Nanosecond Fluorescence Anisotropy of the DNA-Acridine Complexes

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The rotational motions of various acridine dyes bound to DNA were investigated by the measurements of nanosecond fluorescence anisotropy. It was found that the total decay of fluorescence is a single exponential, whereas the decay of fluorescence anisotropy is a sum of an exponential function and a constant. The apparent values of the rotational relaxation time of the complexes calculated from anisotropy data range from 21 to 31 ns. The results are discussed by comparing with those obtained from the measurements of steady-state fluorescence depolarization.

Since Lerman[1,2] proposed the intercalation model in which the dye molecule is sandwiched between the adjacent base pairs of DNA by extension and local unwinding of the helix, the general features of the model have been widely accepted.[3,4] However, the exact location of the intercalated dye is not yet definitely known. One of the approaches to elucidate the possible location of the dye is a systematic investigation of the dependence of the binding nature on the dye structure.[5-7]

In a previous paper,[6] it was found that the mean rotational relaxation times of the DNA-acridine complexes obtained from the measurements of steady-state fluorescence depolarization depend on the dye structure. In this paper, nanosecond fluorescence anisotropy and viscosity studies have been undertaken to obtain further information on the rotational motions of the dye bound to DNA, using various acridine dyes.

Experimental

Materials. Calf thymus DNA was purchased from Worthington Biochemical Corporation. The concentration of DNA was determined spectrophotometrically at 260 nm with the extinction coefficient per mol of DNA phosphate (ε_p=6600 M^{-1} cm^{-1}).[8] Acridine Orange (AO), Proflavine (PF), 3,6-bis(methylamino)acridine (Ac[NHMe]$_2$), 3,6-bis(ethylamino)acridine (Ac[NHEt]$_2$), 3,6-bis(diethylamino)acridine (Ac[NEt$_2$]$_2$), 3,6-bis(dimethylamino)-10-propylacridinium chloride (AO-propyl) and 3,6-bis(dimethylamino)-10-isopropylacridinium chloride (AO-isopropyl) were the same as previously reported.[6,7]

Nanosecond Fluorescence Anisotropy. Fluorescence decay curves were measured with an Ortec 9200 single photon counting nanosecond fluorometer.[9] In the measurements of fluorescence anisotropy, the exciting light was vertically polarized. A polarizer was also placed in the emission beam, which could be rotated by 90 ° to permit the measurements of the intensities of the vertically and horizontally polarized components. The time-dependent anisotropy $r(t)$ is defined as:

$$r(t) = \frac{I_{//}(t) - I_{\perp}(t)}{I_{//}(t) + 2I_{\perp}(t)} = \frac{d(t)}{s(t)}, \quad (1)$$

where $I_{//}(t)$ and $I_{\perp}(t)$ are the fluorescence intensities observed with the analyzer parallel and perpendicular to the direction of polarization of the exciting light. Unequal response of the detector system to the polarized light was corrected using an aqueous dilute solution of 9-aminoacridine.[9] The total decay of fluorescence $s(t)$ was determined independently. This was achieved by orienting the polarization axis of the analyzer at 35.3 ° from the vertical direction and using unpolarized exciting light.[10] In the measurements of the fluorescence decay curves, there was a small contribution of the scattered light; this was subtracted from the decay curves prior to analysis.

In agreement with previous observations,[6,11] the polarization of fluorescence was constant above *ca.* 400 nm and showed a minimum around 320—340 nm. Therefore, the complexes were excited at 430 nm for the measurements of nanosecond anisotropy.

All the measurements were carried out in 5 mM phosphate buffer (pH 6.9, ionic strength of 0.01) at room temperature (23±1 °C). The molar ratio of DNA phosphate to dye (P/D) was about 200, and the dye concentration ranged from 5×10^{-6} to 10^{-5} M. In such conditions, the concentration of free dye and energy transfer between bound dye molecules were negligible.[7,11]

Analysis of Nanosecond Anisotropy Decay Curves. The fluorescence decay functions $S(t)$ and $D(t)$ are related to the observed decay curves $s(t)$ and $d(t)$ by the following convolution integrals;

$$s(t) = \int_0^t S(t-u)E(u)\mathrm{d}u, \quad (2)$$

$$d(t) = \int_0^t D(t-u)E(u)\mathrm{d}u, \quad (3)$$

where $E(t)$ is the response function of the apparatus to the exciting pulse. First, $S(t)$ was determined by deconvolution of Eq. 2. In every case, $S(t)$ was found to be a single exponetial;

$$S(t) = S_0 e^{-t/\tau}, \quad (4)$$

where τ is the fluorescence lifetime. Next, the result was checked by a synthetic method.[12] The anisotropy decay function is given by

$$R(t) = \frac{D(t)}{S(t)}, \quad (5)$$

where $S(t)$ is the function given by Eq. 4. Several $R(t)$ functions were tested so that the numerically computed convolution of $D(t)$ with $E(t)$ may fit the experimental decay curve. The computed curve, $f^{\circ}(k)$, was then visually compared with the experimental curve, $f(k)$, and the weighed residue (χ^2) was calculated according to the method of Knight and Selinger.[13] Here, k denotes channel number.

$$\chi^2 = \frac{1}{n}\sum_{k=1}^{n}\frac{1}{f(k)}[f(k)-f^{\circ}(k)]^2. \quad (6)$$

The best fit between the computed and experimental decay curves was achieved by minimizing the χ^2 value.

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Deconvolution was made with the aid of the methods of moments[14,15] and Laplace transformation.[16] Both methods yielded very similar results. Copies of the computer programs for these methods were kindly supplied to us by Dr. D. R. Dyson and Dr. L. Brand. All the analyses were carried out with a Univac 1108 computer.

Viscosity Measurements. Viscosity measurements of the sonicated DNA–acridine complexes were performed at 25± 0.05 °C by means of a Ubbelohde viscometer. The flow time of the solvent (5 mM phosphate buffer) was 270 s. We limited our study to r values smaller than 0.12 to avoid the errors due to the possible presence of the weakly bound dye molecules; r represents the number of moles of dye bound per DNA phosphate. The r value was determined by using data of equilibrium dialysis reported previously.[7]

Sonication of DNA was carried out with a Kubota ultrasonicator (200 W, 9 kHz). Immediately prior to irradiation, the sample solution was flushed with nitrogen gas, and during irradiation, ice-cold water was circulated through the jacket sorrounding the steel cup of a sonic oscillator. The molecular weight of sonicated DNA was determined to be 5.2×10^5 from the viscosity measurements; this value means that the sonicated DNA behaves almost like a rigid rod.[17]

Results and Discussion

A typical set of anisotropy data is shown in Fig. 1 for the DNA–AO complex, where the observed $s(t)$, $d(t)$, and $E(t)$ values are plotted every five channels. After deconvolution using the methods of moments[14,15] and Laplace transformation,[16] the function $S(t)$ was found to be a single exponential. On the other hand, the function $D(t)$ was found to be a sum of two exponential functions whose decay constants are very close. Figure 1 shows that, at large channel numbers, $s(t)$ and $d(t)$ are superposable on each other upon translation. As can be seen in Figs. 1 and 2, the anisotropy curves $r(t)=d(t)/s(t)$ obtained by point by point division exhibit non-exponential decays. They tend to decay exponentially at the initial stage and then to become constant, although the calculated points are somewhat scattered after channel 200. In view of these findings, it seems reasonable to assume that $R(t)$ and $D(t)$ are expressed by the following equations:[12,18]

$$R(t) = R_0(\alpha e^{-t/\phi}+1-\alpha), \quad (7)$$

$$D(t) = R(t)S(t) = S_0R_0\alpha e^{-t/\tau'} + S_0R_0(1-\alpha)e^{-t/\tau}, \quad (8)$$

where

$$\tau' = \frac{\phi\tau}{\phi+\tau}, \quad (9)$$

and where ϕ corresponds to the rotational correlation time of the dye. This is a rather general result for the dye–macromolecule and dye–membrane systems in which the motion of the dye is locally limited.[12,18,19] Since two decay constants are very close in our case ($\tau/\tau'\simeq1.5$), we can not accurately deconvolute the observed $d(t)$ curves.[16,20] Therefore, using the deconvoluted $S(t)$ function which is a single exponential like Eq. 4 and assuming appropriate α and ϕ in Eq. 7, several $R(t)$ functions were tested so that the numerically computed convolution of $D(t)$ with $E(t)$ may fit the experimental curve. The solid line for $d(t)$ in Fig. 1 shows the calculated best-fit curves.

Table 1 summarizes the results of nanosecond anisotropy. The two different methods were used for deconvolution of the observed $s(t)$ curves. Both methods give almost the same lifetimes, which are in fairly good agreement with the lifetimes obtained by phase-shift method.[6] All anisotropy decay curves for the complexes are expressed by a function like Eq. 7. It should be noted that acridine dyes bound to DNA behave similarly to ethidium bromide (another intercalative dye) with respect to the anisotropy decay.[18] The values of limiting anisotropy (R_0=0.31—0.36) are comparable to those obtained from the measurements of steady-state depolarization.[6,21] The apparent values of the rotational relaxation time ($\rho_a=3\phi$) were calculated using decon-

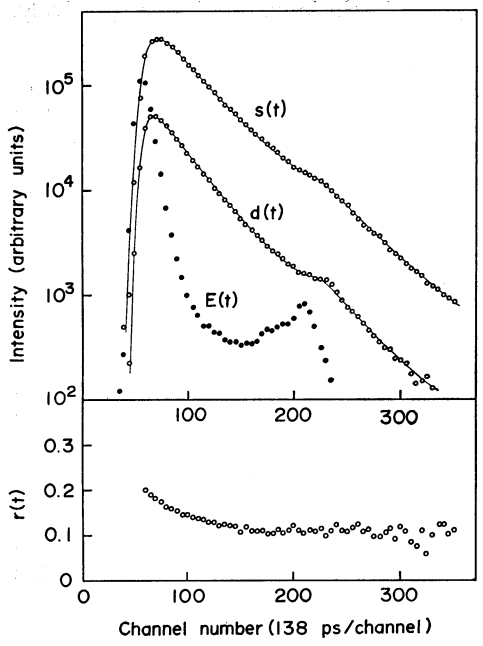

Fig. 1. Fluorescence decay curves of DNA–AO complex (P/D=204) in 5 mM phosphate buffer (pH 6.9) at 22°C. Open circles are observed decay curves, and solid lines are the best-fit curves. Excitation wavelength: 430 nm. Emission wavelength: 520 nm.

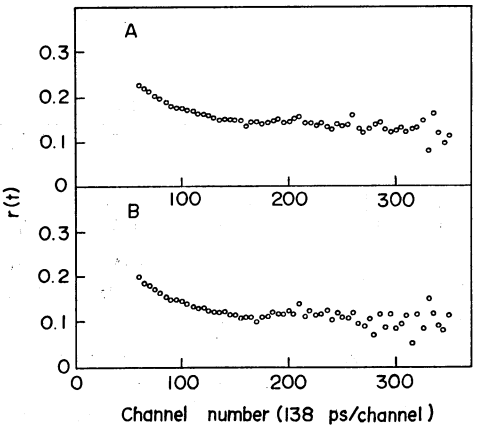

Fig. 2. Observed anisotropy decay curves at 22°C. (A) DNA–PF complex (P/D=204). Excitation wavelength: 430 nm. Emission wavelength: 500 nm. (B) DNA–Ac[NEt_2]$_2$ complex (P/D=242). Excitation wavelength: 430 nm. Emission wavelength: 520 nm.

TABLE 1. RESULTS OF NANOSECOND ANISOTROPY AND VISCOSITY

Dye	P/D	τ/ns a)	τ/ns b)	R(t)	ρa/ns c)	ρa/ns d)	ρa/ns e)	β
PF	204	6.5_0	6.5_4	$0.34\ (0.62e^{-t/10.0}+0.38)$	31	60	52	1.30
Ac[NHMe]$_2$	201	4.1_9	4.1_5	$0.36\ (0.60e^{-t/10.0}+0.40)$	28	55	48	1.25
Ac[NHEt]$_2$	205	4.4_9	4.4_4	$0.34\ (0.60e^{-t/9.47}+0.40)$	27	52	45	1.05
Ac[NEt$_2$]$_2$	242	4.7_3	4.7_1	$0.32\ (0.72e^{-t/7.51}+0.28)$	21	35	32	0.60
AO	204	5.6_3	5.6_1	$0.33\ (0.71e^{-t/10.1}+0.29)$	28	50	48	1.30
AO-propyl	201	4.9_7	4.9_7	$0.32\ (0.71e^{-t/10.0}+0.29)$	28	48		1.15
AO-isopropyl	201	4.6_4	4.6_3	$0.31\ (0.70e^{-t/7.91}+0.30)$	22	37		0.70

a) Analyzed by the method of moments.[14,15] b) Analyzed by the method of Laplace transformation.[16]
c) The apparent value of the rotational relaxation time ($\rho_a=3\phi$) for the aqueous solution at 25 °C.
d) Calculated using Eq.14 ($\rho_a=3\phi'$). e) Obtained from the measurements of steady-state depolarization.

voluted $R(t)$ functions; the results are listed in Table 1. The ρ_a values are much smaller when compared to the values obtained by a steady-state study (Table 1).[6]

A continuous light source may be considered as a sum of an infinite number of pulses. Therefore, the observed static anisotropy is the mean value of $R(t)$.

$$\langle R\rangle = \frac{\int_0^\infty D(t)\mathrm{d}t}{\int_0^\infty S(t)\mathrm{d}t}. \tag{10}$$

If $S(t)$ is a single exponential like Eq. 4, Eq. 10 gives

$$\langle R\rangle = \frac{1}{\tau}\int_0^\infty R(t)e^{-t/\tau}\mathrm{d}t. \tag{11}$$

Put the case that $R(t)$ is an exponential function like $R(t)=R_0e^{-t/\phi}$, Eq. 11 leads to

$$\langle R\rangle = \frac{R_0}{1+\tau/\phi}. \tag{12}$$

Equation 12 is equivalent to Perrin's well-known formula.[22] When $R(t)$ is a function like Eq. 7, which is the present case, one obtains

$$\langle R\rangle = \frac{R_0\{\phi+(1-\alpha)\tau\}}{\phi+\tau} = \frac{R_0}{1+\tau/\phi'}, \tag{13}$$

where

$$\phi' = \frac{1}{\alpha}\{\phi+(1-\alpha)\tau\}. \tag{14}$$

According to Eq. 13, Perrin's plot in the steady-state study gives us ϕ' instead of ϕ. If Eq. 14 is applied to the deconvoluted $R(t)$ functions for the DNA–acridine complexes, the ρ_a values ($\rho_a=3\phi'$) listed in Table 1 are obtained. These values are in agreement with those obtained from the steady-state measurements (Table 1).[6] Thus, the discrepancy between steady-state and nanosecond anisotropy results can be understood.

It should be noted that the ρ_a values reported here are comparable to the rotational correlation times for the DNA–ethidium bromide[18] and *Cl. perfringens* DNA–PF[23] complexes which were obtained by nanosecond fluorometry. However, the ρ_a values are too much small to correspond to the rotation of the whole DNA molecule.[24] The most probable origin for the observed depolarization appears to be a local deformation motion of DNA base pairs.[18,21]

As is seen in Table 1, the ρ_a values for acridine dyes with bulky substituents (Ac[NEt$_2$]$_2$ and AO-isopropyl) are a little smaller than those obtained with the other dyes. Further, fluorescence quenching[5] and equilibrium dialysis[7] studies showed that the binding constant and the maximum number of binding sites per DNA phosphate are much smaller for acridine dyes with bulky substituents. This implies that the bulky groups attached to the acridine ring (*e.g.*, diethylamino and isopropyl) produce some steric hindrance to the binding.

Due to the presence of bulky substituents on the acridine ring, some changes in hydrodynamic properties of DNA may arise when acridine dyes are bound to it. Cohen and Eisenberg[25] showed that for short, almost rodlike fragments of DNA, the ratio of the contour length at the binding ratio r to that in the absence of dye (L/L_0) can be calculated from the viscosity data:

$$\frac{L}{L_0} = \left\{\frac{[\eta]f(p)_0}{[\eta]_0 f(p)}\right\}^{1/3}. \tag{15}$$

where $f(p)$, a function of the axial ratio p of the DNA rod, is insensitive to variations in p for large p, and $[\eta]$ and $[\eta]_0$ are intrinsic viscosities, respectively, in the

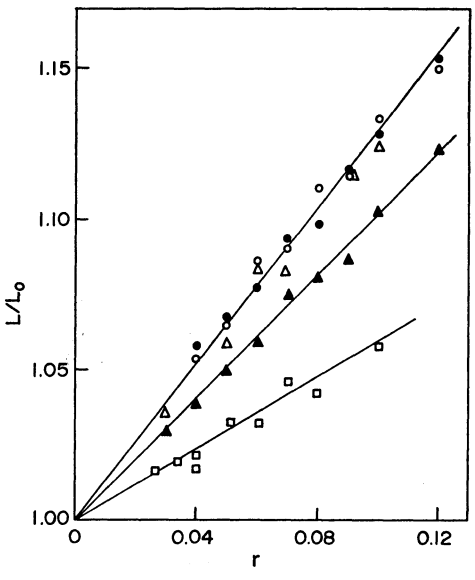

Fig. 3. Variation of L/L_0 as a function of r for sonicated DNA–acridine complexes. The molar concentration of DNA phosphate: 3.7×10^{-4} M. Acridine dyes: ○PF, ● AO, △ Ac[NHMe]$_2$, ▲ Ac[NHEt]$_2$, □ Ac[NEt$_2$]$_2$.

presence and absence of dye. The results obtained with various acridines are plotted in Fig. 3. In all cases, L/L_0 varies linearly with the binding ratio r.

$$\frac{L}{L_0} = 1 + \beta r. \tag{16}$$

The values of the slope β are listed in Table 1. The result obtained with PF is in good agreement with that previously reported.[25] As is clearly seen in Fig. 3 and Table 1, β is dependent on the dye structure; the β values for $Ac[NEt_2]_2$ and AO-isopropyl are much smaller than those for the other dyes.

Lerman's intercalation model[1,2] predicts that the DNA molecule is lengthened by 3.35 Å per intercalated dye molecule. If all bound molecules are completely intercalated, the slope of the L/L_0 *vs.* r plot should be 2.[1,25] From temperature jump relaxation studies of the DNA–PF complex, Crothers *et al.*[26,27] showed that most of bound PF molecules are intercalated, but some of those are bound outside of the DNA helix even at low binding ratios. On the other hand, Müller *et al.*[28] and Bontemps *et al.*[29] showed that a derivative of PF with bulky side chains, 3,6-diamino-2,7-di-*t*-butylacridine, binds to DNA but not by intercalation; they concluded that an outside bound complex is formed. In view of these, our viscosity results can be interpreted as follows: (1) the β values smaller than 2 result from the existence of the outside bound complex which would not contribute to an increase in the length of DNA[25–27] and (2) there exists a higher fraction of the outside bound complex in the case of $Ac[NEt_2]_2$ and AO-isopropyl because of the presence of bulky substituents.

Hydrodynamic and thermodynamic properties of outside bound dye are different from those of intercalated dye, while their optical properties are very similar.[26,27] In practice, the fluorescence decay of the complex could be characterized by a single exponential function (Fig. 1; Table 1). However, the structure of the outside bound complex is not yet well-defined.[26,27] In conclusion, the interpretation of the anisotropy results may be complicated by the heterogeneity of binding sites (intercalating and outside binding sites). If we assume that the local movement of the outside bound dye is more rapid compared to that of the intercalated dye which is restricted between adjacent base pairs, the smaller ρ_a values obtained with $Ac[NEt_2]_2$ and AO-isopropyl may be qualitatively understood. Further study would be necessary to clarify the origin for depolarization of the DNA–acridine complexes.

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