Proceedings of the National Academy of Sciences
Vol. 65, No. 2, pp. 417–421, February 1970

Decay of Fluorescence Emission Anisotropy of the Ethidium Bromide-DNA Complex Evidence for an Internal Motion in DNA

Ph. Wahl, J. Paoletti, and J.-B. Le Pecq

CENTRE DE BIOPHYSIQUE MOLÉCULAIRE DU C.N.R.S., LA SOURCE, 45 ORLEANS, FRANCE, AND UNITÉ DE PHYSICO-CHIMIE, INSTITUT GUSTAVE ROUSSY, 94 VILLEJUIF, FRANCE

Communicated by Norman Davidson, October 23, 1969

Abstract. Evidence for an internal oscillatory Brownian motion in the DNA helix is obtained from the measurement of the decay of the fluorescence emission anisotropy of the ethidium bromide-DNA complex. The amplitude of the oscillation is found to be equal to 35° and the relaxation time equal to 28 nanoseconds.

The dye ethidium bromide has been found to bind specifically to double-stranded nucleic acid helix. The interesting characteristics of this binding are:

(1) The fluorescence quantum efficiency of the dye increases considerably on binding to double-stranded nucleic acids.[1, 2]

(2) Binding occurs by intercalation.[2, 3] Then ethidium bromide is rigidly bound to the double-stranded helix and the direction of the absorption and emission transition moments which are in the plane of the dye have a known direction with reference to the helix axis.[2]

Then, ethidium bromide is an ideal candidate as a fluorescence label for fluorescence depolarization measurements since the ethidium bromide lifetime of fluorescence is relatively large.[4]

It was first observed[5] that the polarization fluorescence coefficient for ethidium bromide bound to DNA was dependent on the viscosity of the solution. In other words, in aqueous solvent a depolarization of fluorescence was observed. Since the DNA is a large molecule, the extent of its rotation during the time of the ethidium bromide excited state can be assumed negligible. Then, two hypotheses had to be postulated, either the DNA was not rigid and was able to undergo some kind of internal motion, or ethidium bromide was not really rigidly bound as predicted by the intercalation model.[6] In these conditions it was of interest to further study this problem by measuring the emission anisotropy during the fluorescence decay. This method has been already used with macromolecules bearing covalently bound fluorescent chromophores. Detailed information on the Brownian motion and the internal deformation of these macromolecules have been obtained.[7–10]

Methods. Let $I\|(t)$, $I\perp(t)$, be, respectively, the decay of fluorescence intensity of the horizontal and perpendicular components when fluorescence excitation is made by an infinitely short flash of natural light. The time of the flash is taken as the origin.

The anisotropy decay is then defined as[11]

$$r_n(t) = \frac{D(t)}{S(t)}, \tag{1}$$

where $D(t)$ and $S(t)$ are:

$$D(t) = I\|(t) - I\perp(t) \tag{2}$$

and

$$S(t) = 2I\|(t) + I\perp(t). \tag{3}$$

$r_n(t)$ is related to the direction of the transition moments of the excited molecules by the following expression[11]

$$r_n(t) = r_{n0}\,\frac{\overline{3\cos^2\alpha(t)} - 1}{2}, \tag{4}$$

$r_n(t)$ and r_{n0} being the anisotropy at time t and at time 0, respectively. $\alpha(t)$ is the rotation angle of the emission transition moment between time 0 and time t $\overline{\cos^2\alpha(t)}$ is the mean of the function $\cos^2\alpha(t)$.

In the absence of energy transfer between fluorescent molecules, $r_n(t)$ is the sum of exponential functions, the time constants of which are the Brownian relaxation times.

Actually the exciting flash is not infinitely short and experimental functions $i\|(t)$ and $i\perp(t)$ corresponding to ideal functions, $I\|(t)$ and $I\perp(t)$ are determined. From them, the functions $s(t)$ and $d(t)$ corresponding to the ideal functions $S(t)$ and $D(t)$ are calculated. From these functions and from the time response of the apparatus to the flash $g(t)$, $S(t)$, and $D(t)$ are calculated as previously described.[8]

Measurements of $i\|(t)$, $i\perp(t)$, and $g(t)$ are made using instruments which have been extensively described earlier.[8]

Result. The functions $s(t)$, $d(t)$, and $g(t)$ are shown in Figure 1.

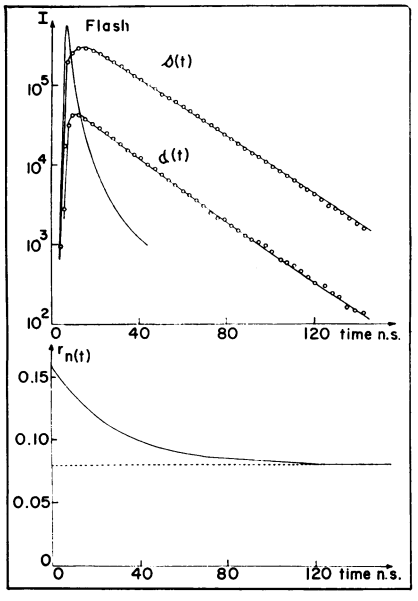

FIG. 1.—Decay of the fluorescence of the DNA-ethidium bromide complex for polarized light and variation of the function $r_n(t) = f(t)$ according to equation (7). Calf thymus DNA prepared in our laboratory was used in this experiment. Molecular weigh was 6.3×10^6 and extinction coefficient $\epsilon(P)$ 6.710. Ethidium bromide was a gift from the Boots Pure Drug Co. DNA concentration measured by spectrophotometry at 260 mμ was 230 μg/ml, ethidium bromide was 3 μg/ml in Tris-HCl buffer, pH 7.7, 0.1 M, NaCl 0.15 M. In these conditions P/D (molecular concentration of DNA-P/molar concentration of dye) equals 130. Practically all molecules of ethidium bromide are bound in these conditions.[2] Fluorescent excitation is made at 520 mμ, emission is observed through an optical filter at 600 mμ. Absorbance of the DNA-ethidium bromide complex at the excitating wavelength is 0.030.

The function $S(t)$ can be shown to be a simple exponential function

$$S(t) = S_0 e^{-t/\tau_s} \tag{5}$$

where τ_s is the fluorescence lifetime of the DNA bound ethidium bromide

$$\tau_s = 24 \text{ nsec} \pm 0.5 \text{ nsec}$$

The function $D(t)$ can be decomposed into the sum of two elementary exponential functions. D_0 being the value of $D(t)$ for $t = 0$ it becomes:

$$D(t) = D_0(0.5 \exp -t/\tau_1 + 0.5 \exp -t/\tau_2) \tag{6}$$

with $\tau_1 = 13$ nsec $\pm$ 0.5 nsec, $\tau_2 = \tau_s = 24$ nsec.

From equation (1) it can be written:

$$r_n(t) = r_{n0}(0.5\, e^{-t/\theta} + 0.5) \tag{7}$$

with $r_{n0} = D_0/S_0 = 0.160$. This function is shown in Figure 1.

The relaxation time θ is then:

$$\theta = \frac{\tau_1 \times \tau_2}{\tau_s - \tau_1} = 28 \text{ nsec}$$

the error on this relaxation time can be estimated of the order of 20 per cent.

Considering equation (7), it is easily seen that this function is the sum of an exponential function and a constant. So even at a very long time after the flash excitation the polarization does not tend to 0.

For $t = \infty$, it comes from equations (4) and (7):

$$\frac{\overline{3\cos^2\alpha(\infty)} - 1}{2} = 0.5$$

$$\overline{\cos^2\alpha(\infty)} = 0.666$$

this corresponds for α to an angle of 35° at infinite time.

For $t = 0 r_n(0)$ is the value of r_n in the absence of Brownian motion.

Since the anisotropy is related to the polarization coefficient through $p = \frac{3r_n}{1 + r_n}$, we calculate p_0 corresponding to r_{n0} as $p_0 = 0.415$.

This value can be compared with the polarization coefficient measured with a continuous excitation on an ethidium bromide-DNA solution saturated with sucrose. Under these conditions of very high viscosity where Brownian motion can be neglected, the value for the polarization coefficient is found equal to 0.410. This is in good agreement with p_0.

Discussion. Before discussing the fluorescence depolarization of the DNA-bound ethidium bromide owing to any Brownian motion, three causes of depolarization unrelated to such a motion have to be eliminated.

(*a*) Depolarization could occur by energy transfer between ethidium bromide molecules bound to the same DNA molecule. In the present experimental conditions (Fig. 1), ethidium bromide molecules are very far apart, more than 200 Å on the average and it can be shown that energy transfer is negligible.[12]

(*b*) One could also argue that since ethidium bromide is not covalently bound, the motion off and on the DNA at equilibrium could cause depolarization. This can be very easily ruled out from the known values of the rate constants for intercalation.[13]

(*c*) Ethidium bromide is constituted of two independent rings: one phenanthridine ring, intercalated inside the DNA, and one phenyl ring which stays outside of the helix. One could argue that the rotation of the phenyl ring is not prevented by intercalation. But this hypothesis can be ruled out. It is easy to see on an ethidium bromide model molecule built with space filling atomic model that, owing to steric hindrance, the phenyl ring is blocked in a position such as its plane is perpendicular to the phenanthridine ring. This fact has recently been confirmed by X-ray diffraction study of ethidium bromide.[14]

The results then demonstrate that ethidium bromide when bound to DNA is able to undergo a Brownian motion of rotation with a relaxation time of 28 nsec, the angle of rotation being limited to about 35°. The relaxation time value is much larger than the expected value for the free ethidium bromide in water (a few tenths of 1 nsec). On the other hand, the value of the relaxation time is much too small to correspond to the rotation of the whole DNA molecule for which one could estimate the relaxation time to be at least of the order of the millisecond.[15] Therefore there are only two hypotheses left:

(1) The depolarization is due to a restricted motion of the ethidium bromide molecule in its binding site on DNA without motion of the DNA.

(2) The observed motion of the ethidium bromide is the result of a deformation motion of the DNA.

In the intercalation model, such as the one described by Lerman,[6] a 35° rotation motion of ethidium bromide without motion of the DNA seems impossible. One could argue then that ethidium bromide does not bind to DNA according to such a model. But so many lines of evidence[1-3] argue against this that the only likely hypothesis is that the observed depolarization of the ethidium bromide fluorescence is the result of a motion of the DNA molecule itself.

As previously mentioned in this paper, the rotation of the DNA molecule considered as rigid cannot explain our data. An internal motion of deformation of the DNA has to be postulated. Ethidium bromide in the intercalation model behaves substantially like an additional base pair. Any motion of the contiguous base pairs will bring about a motion of the ethidium bromide molecule. Then ethidium bromide motion would just reflect the motion of the adjacent base pairs inside the DNA; the two motions being probably very similar.

If the order of magnitude of the deformation can be derived from these measurements, the direction of the motion cannot be determined. However, a local rotation of the DNA base pairs around one of the small axis would involve a bending of the chain inconsistent with the known rigidity of the DNA chain. So it is more likely that the bases are able to oscillate around the great axis of the helix leading to a local deformation resulting from a local change in the pitch of the helix.

We are indebted to Dr. Aubel-Sadron for the gift of a DNA sample and for measurement of molecular weight and $\epsilon(P)$ of our DNA samples.

[1] Le Pecq, J. B., P. Yot, and C. Paoletti, *Comp. Rend. Acad. Sci.*, **259**, 1786 (1964).
[2] Le Pecq, J. B., and C. Paoletti, *J. Mol. Biol.*, **27**, 87 (1967).
[3] Fuller, W., and M. J. Waring, *Ber. Busenges. Physik. Chem.*, **68** 805 (1964).
[4] Laustriat, G., personal communication.
[5] Paoletti, J., and J. B. Le Pecq, unpublished results.
[6] Lerman, L. S., *J. Mol. Biol.*, **3**, 18 (1961).
[7] Wahl, Ph., *Comp. Rend. Acad. Sci.*, **263**, série D, 1525 (1966).
[8] Wahl, Ph., *Biochem. Biophys. Acta*, **175**, 55 (1969).
[9] Fayet, M., and Ph. Wahl, *Biochem. Biophys. Acta*, **181**, 373 (1969).
[10] Stryer, L., *Science*, **162**, 526 (1968).
[11] Jablonski, A., *Bull. Acad. Pol. Sci. Ser. Math. Astr. Phys.*, **8**, 258 (1960).
[12] Wahl, Ph., in preparation.
[13] Li, H. J., and D. M. Crothers, *J. Mol. Biol.*, **39**, 461 (1969).
[14] Hospital, M., and B. Busetta, *Comp. Rend. Acad. Sci.*. **268**, série C, 1232 (1969).
[15] Callis, P. K., and N. Davidson, *Biopolymers*, **7**, 335 (1969).