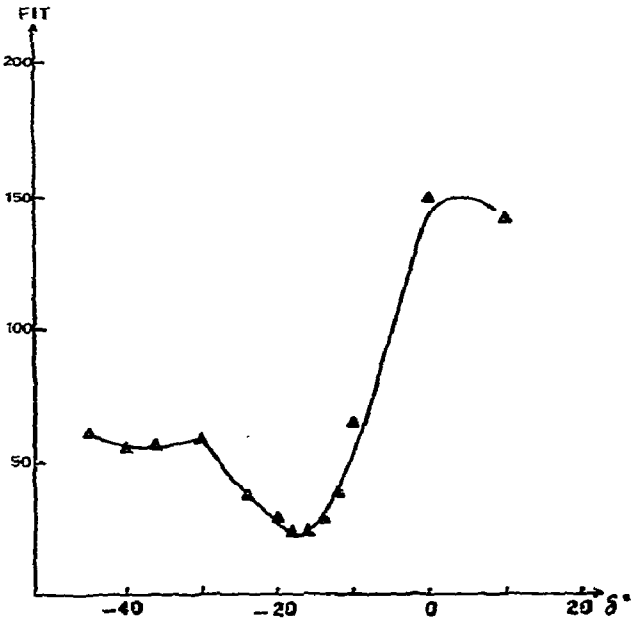

Fig. 2. Average weighted residue F for the different values of δ.

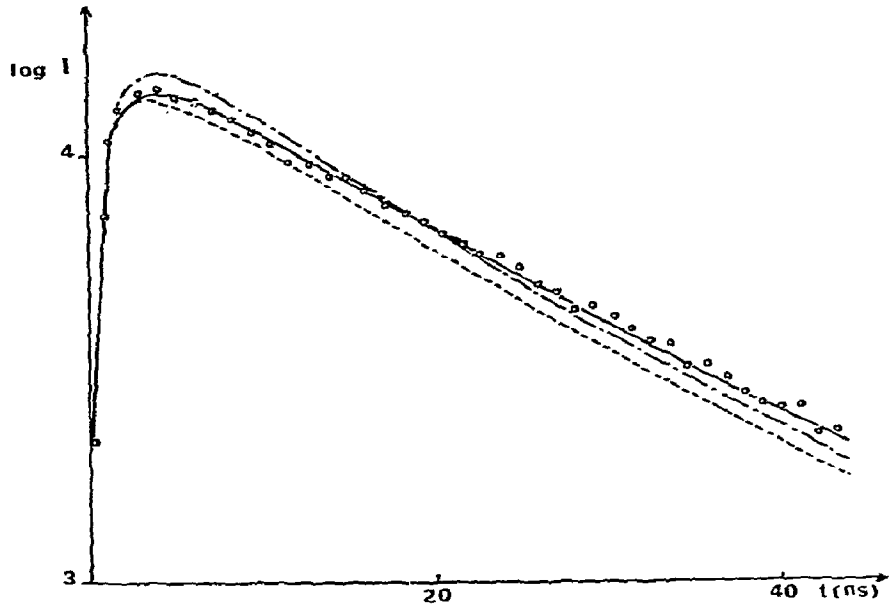

Fig. 3. Comparison of the experimental curve $d(t)$ for P/D = 7.35 (o) with $d'(t)$ curves computed with different values of δ (– –: $\delta = -36°$; —— $\delta = -18°$; – – – $\delta = -10°$).

curve $d(t)$ of sample 2, and a few computed curves $d'(t)$.

4. Discussion

The poly d(A–T) we used is partially denatured [5] but one expects that the dye is bound to the sections of the molecule in double strand, since the binding constant is very low for the single-strand polynucleotides [15]. This is in agreement with the fact that the whole fluorescence decay is a single exponential function.

Intercalation of an EB molecule in poly d(A–T) induces an unwinding of the double helix of about 17°, which is practically identical to the one induced in calf thymus DNA. It seems, therefore, that this angle is independent of the bases nature of the site. In addition, the EB molecule appears to have a well-defined position in its site [1].

Studies about the unwinding of DNA helix by intercalation of ethidium bromide have been performed with the static polarization method [16]. It has been shown that this technique is not accurate enough to allow the determination of the unwinding angle [17]. On the contrary, as shown in this work and in our preceding one, the decay of anisotropy measurement permits us to determine this unwinding angle without ambiguity.

It has been reported [5,18] that the structure in solution of DNA rich in A–T is different from the B structure. We used the B form in our calculations and we obtained satisfactory results. However, our method is sensitive to the polymer structure. A study performed with poly (rA–rU) which adopts the A form of RNA leads to very different results [19]. It can be concluded that there is not much difference between the structure of poly d(A–T) and the structure of calf thymus DNA in solution.

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