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FLUORESCENCE ANISOTROPY DECAY OF ETHIDIUM BOUND TO CHROMATIN

Daniel GENEST, Georgette SABEUR, Philippe WAHL and Jean-Claude AUCHET*
Centre de Biophysique Moléculaire, C.N.R.S., 45045 Orleans Cedex, France

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The fluorescence anisotropy decays of the chromatin ethidium complexes have been measured in solutions in which the dye was bound to the high affinity sites of the nucleosome DNA. Energy transfers between chromatin-bound ethidium molecules cause an increase of the anisotropy decay rate for much smaller values of the concentration ratio of dye to nucleotide than in the case of nacked DNA–ethidium complexes. This result implies that the high affinity sites are clustered on a short nucleosomal DNA segment. Quantitative analysis of the experimental data by computer simulations of the energy transfer process, shows that these sites are gathered on a single nucleosomal DNA segment, 28 base pairs long. Such a segment probably belongs to the nucleosome "linker", contributing about half of it.

1. Introduction

A number of recent investigations brought about important knowledge about the organization of DNA and histones in chromatin. It is now well established that chromatin consists of repeating subunits containing approximatively 200 base pairs of DNA, called nucleosomes [1–11]. 140 base pairs of the nucleosomal DNA are wrapped around a core of histones, the rest forming a linker between the cores. The length of the linker depends on the nature of the tissue from which chromatin is extracted [5], while the number of base pairs of the core seems very stable.

Aromatic compounds such as ethidium bromide, which intercalate into nacked DNA, interacts also with DNA in chromatin. The study of this interaction should give informations about the accessibility of DNA in chromatin [12–22]. Measurements of binding isotherms indicate that ethidium bromide forms two kinds of intercalation complexes with chromatin, instead of one with nacked DNA. However, there is no complete agreement between the authors about the values of association constants and the values of the total number of sites which characterize these complexes. Furthermore, many of these works have been performed with chromatin obtained by shearing. This procedure has been found to destroy the superstructure of chromatin [7] and to modify the binding isotherms of ethidium chromatin complex [20]. Paoletti et al. used fluorescence titration to show that chromatin extracted by enzymatic digestion forms only one type of complex at low ethidium bromide concentration [20]. Increasing the ethidium bromide concentration induces a change in chromatin conformation and a second type of complex appears with a lower affinity of ethidium for chromatin. Furthermore static fluorescence polarization measurements indicate that in the first type of complex, the ethidium molecules are clustered together. Accurate quantitative data however cannot be obtained by these studies because of the difficulty of analyzing complex Scatchard's plots, and the lack of information brought about by fluorescence static polarization measurements.

In previous works we studied [23–27] ethidium acid nucleic complexes by pulse fluorimetry in polarized light. By this technique, we were able to follow the kinetics of excitation energy transfers between ethidium molecules bound to nucleic acids. Analyse of these data by computer simulation provided structural data on these complexes and espe-

* Technical assistance.

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cially accurate informations on the dye distribution along the nucleic acid double helix.

In the present work, this method has been applied to the study of the ethidium bromide complex of high affinity.

2. Materials and methods

2.1. Biochemicals

Three samples of chromatin obtained by enzymatic digestion have been used in this work. One preparation has been performed from Ehrlich ascite cells of infected mice according to a technique already described [20,65]. Both other preparations have been made from calf thymus stored at −70°C according to the technique described by Noll et al. [7]. The characterization of our chromatin preparations was done as follows: 1) The circular dichroism gave always an ellipticity at 283.5 nm equal to 2200 ± 200 deg cm^2 $dmole^{-1}$ which is characteristic of a native chromatin [66]. 2) The 3.5% polyacrylamid gels electrophoresis of the chromatin DNA, stained by ethidium bromide revealed no migration whereas a further digestion of our chromatin gave discrete bands of DNA under the same conditions, characteristic of mononucleosomes, dinucleosomes, etc. (fig. 1). 3) Electrophoresis of histones were done as previously described [65] and showed that all histones were present (see next paper, this issue). 4) The composition in DNA, protein and RNA was determined as previously described [65]. All these controls revealed that chromatin was native and present as large polynucleosomes.

The sample of ethidium bromide used was the same as previously [23–27]. All these solutions have been prepared in 2×10^{-4} M EDTA pH 7.7, from stock solutions of chromatin and ethidium bromide kept at 4°C in the dark. Concentration have been determined by absorption taking as molar absorptivities $\epsilon = 7300$ cm^2/mole at 260 nm for chromatin and $\epsilon = 5600$ cm^2/mole for ethidium at 480 nm. The ratio of molar concentrations of nucleotides and dye (P/D) varied from 27 to 2000. In any case the nucleotide concentration was greater than 10^{-3} M. As the affinity of ethidium for DNA is high, practically all the dye was bound to the chromatin under our experimental conditions and the number of ligands bound per nucleotide, r, was equal to D/P. All measurements were performed at 20°C, in 2×10^{-4} M EDTA (pH 7.7).

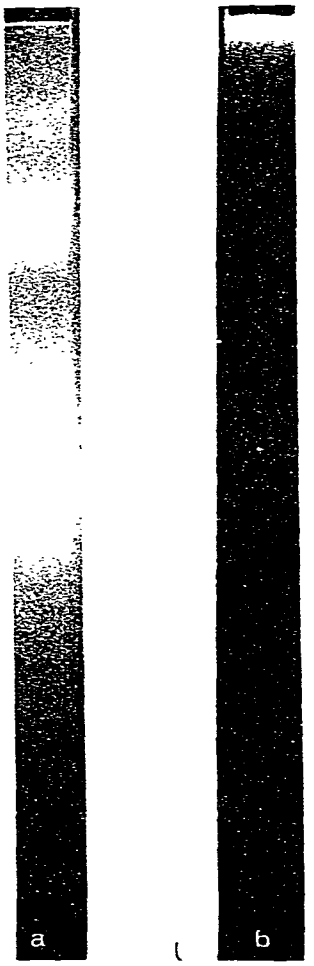

Fig. 1. 3.5% acrylamide electrophoresis gel of a sample of the chromatin used in our study (b) and of a further digestion of this chromatin (a).

2.2. Fluorescence measurements

Transient fluorescence measurements have been performed with an apparatus already described [23, 27–29]. Luminescent solutions were excited with

a short light pulse (1.6 ns at half-height) produced in N_2 at 15 kg/cm^2 pressure, and vertically polarized. The vertical and horizontal components of emission $i_v(t)$ and $i_h(t)$ were alternatively measured a great number of times, and the following transient curves were calculated:

$$s(t) = i_v(t) + 2i_h(t), \tag{1}$$

$$d(t) = i_v(t) - i_h(t). \tag{2}$$

The corresponding decays $S(t)$ and $D(t)$ are solutions of the following convolution equations:

$$s(t) = \int_0^t S(t - T)g(T)\,dT, \tag{3}$$

$$d(t) = \int_0^t D(t - T)g(T)\,dT, \tag{4}$$

while the fluorescence anisotropy decay is defined by:

$$A(t) = D(t)/S(t). \tag{5}$$

$g(t)$ was obtained by using an ethidium bromide solution as a reference [30], the lifetime of which was 1.65 ns [31]. $S(t)$ was obtained by a grid research of decay parameters [32].

For very small values of r ($\simeq 5 \times 10^{-4}$), depolarization was only due to rotational brownian motions, and $A(t)$ was a sum of exponential terms [33], the time constants of which being the correlation times of the motion. The parameters of these exponential sums were obtained by using a least square method recently described [34]. For higher values of r, energy migration introduced a new cause of depolarization [35,36]. The anisotropy was then assumed to be of the form: $A(t) = A_B(t)A_T(t)$ [24,37], where $A_B(t)$ and $A_T(t)$ were the brownian and the energy transfers contributions respectively. $A_B(t)$ was assumed to be identical to the anisotropy determined at low r values. $A_T(t)$ was determined by computer simulations as described in the following paragraph.

2.3. Computer simulation of fluorescence anisotropy decay

We shall briefly recall here the method of analysis set up and the results obtained in preceding works in which we studied the fluorescence anisotropy decay of ethidium-nucleic acids complexes [24–27]. We shall then describe how this method has been modified and applied in the present work.

The analysis of the anisotropy decay of ethidium-nucleic acids complexes was based on a comparison between experimental curves and computed curves obtained by a simulation of the excitation energy migration among the ethidium molecules included in a geometrical model of these complexes. This procedure was very similar to the method of Paoletti and Le Pecq which was set up by these authors in order to compute the steady-state polarization of ethidium–DNA complexes [46]. But there was an important difference between their computations and ours since we had to simulate the time course of the energy migration in order to describe the anisotropy decay of fluorescence [27], whereas in the Paoletti and Le Pecq simulation the steady-state polarization was directly computed.

According to the Förster's theory relative to the "very weak resonance interaction", the rate of excitation energy transfer is given by the following relation [35,47]:

$$k_T = \frac{C J \kappa^2}{n^4 \tau R^6}, \tag{6}$$

where C is a numerical constant, n the refractive index of the propagating medium (here the nucleic acid), J the overlap integral between absorption and emission spectra of the chromophore molecules, τ their fluorescence decay time, R the distance between the donor and acceptor molecule, and κ an angular factor.

In the case of the ethidium–DNA complexes, the Lerman's model [59] was chosen in which the DNA adopts the B structure, and the ethidium molecules intercalate between two base planes, lengthening their distance by 3.4 Å and unwinding their angle by δ. The ethidium molecule plane is perpendicular to the DNA longitudinal axis. In the simplest case studied, the position of the ethidium molecules in their sites was identical for all molecules, and the position of two chromophores was obtained from one another by a helical displacement around the longitudinal DNA axis. The angular factor κ^2 was reduced to $\cos^2\theta$ where θ was the rotation associated with this displacement. θ was simply related to δ. The distribution of the ethidium bromide molecules along a 200

base pairs DNA segment was obtained by a first type of simulation in which the probability of site occupancy was given by [46,27]:

$$p = r/(0.5 - Kr). \tag{7}$$

K had one of the values 0, 1 or 2 respectively, according to the following assumptions: 1) all the sites had the same probability of occupancy; 2) a site adjacent to an occupied one was excluded; 3) two adjacent sites were excluded.

The energy migration among the ethidium molecules bound to the same nucleic acid molecule was simulated by choosing the most central ethidium of the simulated distribution as the primary excited molecule.

The number of times that the excitation energy reached the most distal dyes bound to the 200 base pairs DNA segment was negligible. Therefore the calculation could be considered to correspond to an infinitely long DNA molecule. It was found that, among the three distribution laws considered, only the law based on the single excluded adjacent site was able to represent the experimental data ($K = 1$ in formula (7)). The validity of this last distribution, which had been proposed in order to describe the binding isotherms of dye intercalation into DNA [38], was then confirmed by our studies. The value δ which fitted the experimental data depended on refractive index n assumed for DNA. Taking $n = 1.6$, one found $\delta = -18°$.

In the present work, we were concerned with the high affinity complex formed between ethidium and chromatin. Chromatin is made of identical subunits containing a DNA segment of N_0 base pairs ($N_0 = 200$).

Static fluorescence polarization measurements and qualitative transient fluorescence measurements indicated that the ethidium high affinity sites were clustered on a fraction of the nucleosomal DNA. We assumed that these sites were identical to the nacked DNA sites, but that they were present only on DNA segments of equal length containing N base pairs each ($N < N_0$). Therefore we expected that the anisotropy depended on N and that the quantitative analysis of this curve would provide the value of N. The model of the complex used in this work was the one described above. δ was taken equal to $-18°$, the value which best fitted the experimental anisotropy decays of ethidium with naked DNA, when assuming $n = 1.6$; the distribution of the ethidium molecules along the segment of DNA was assumed to follow the rule of excluded adjacent sites. But the probability of site occupancy must be written now:

$$p = r'/(0.5 - r'), \tag{8}$$

where $r' = r/\alpha$, with $\alpha < 1$. α should satisfy the following equation:

$$\alpha = qN/N_0, \tag{9}$$

where q is the number of DNA segments per nucleosome which contained the high affinity sites.

The simulation of energy migration had to take into account a specific effect coming from the boundaries of the DNA segments. That meant that for a given r' value:

1) The primary excited molecules should be randomly chosen among the ethidium molecules bound to the DNA segment.

2) When the excitation reached one of the most distal molecule, the next possible migration steps were restricted to the nearest neighbour situated on the side of the DNA segment center.

The calculated time dependent anisotropy due to energy transfer could be considered as a function of r' and N. This function will be represented by $A_T(t, r', N)$. The determination of N was obtained in the following way: For one arbitrary value of N, and several values of r', we determined the functions $A_T(t, r', N)$ by computer simulation. These functions were compared with the experimental results (characterized by a binding ratio r), by computation of the residuals [39]:

$$R(r', N, r) = \frac{1}{n}\sum_{i=1}^{n} \frac{[d_c(t_i, r', N) - d_{ex}(t_i, r)]^2}{d_{ex}(t_i, r)},$$

where $d_c(t, r', N)$ was the computed convolution defined by:

$$d_c(t, r', N) = g(t) * [A_B(t)\, A_T(t, r', N) S(t)]$$

and $d_{ex}(t, r)$ the experimental transient fluorescence defined by the equation (2).

For each solution (r fixed) one plotted $R(r', N, r)$ as a function of r', and took the abscissa of the minimum which was designated by $\hat{r}'(N, r)$. The quality

Table 1
Fluorescence decay parameters $[S(t) = a_1 \exp(-t/\tau_1) + (1 - a_1)\exp(-t/\tau_2]$ of ethidium–chromatin complex for the three different preparations studied, respectively Ehrlich ascite, calf thymus and calf thymus. The fluorescence lifetimes are given with an accuracy of 0.5 ns

P/D	1515	380	200	135	103	81	64	50	40	33	27
a_1	0.12	0.08	0.09	0.12	0.07	0.10	0.12	0.06	0.08	0.13	0.14
τ_1 (ns)	9.5	7.0	9.5	9.5	8.0	9.0	9.5	8.0	7.5	9.5	9.5
τ_2 (ns)	24.0	23.5	23.5	23.5	23.0	23.0	23.0	22.5	22.5	22.5	22.5

P/D	1500	346	234	177	118	88	66	27
a_1	0.13	0.13	0.13	0.12	0.12	0.15	0.11	0.13
τ_1 (ns)	7.5	5.5	8.5	5.5	6.5	8.0	6.5	6.5
τ_2 (ns)	24.5	24.0	24.0	23.5	23.5	23.5	23.0	23.0

P/D	2000	1000	150	117	98	84	68
a_1	0.07	0.09	0.11	0.10	0.13	0.13	0.16
τ_1 (ns)	6.5	8.0	7.5	7.0	9.0	6.0	6.5
τ_2 (ns)	23.5	23.0	22.5	22.5	22.5	22.0	22.0

of this fit was checked by examining the following deviation functions [40]:

$$Dv(t_i, \hat{r}', N, r) = \frac{d_c(t_i, \hat{r}', N) - d_{ex}(t_i, r)}{[d_{ex}(t_i, r)]^{1/2}} .$$

Plotting $\hat{r}' = (N, r)$ as a function of r yielded a straight line, the slope of which was the inverse of α, a function of N. According to the equation (9) the estimate $\hat{N}$ of the number of base pairs in a DNA segment was the abscissa of the intersection point between the curve $\alpha(N)$ and the straight line defined by:

$$y = qN/N_0$$

and with q equal successively to $1, 2, \ldots$

3. Results

3.1. Fluorescence decays

We measured the transient fluorescence of ethidium–chromatin complex solutions having binding ratios (r = D/P) varying from 5×10^{-4} to 0.037. The fluorescence decay of ethidium bound to chromatin was fitted with a sum of two exponential functions. One of this function had a relative amplitude equal to 0.1 and a short lifetime of 8 ns, while the other function had an amplitude of 0.9 and a longer lifetime slowly decreasing with increasing r values (table 1).

Fig. 2 shows a transient fluorescence $S(t)$ and the deviation function obtained with the best two exponential decays, These results are very similar to those obtained with naked DNA [23] except that in the latter case, the higher lifetime was independent of r.

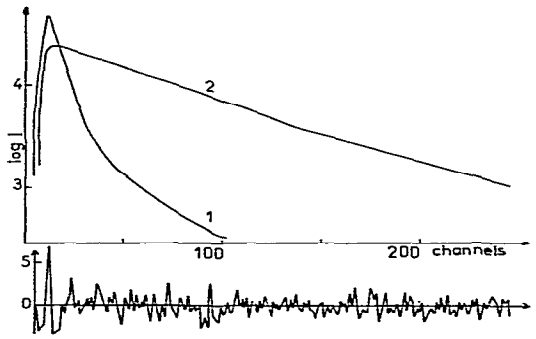

Fig. 2. Experimental transient fluorescence of ethidium bromide in aqueous solution, used as standard for the determination of the response $g(t)$ (1) and a $s(t)$ curve corresponding to ethidium calf thymus chromatin complex with P/D = 1500 (2). The lower curve represents the deviation function between $s(t)$ and a convolution computed with $a_1 = 0.13$, $\tau_1 = 7.5$ ns and $\tau_2 = 24.5$ ns (1 channel = 0.416 ns).

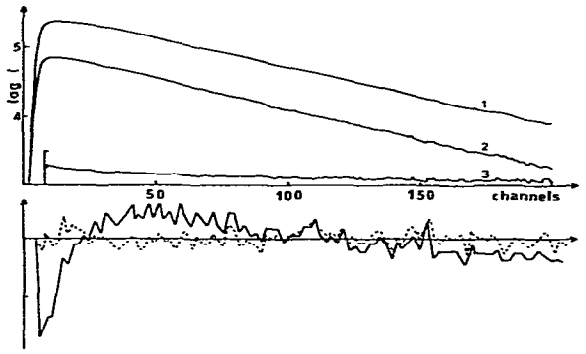

Fig. 3. Experimental $s(t)$ (1), $d(t)$ (2) and $A(t)$ (3) transient fluorescence of ethidium Ehrlich ascite chromatin complex with P/D = 1515 and deviation functions between the anisotropy decay curve and curves computed with the best monoexponential anisotropy decay characterized by θ = 260 ns (——) and the best biexponential anisotropy decay characterized by c_1 = 0.13, θ_1 = 7.6 ns, θ_2 = 421 ns and r_0 = 0.314 (– – –) (1 channel = 0.5 ns).

3.2. Fluorescence anisotropy decay

The brownian anisotropy decay $A_B(t)$ has been determined by measuring the transient fluorescence of solutions with a r value comprised between 5×10^{-4} and 7×10^{-4}. The anisotropy decay of the three chromatin preparations studied, obeyed the relation:

$$A_B(t) = A_0 [c_1 \exp(-t/\theta_1) + (1 - c_1) \exp(-t/\theta_2)].$$

where $c_1 = 0.125 \pm 0.005$, $\theta_1 = 6.5 \pm 1$ ns and $\theta_2 = 435 \pm 15$ ns. A_0 was found to vary with the preparations from 0.30 to 0.33. Fig. 3 shows an example of the analysis of $A_B(t)$.

Fig. 4 shows experimental transient fluorescence $d(t)$ of ethidium bromide–chromatin complex solutions having various D/P values. It can be seen on this figure that the slope of the decaying part of the curve increases with r, whereas in the case of DNA–ethidium complex, it has been previously found that the $d(t)$ curves were independent of r in most of this range of r values. For its greater part this variation is the result of the anisotropy change, a remaining small part coming from the variation of $S(t)$.

These experimental curves have been compared to computed curves based on energy transfers simulations, as explained in the materials and methods

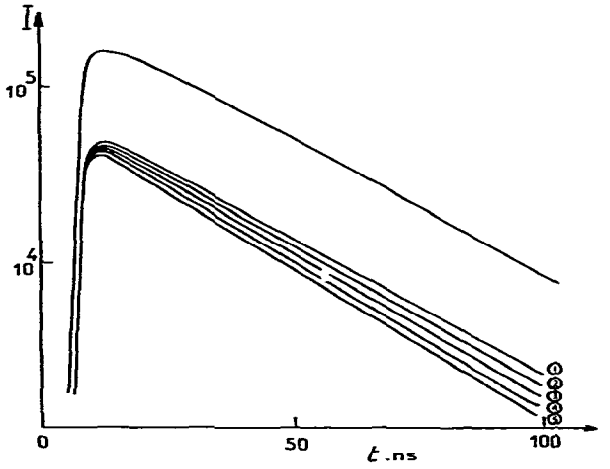

Fig. 4. Experimental $s(t)$ curve (upper curve) with P/D = 1515 and $d(t)$ curves characterized by different values of P/D: 1515 (1), 200 (2), 135 (3), 81 (4) and 50 (5) for ethidium Ehrlich ascite chromatin complex.

section. The curves $A_T(t, r', N)$ have been computed for values of N equal to 10, 15, 25, 40 and for a very long DNA molecule ($N = \infty$).

Fig. 5 shows an example of the variation of the residual $R(r', N, r)$ with r', for $N = 25$, and an ethidium–chromatin complex solution characterized by a binding ratio $r \approx 0.0085$. The abscissa of the minimum of R defines $\hat{r}'(N, r) = 0.0575$.

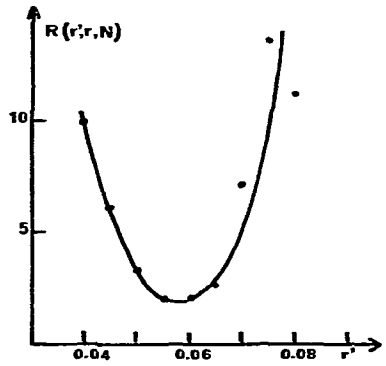

Fig. 5. Example of variation of the residual $R(r', r, N)$ with r' for $N = 25$. This curve corresponds to an ethidium calf thymus chromatin complex characterized by $r = 0.0085$. The abscissa of its minimum gives $\hat{r}' = 0.0575$.

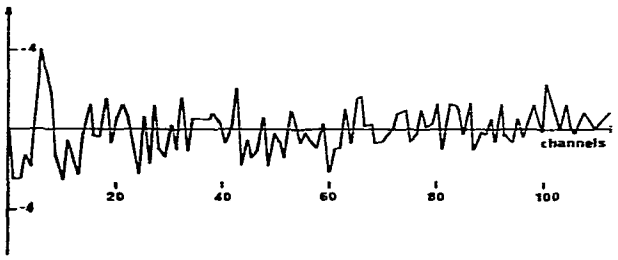

Fig. 6. Deviation function between an experimental $d(t)$ curve and a computed one in the case of $N = 25$. The experimental curve and the value of $\hat{r}'$ in the computation are the same as in fig. 5 (1 channel = 0.5 ns).

Fig. 6 shows the deviation function of $d(t)$ corresponding to the computed anisotropy decay. Similar results have been obtained for other values of N, except for $N = 10$, where we found that the minimum values of the residuals $R(\hat{r}', 10, r)$ were twice as high as for the other values of N. Therefore we concluded that N must be higher than 10.

Fig. 7 shows the curves representing the variation of $\hat{r}'(N, r)$ with r, for the different possible values of N. For small values of r, $\hat{r}'$ appears to be proportional to r, with a coefficient $1/\alpha(N)$ defined by the initial slope of the curve $\hat{r}'(N, r)$.

Fig. 8 shows the variation of $\alpha(N)$. According to

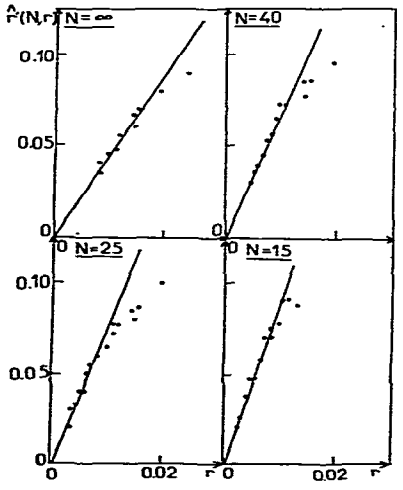

Fig. 7. Variations of $\hat{r}'$ with r for different values of N.

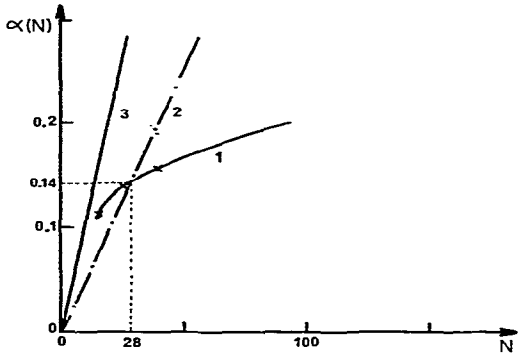

Fig. 8. Curve representing the variation of α with N(1) and the lines $y_1 = N/200$ (2) and $y_2 = 2N/200$ (3). The intersection between $\alpha(N)$ and y_1 defines the length of the DNA segments which contains the sites of high affinity for ethidium bromide.

the discussion above, this curve is not defined for values of N smaller than 10. The intersection of this curve with $y = N/N_0$ ($q = 1$ in formula (9)) defines a value $\hat{N} = 28$, corresponding to one binding segment per nucleosome. Fig. 8 also shows that the line $y = (2N/N_0)$ ($q = 2$) does not cut the curve $\alpha(N)$. Therefore, the only solution which fits the data is $q = 1, N = 28$.

4. Discussion

In this work we measured the transient polarized fluorescence of the high affinity complex formed by ethidium with three different chromatin preparations (two from calf thymus and one from Ehrlich ascite cells).

The fluorescence decay of ethidium bound to chromatin was found to be a sum of two exponential functions, with values of parameters practically identical to those already found with ethidium–DNA complex [41]. The longest lifetime however decreased slightly when the binding ratio r was increased, whereas in the case of ethidium–naked DNA complex in 0.2 M NaCl buffer, the decay and the quantum yield was independent of r. This variation might be due to a weak change of conformation of DNA included in chromatin. We already reported that the longest fluorescence lifetime of ethidium being about 28 ns

for a A-form nucleic acid [25,42] and 23 ns for a B-form one [26,27] depends on the nucleic acids structure. Anyway it must be emphasized that the fluorescence decay of the ethidium–chromatin complexes remains very close to the decay observed with ethidium–naked DNA complex, which suggest that the structure of the nucleic acid is very similar in both complexes.

At a very low value of the binding ratio ($r = 5 \times 10^{-4}$), the energy transfers between ethidium molecules were negligible and the anisotropy decay was only due to the brownian motion of the intercalated chromophores. The corresponding decay rate was found smaller for the chromatin complex than for the naked DNA one [41,43], which indicates that the high affinity sites in chromatin are situated on a DNA fraction having a higher rigidity than the naked DNA. To the high correlation time (θ_2 = 435 ns) (which is predominant in chromatin anisotropy), corresponds an equivalent sphere having a diameter of 145 Å. This is approximatively the value expected for a rigid sphere of the nucleosome size. It seems then reasonable to attribute θ_2 to the motion of the whole nucleosome particle.

It has been previously reported that the fluorescence static anisotropy decreased with r more rapidly in the case of ethidium bromide–chromatin complexes than in the case of ethidium–naked DNA complex [44,15,20]. This difference persisted in viscous solutions, which implied that energy transfers are more efficient in chromatin than in DNA complexes. Our measurements confirm that observation.

We have analyzed our data quantitatively by comparing them with simulations of energy migration based on the one hand on the Förster's theory of energy transfers and on the other hand on a model of the complex similar to the model first proposed by Lerman. Let us discuss both aspects of the computation.

The formula (6) giving the transfer rate has been established by Förster in 1948 [35]. The validity of the Förster's theory has been then questioned [48, 49] in the case of transfers between like molecules. In 1965, doing a complete reexamination of this theory, Förster [47] concluded that his formula was valid even in this case.

The Förster's formula has been used by Paoletti and Le Pecq [46] in their study of the steady-state polarization of the ethidium–DNA complexes. From the comparison between their computer simulation and the experimental variation of polarization with the ligand fixation ratio, they determined that DNA was wound by $\delta = +14°$. This result disagreed with the determination of δ obtained by other methods (see ref. [50] for a review). The computation of Paoletti and Le Pecq have been remade by Pigram et al. [60] who concluded that steady-state polarization was not accurate enough to yield the value of δ.

Nevertheless, Le Bret et al. [51] have attributed this discrepancy to the failure of Förster's theory for like molecules. The main experimental argument against this theory presented by these authors was based on anisotropy decay measurements of diacridine molecules dissolved in glycerol. These molecules contained two identical aromatic chromophores linked by a chain of covalent bonds. The rate of energy transfers between the two chromophores of a molecule was determined by fitting an exponential function with the measured decay. A critical distance of transfer was deduced from the obtained decay time, and found smaller than the critical distance predicted by the Förster's theory. The way of interpreting these experiments calls for the following comments:

The exponential anisotropy decay due to homologous transfers is theoretically justified if all molecules have the same rigid conformation [35]. The formula might be extended to the case in which the two chromophores are at fixed distance identical for all molecules, and are free to rotate in all directions with a much higher rate than the transfer rate (dynamic limit) [52]. None of these conditions are fulfilled in the experiments of Le Bret et al. since, according to the chemical structure on the one hand and the NMR measurements [53] on the other hand the molecules are flexible, and since the viscous solvent used did not allow fast rotation of the chromophores. In addition, one may wonder whether a part of the decay anisotropy due to fast energy transfers is not missed in the experiments analysis, since according to fig. 8 of the work of Le Bret et al., the anisotropy starts decaying from 0.15, a very small value compared to the fundamental anisotropy of this type of aromatic molecules. The initial decay was apparently too fast to be analyzed with the experiment resolution, all the more as the authors do not seem to

have deconvoluted their data.

In conclusion, the experimental argument of Le Bret et al. did not bring about a valid proof against the Förster's theory relative to energy transfer between like molecules. The model of the ethidium–DNA complex used in this work was first proposed by Lehrman [59]. This model has been recently questioned. It has been proposed that the ethidium molecules were not perpendicular to the DNA longitudinal axis and that the lengthening of the distance between the adjacent base pairs was only 2.7 Å [54]. The two features however disagree with the crystallographic studies of dinucleotide ethidium complexes [55].

It has been also assumed in our present model that the positions of two ethidium molecules intercalated in the same DNA molecule, are related to each other by a helical displacement. In a preceding work [27] this model was compared to another one in which the ethidium molecules in their sites had two positions differing from one another by a rotation of 180° around the small molecular axis. In this case the fitted value of δ depended strongly on the assumed value of the angle made by the transition moment direction and the long axis of the ethidium molecule. Several different values of this angle have been proposed in the literature (33° [56], 45° [57], 65° [58], 78° [64]). Therefore as earlier discussed, the value of this angle must be ascertained before δ is exactly determined by our method [27]. The value $\delta = -18°$ must be considered as the value which best fits our model to the experimental data.

According to recent studies [61–63] of the tertiary structure of circular DNA, the present accepted value of δ is $-26°$. As described above, we used this value in some of our calculations and obtained the same results as with $\delta = -18°$.

Let us recall that our computations, although based on simplified assumptions, account quantitatively for the following ethidium-nucleic acid complex properties:

1) The anisotropy decays of ethidium–poly(rA-rU) complexes can be fitted to a simulation in which specific features of the double helix of type A are introduced, whereas this is not possible with the B structure which fits the ethidium DNA anisotropy decays [25].

2) In agreement with the binding isotherms, all the ethidium sites have the same affinity, except the two adjacent sites of an occupied one which are excluded [25,27].

3) The variation of the anisotropy decay with the binding ratio is correctly described [23,24].

In the present work the nucleosomal DNA fraction which binds ethidium with high affinity, was determined by the ratio of the local (r') and the overall (r) binding ratios.

Under our experimental conditions, r was equal to the ratio of the dye to nucleotide concentration, whereas r' was the equivalent value obtained by fitting the experimental anisotropy decay of ethidium–chromatin complex to decays simulated for ethidium–DNA complex and taking into account the "boundary effects".

That means that the binding DNA segment of chromatin was limited by boundary nucleotides which play the role of reflective mirrors for the migration process.

It appears then, that the main condition of validity of our approach is that the high affinity complex has the same structure as the ethidium–DNA complex. Even this assumption is not arbitrary, since as recalled above, the model must include some important characteristics of the actual structure in order to fit the experiments. Furthermore one currently admits that the structure of the chromatin–DNA appears similar to the B structure [45].

In the range of small r values the local binding ratio $\bar{r}'$ was found to be proportional to r (with proportionality coefficient $1/\alpha$), which confirms that only the high affinity complex existed under these conditions. At higher r values, new binding sites appeared, which explained the curvature of the curve representing $\bar{r}'$ as a function of r.

From the values of α, one obtains the number of nucleotide base pairs N contained in a binding DNA segment, if one knows the number q of these segments per nucleosome. On the other hand, N has a lower limit of 10; for this value, the residual characterizing the fit of the computed anisotropy decay to the experimental data begins to increase. Then the only q and N values which agree with experiments are $q = 1$ and $N = 28$. The fraction of the nucleosomal DNA which constitutes the high affinity is then equal to 14%. This agrees fairly well with the binding results in which the high affinity sites were found to

represent 13% of sheared chromatin [19] and 12.5% of chromatin prepared by enzymatic digestion [20].

Our method confirms these studies and in addition it shows that these high affinity sites are situated on the same continuous segment of nucleosomal DNA.

Finally there arises the questions of the DNA segment location in the nucleosome. It seems most probable that this segment forms a part of the linker since the core DNA, which is tightly wrapped around the histones, could hardly contain a segment 28 base pairs long freely accessible to ethidium bromide. This segment represents about half of the linker DNA.

The results obtained in this work will be used in a following paper where we studied the influence of histone H_1 on the binding of ethidium to chromatin.

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References

[1] D.R. Hewish and L.A. Burgoyne, Biochem. Biophys. Res. Comm. 52 (1973) 504.
[2] P. Oudet, M. Gross-Bellard and P. Chambon, Cell 4 (1975) 281.
[3] A.L. Olins and D.E. Olins, Science 183 (1974) 330.
[4] R.D. Kornberg, Science 184 (1974) 868.
[5] E. Van Holde, C.G. Sahasrabuddhe and B.R. Shaw, Nucl. Acids Res. 11 (1974) 1579.
[6] M. Noll, Nature 251 (1974) 249.
[7] M. Noll, J.O. Thomas and R.D. Kornberg, Science 187 (1975) 1203.
[8] J.D. Griffith, Science 187 (1975) 1202.
[9] K.E. Van Holde and I. Isenberg, Accounts Chem. Res. 8 (1975) 327.
[10] M. Noll, Nucl. Acids Res. 1 (1974) 1573.
[11] J.T. Finch, L.C. Lutter, D. Rhodes, R.S. Brown, B. Rushton, M. Levitt and A. Klug, Nature 269 (1977) 29.
[12] P.F. Lurquin and V.L. Seligy, Biochem. Biophys. Res. Comm. 46 (1972) 1399.
[13] P.F. Lurquin and V.L. Seligy, Chem. Biol. Inter. 13 (1976) 27.
[14] L.M. Angerer and E.N. Moudrianakis, J. Mol. Biol. 63 (1972) 505.
[15] L.M. Angerer, S. Georghiou and E.N. Moudrianakis, Biochem. 13 (1974) 1075.
[16] J.J. Lawrence and M. Louis, Biochim. Biophys. Acta 272 (1972) 231.
[17] J.J. Lawrence and M. Louis, FEBS Letters 40 (1974) 9.
[18] J.J. Lawrence, D.C.F. Chan and L.H. Piette, Nucl. Acids Res. 3 (1976) 2879.
[19] J.J. Lawrence and M. Daune, Biochem. (1976) 2201.
[20] J. Paoletti, B.B. Magee and P.T. Magee, Biochemistry 16 (1976) 351.
[21] J. Paoletti, Biochem. Biophys. Res. Comm. 81 (1978) 193.
[22] H. La Rue and D. Pallota, Nucl. Acids Res. 3 (1976) 2193.
[23] D. Genest and Ph. Wahl, Biochim. Biophys. Acta 259 (1972) 175.
[24] D. Genest, P. Wahl and J.C. Auchet, Biophys. Chem. 1 (1974) 266.
[25] Ph. Wahl, D. Genest and J.L. Tichadou, Biophys. Chem. 6 (1977) 311.
[26] J.L. Tichadou, D. Genest, Ph. Wahl and G. Aubel-Sadron, Biophys. Chem. 3 (1975) 142.
[27] D. Genest and Ph. Wahl, Biophys. Chem. 7 (1978) 317.
[28] Ph. Wahl, in: New technics in biophysics and cell biology, Vol. 2, eds. H. Pain and Smith (Wiley, London), 1975) p. 233.
[29] J.C. Auchet, Thesis, Orléans (1977).
[30] Ph. Wahl, J.C. Auchet and B. Donzel, Rev. Sci. Ins. 45 (1974) 28.
[31] D. Genest, J.L. Tichadou and Ph. Wahl, unpublished results.
[32] J.C. Brochon and Ph. Wahl, Eur. J. Biochem. 25 (1972) 20.
[33] F. Perrin, J. de Physique 6 (1926) 390.
[34] K. Tawada, Ph. Wahl and J.C. Auchet, Eur. J. Biochem. 88 (1978) 411.
[35] T. Förster, Ann. Physik 2 (1948) 55.
[36] A. Jablonsky, Acta Phys. Pol. 14 (1955) 295.
[37] D. Genest and Ph. Wahl, in: Dynamic aspects of conformation changes in biological macromolecules, ed. Ch. Sadron (D. Reidel Publishing Company, Dordrech-Holland, 1973) p. 367.
[38] D.M. Crothers, Biopolymers 6 (1968) 575.
[39] A.E.W. Knight and B.J. Selinger, Spect. Acta 27A (1971) 1223.
[40] A. Grinwald and I.Z. Steinberg, Analyt. Biochem. 59 (1974) 583.
[41] D. Genest and Ph. Wahl, Biochim. Biophys. Acta 521 (1978) 502.
[42] M. Leng, M. Guigues and D. Genest, Biochemistry 17 (1978) 3215.
[43] Ph. Wahl, J. Paoletti and J.B. Le Pecq, Proc. Nat. Acad. Sci. 65 (1970) 417.
[44] J. Bontemps and E. Fredericq, Biophys. Chem. 2 (1974) 1.
[45] D.C. Goodwin and J. Brahms, Nucl. Acids Res. 5 (1978) 835.
[46] J. Paoletti and J.B. Le Pecq, J. Mol. Biol. 59 (1971) 43.
[47] T. Förster, in: Modern quantum chemistry, part III, ed. O. Sinanoglu (Academic Press, New York and London, 1965) p. 93.

[48] G.W. Robinson and R.P. Frisch, J. Chem. Phys. 38 (1963) 1187.
[49] W.T. Simpson and D.L. Peterson, J. Chem. Phys. 26 (1957) 588.
[50] W.R. Bauer, Ann. Rev. of Biophys. and Bioeng. 7 (1978) 287.
[51] M. Le Bret, J.B. Le Pecq, J. Barbet and B.P. Roques, Nucl. Acids Res. 4 (1977) 1361.
[52] R.F. Dale and J. Eisenberg, Biopolymers 13 (1975) 1573.
[53] J. Barbet, B.P. Roques, S. Combrisson and J.B. Le Pecq, Biochemistry 15 (1976) 2642.
[54] M. Hogan, N. Dattagupta and D.M. Crothers, Biochemistry 18 (1979) 280.
[55] C.C. Tsai, S.C. Jain and H.M. Sobell, Proc. Nat. Acad. Sci. USA 72 (1975) 628.
[56] P.V. Giacomoni and M. Le Bret, FEBS Letters 29 (1973) 227.
[57] M. Le Bret and O. Chalvet, J. of Mol. Struct. 37 (1977) 299.
[58] C. Houssier, B. Hardy and E. Fredericq, Biopolymers 13 (1974) 1141.
[59] L.S. Lerman, J. Mol. Biol. 3 (1961) 18.
[60] W.J. Pigram, W. Fuller and M.E. Davies, J. Mol. Biol. 80 (1973) 361.
[61] J.C. Wang, J. Mol. Biol. 89 (1974) 783.
[62] L.F. Liu and J.C. Wang, Biochim. Biophys. Acta 395 (1975) 405.
[63] W.A. Baase and W.C. Johnson, Nucl. Acids Res. 6 (1979) 797.
[64] I. Zimmermann and H.W. Zimmermann, Ber. Bunsen-Gesell. 81 (1977) 81.
[65] G. Sabeur, D. Genest and G. Aubel-Sadron, Biochem. Biophys. Res. Comm. 88 (1979) 722.
[66] G. De Murcia, G.C. Das, M. Erard and M. Daune, Nucleic Acids Res. 5 (1978) 523.