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Fluorescence lifetime distributions of DNA–4′,6-diamidino-2-phenylindole complex

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Time-resolved fluorescence of 4′,6-diamidino-2-phenylindole (DAPI) complexes show that for a homogeneous polymer (polyd(AT) or polyd(A) · polyd(T)) at high *P/D* (phosphate/dye) ratio, a single exponential component adequately describes the fluorescence decay. For the AT polymers at low *P/D* ratio or for native DNA, the decay cannot be described by a single-exponential term. A continuous distribution of lifetime values of Gaussian shape gives a good fit to the decay data. We propose that the lifetime distribution method for the analysis of the fluorescence decay of DNA–DAPI complexes provides a useful method of characterizing the microheterogeneity of site binding.

Introduction

DAPI (4′,6-diamidino-2-phenylindole) interacts reversibly with natural and synthetic polydeoxynucleotides. The binding is accompanied by a large increase in fluorescence intensity [1–3]. DAPI is a fluorescent probe well known to the biological community, used for the selective visualization of the paracentromeric constrictions of human chromosomes 1, 9 and 16 and for a Q-banding-like pattern of the short arm of chromosome 15 and the distal part of the Y chromosome, and for the assessment of all chromosomal DNA in flow systems [2–22]. DAPI also probes the molecular environment of restriction endonuclease cleavage sites [23,24]. Previous studies have shown that only complexes with AT, AU and IC clusters are responsible for the fluorescence enhancement [4]. From a number of studies using different techniques, a mechanism for DNA–DAPI interaction has been proposed. The interaction occurs through two different kinds of mechanisms: one, highly energetic (−4.5 kcal/mol of DAPI), being characterized by high specificity for A-T and A-U bases; the second, non-specific, giving rise to an unenhanced fluorescent complex characterized by a much lower affinity constant. A detailed molecular mechanism for the binding process has been postulated and it accounts for most of the experimental observations. In this model, high-affinity binding results from the formation of two hydrogen bonds in the minor groove between the N3 and O2 of adenine and thymine, respectively, and the donor groups of the dye. Electrostatic interactions also contribute to the overall binding energy and are responsible for the low-affinity binding characteristic of the interaction of DAPI with GC clusters [5–8,25–27]. The two modes of binding of DAPI, to AT and GC clusters, are clearly distinguishable using fluorescence intensity measurements.

In the work reported in this paper, we have explored the possibility of discriminating the various contributions to binding of the probe to polydeoxynucleotides of different base content and sequence, using the different fluorescence-decay behavior associated with binding to AT and GC clusters. Furthermore, since natural polydeoxynucleotides should provide a less homogeneous substrate for DAPI than to synthetic polydeoxynucleotides, there arises the possibility of having an appreciable degress of heterogeneity of lifetimes, which cannot be resolved by fluorescence intensity measurements. We report here a study performed on the DNA–DAPI complex using frequency-domain fluorometry which has been successfully used to investigate the microheterogeneity of other complex biological systems [28–32]. The fluorescence decay has been analyzed using two models. One assumes the existence of two species, each characterized by a well-defined lifetime value. In the

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; POPOP, 1,4-bis(2-(5-phenyloxazolyl))benzene.

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limiting case, apparently appropriate for the homogeneous synthetic polymers, these two species should be identified with DAPI molecules shielded from the solvent and DAPI molecules in or exposed to the solvent, respectively. The second model assumes the existence of a large number of molecular species characterized by continuous distributions of lifetime values, and appears to be more appropriate for the analysis of the decay of DAPI bound to natural polydeoxynucleotides where different binding situations can occur. In this latter approach, the degree of heterogeneity of the system may be assessed by the spread of the lifetime distribution.

Materials and Methods

Calf thymus DNA (*t*DNA) (Type I, Sigma, St. Louis, MO) was found to contain less than 0.5% protein contamination and was used without further purification. Col E_1 plasmid DNA (ccDNA) was from Boehringer (Indianapolis, IN). Two DNA fractions were separated by a CsCl-Eb buoyant density gradient (refractive index = 1.39–1.4), in a vertical rotor at 278 000 × *g* over night at 20 °C and detected by means of ultraviolet light inspection. The lower band is closed circular plasmid DNA [33]. It was collected and the ethidium bromide was extracted with *n*-butanol, and the DNA was precipitated with 80% ethanol on ice. Polyd(AT), polyd(A) · polyd(T) and polyd(GC) were from Boehringer. DAPI was purchased from Serva Biochemicals (Heidelberg, F.R.G.) and checked for purity by thin-layer chromatography. All organic solvents were of the highest purity available and doubly distilled. Millipore filtered water was used throughout. Inorganic chemicals were reagent grade. Highly polymerized linear DNA was sonicated several times at ice-bath temperature to prevent intermolecular aggregation. The concentration of the solutions was determined by using the following molar absorption coefficients: calf thymus DNA, ccDNA and polyd(AT), 6600; polyd(GC), 8400; polyd(A) · polyd(T), 6000. A molar absorption coefficient of 23 000 $M^{-1} \cdot cm^{-1}$ at 342 nm was used to determine the concentration of DAPI solutions. All samples were dissolved in aqueous buffered solutions containing 0.01 M NaCl/0.1 M Tris (pH = 7.2) (buffer A). Depending on the final concentration of DNA, different ratios of deoxyribonucleic acid, as moles of phosphate (P) to dye (D) were used. For the polydeoxynucleotide/DAPI experiments the final concentration was obtained by adding increasing amounts of polymer to give the desired P/D ratio. Constancy of DAPI concentration was achieved by adding a polymer solution containing DAPI at the same molarity as that of the initial polymer free solution. All the measurements were performed at 24 °C. In all experiments each sample was allowed to equilibrate at room temperature for at least 5 min before measurement. Steady-state spectrofluorimetric experiments were carried out with the microprocessor-controlled photon-counting apparatus described by Gratton and Limkeman [34]. Lifetime measurements were performed on the multifrequency phase and modulation fluorometer described by Gratton and Limkeman [35], equipped with an ISS1ADC interface (ISS, Champaign, IL) for data acquisition and analysis. The excitation source was a HeCd laser (Liconix model 4240N, Sunnyvale, CA) emitting at 325 nm. In each experiment a set of 10 to 12 different modulation frequencies was employed in the range 10 to 200 MHz. The emission was observed using a RG370 band-pass filter (Janos Technology, Townshend, VT). A solution of POPOP in ethyl alcohol was used as a reference with a lifetime of 1.35 ns. Phase and modulation data were analyzed using a sum of exponentials by a non-linear least-squares routine described elsewhere [36,37]. The analysis using continuous lifetime distributions was performed using a program provided by ISS, for an IBM-PC computer. This program minimized the reduced chi-square defined by:

$$\chi^2 = \frac{1}{(2*N-\mu)} \sum_{n=1}^{N} \left[\frac{(\phi_c - \phi_m)^2}{\sigma_\phi^2} + \frac{(M_c - M_m)^2}{\sigma_M^2} \right]$$

using the Simplex algorithm, where N is the number of measurement frequencies and μ the number of free parameters used for the fit. The values of ϕ_c and M_c are calculated using the following expression:

$$\phi_c = \tan^{-1}(S/G)$$

$$M_c = \sqrt{S^2 + G^2}$$

where

$$S(\omega,\tau) = \int_0^\infty f(\tau) \sin(\omega t)\, e^{-t/\tau_d}$$

$$G(\omega,\tau) = \int_0^\infty f(\tau) \cos(\omega t)\, e^{-t/\tau_d}$$

and ϕ_m and M_m are the measured values of phase and modulation at the frequency, ω. The standard deviation of the phase determination σ_ϕ and of the modulation determination σ_M were assumed to be the same at each measurement frequency with values of 0.2 degrees and 0.004 for the phase and modulation standard deviation, respectively. For the measurements made in this work, these values slightly overestimate the actual standard deviations, giving values of the χ^2 smaller than 1. However, we use the χ^2 values obtained for the fit using different models only as a term of comparison. The function, $f(\tau)$, describes the lifetime distribution

and can have the following different forms:

Uniform: $f(\tau) = 1$ between $C - (W/2)$ and $C + (W/2)$, $f(\tau) = 0$ elsewhere

Lorentzian: $f(\tau) = \dfrac{1}{1 + \left[\dfrac{(\tau - C)}{W/2}\right]^2}$

Gaussian: $f(\tau) = e^{\ln 2/(4W(\tau - C)^2)}$

Where C is the center of the distribution, and W the full width at half-maximum. The errors quoted for the recovered parameters have been determined using a rigorous error analysis routine [38]. This estimation of the error must not be confused with the resolvability of the lifetime distribution, i.e., the minimum value of the distribution width that can be distinguished from a single-exponential decay. The resolvability problem has been extensively studied in the context of phase fluorometry. Two independent estimations [39,40] provide the same results with respect to the resolvability of different distribution shapes. Generally speaking, the uniform distribution has the lower resolvability, followed by the Gaussian and the Lorentzian shape. With regard to the interpretations of the fits reported in this work, for an average lifetime of about 4 ns the minimum width that can be resolved is about 0.05, 0.6 and 1 ns of the Lorentzian, Gaussian, and uniform distribution respectively, based on the results of the resolvability studies [39,40]. We will consider the fits with a width less than the minimum resolvable as an indication that the distribution recovered can be equally well represented by an exponential decay.

Results

The emission spectra of DAPI free and bound to several different polydeoxynucleotides are shown in Fig. 1. The fluorescence enhancement for the a, b and c

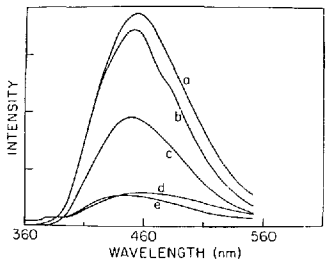

Fig. 1. Emission spectra of DAPI bound to (a) poly(AT), (b) *l*DNA, (c) ccDNA (spectrum ×10), (d) poly(GC) (spectrum ×20) and 9e) free DAPI (spectrum ×20).

spectra is evident. Also spectrum d differs significantly from spectrum e, indicating that DAPI interacts with poly(GC). Table I reports the results of the analysis of the fluorescence decay of polydeoxynucleotide–DAPI complexes using a sum of exponentials and using a continuous distribution of lifetime values of gaussian shape. For the complexes with fully AT containing polymers, either homo or alternating sequence, at least 93 to 98% of the decay is associated with one of the two exponential components (for P/D ratios as low as 7) and its distributional analysis gives a very narrow distribution virtually irresolvable from a single exponential decay. The fluorescence decay for linear and circular DNAs at large P/D ratio is similarly dominated by a single exponential, but at P/D ratio of about 6 the decay has 2 more equal components. The analysis using two exponential components and the analysis using two distributional components gives very similar results with respect to the position of the center of the distribution which is similar to the value of the two discrete lifetimes. The distribution analysis shows a larger value of the distribution width at low P/D ratios (Fig. 2a),

TABLE I

Fluorescence decay parameters of natural and synthetic polydeoxynucleotides – DAPI complexes at different P/D ratios analyzed using two exponential components and using two distributional components of Gaussian shape.

*l*DNA, calf thymus DNA; ccDNA, Col E1 plasmid DNA; (A)·(T), poly(A)·poly(T); (A·T), poly(A·T); (G·C), poly(G·C); τ, lifetime of the exponential component; C, center of the Gaussian component; W, full width at half-maximum of the Gaussian component. Units of τ, C and W are nanoseconds. f_1 is the fractional contribution of the component to the total fluorescence intensity.

Complex	P/D	τ_1	τ_2	f_1	χ^2	C_1	W_1	f_1	C_2	W_2	χ^2
*l*DNA	60	3.50	0.44	0.96	0.99	3.50	1.09	0.97	0.20	1.65	0.87
*l*DNA	6	3.01	0.46	0.82	3.31	2.88	2.23	0.91	0.20	0.05	0.74
ccDNA	54	3.66	0.78	0.95	0.60	3.65	1.05	0.96	0.64	0.05	0.59
ccDNA	6	2.99	0.54	0.82	0.98	2.94	1.37	0.86	0.46	0.18	0.75
(A)·(T)	7	3.98	0.21	0.98	0.35	3.99	0.57	0.98	0.20	0.68	0.34
(A·T)	7	3.90	0.26	0.93	1.18	3.89	0.61	0.93	0.12	0.94	1.04
(G·C)	67	3.04	0.25	0.37	0.83	3.01	1.68	0.38	0.24	0.05	0.74
DAPI (pH 7)		2.81	0.19	0.23	1.51	2.59	1.25	0.25	0.19	0.23	1.12

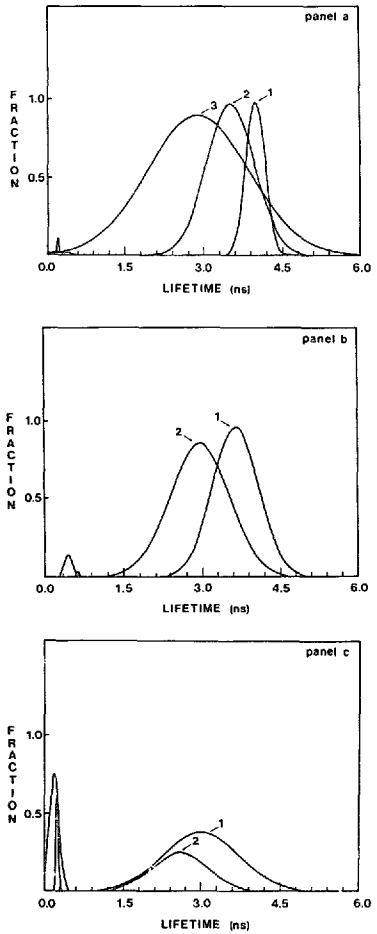

Fig. 2. Lifetime distribution analysis using Gaussian components. (a) 1, polyd(A)-polyd(T); 2, lDNA at P/D ratio of 60; 3, lDNA at P/D ratio of 6. (b) 1, ccDNA at P/D ratio of 54; 2, ccDNA at P/D ratio of 6. (c) 1, polyd(GC) at P/D ratio of 67; 2, DAPI in buffer A at pH 7. The ordinate scale represents the fractional contribution to the total fluorescence intensity.

indicating a larger heterogeneity of the system at high dye concentrations. In the same figure the lifetime distribution obtained for the complexes with polyd(A)·polyd(T) is also shown for comparison. Fig. 2b shows the distribution obtained for ccDNA–DAPI complex at high and low P/D ratios. Fig. 2c shows the distribution obtained for the polyd(GC)–DAPI complex at high P/D ratio. In the same figure, the distribution obtained for DAPI in Tris buffer 0.1 M at pH 7 is shown for comparison. For the distributions reported in Fig. 2, the distribution shape used was Gaussian. In Table II, we show a comparison of the analysis of the decay of lDNA–DAPI complex at P/D ratio of 6, using different distribution shapes. The shape that consistently gives a lower χ^2 is the Gaussian shape. When the distribution is very narrow, the analysis using different shapes gives very similar χ^2 values, as should be expected.

Discussion

The single-exponential decay characterized by a relative longer value of the lifetime (approx. 4 ns) observed for the AT containing polymers indicates that under conditions of a homogeneous polymer there is essentially only one molecular species (for P/D ratio substantially larger than 6), as judged by the lifetime analysis. Furthermore, the distributional analysis in this case recovered a very narrow shape (width 0.5–0.6 ns), providing further evidence that for a homogeneous polymer, where only a single molecular species should be present, the distributional analysis coincides with the exponential analysis. On the other hand, for the linear DNA at low P/D ratio, the distributional analysis reveals the heterogeneity of the system. The width of the distribution and the χ^2 changes are significant [39,40] for the long lifetime component. At high P/D ratios, at least 96% of the decay is associated with a distributional component centered at relatively long lifetime values. The χ^2 change and width of this component are not significant at high P/D ratios, indicating that the heterogeneity is dependent on the degree of site saturation. This result is consistent with previous work [2,6–8,25,27] that demonstrated that at high P/D ratios essentially one kind of binding occurs. Also at low P/D ratios, for both linear and circular DNAs, the fractional intensity of the longer lifetime component decreases, indicating a more heterogeneous environment of the DAPI molecule. This result is also consistent with previous work [2,6–8] that showed that under these conditions, at least two different forms of binding occur.

The distribution of lifetime values originates from different kinds of interactions between DAPI and DNA which result in a population of different molecular species. The possibility exists that the different molecular species persist for a time much longer than the decay time. In this case the distribution observed reflects the degree of heterogeneity of the system. Alternatively, if

TABLE II

Fluorescence decay parameters of IDNA–DAPI complex at P/D = 6, analyzed using two discrete exponential components and using different distribution shapes

C, center of the Gaussian component or lifetime; *W*, full width at half-maximum of the Gaussian components. Units of *C* and *W* are nanoseconds. f_1 is the fractional contribution of the component to the total fluorescence intensity.

Distribution	C_1	W_1	f_1	C_2	W_2	χ^2
Discrete	3.01 ± 0.00		0.82 ± 0.00	0.46 ± 0.00		3.31
Lorentzian	2.84 ± 0.02	0.51 ± 0.04	0.85 ± 0.00	0.28 ± 0.02	0.44 ± 0.02	0.95
Uniform	2.94 ± 0.01	3.00 ± 0.06	0.88 ± 0.00	0.00 ± 0.02	1.07 ± 0.04	0.76
Gaussian	2.88 ± 0.01	2.23 ± 0.03	0.91 ± 0.00	0.20 ± 0.01	0.05 ± 0.50	0.74

the lifetime of the molecular species is comparable to the fluorescence decay time, the lifetime distribution can be altered by the dynamics of the system. Given the relatively strong interaction energy between DNA and DAPI, it is likely that the lifetime of the molecular complex is relatively long. In this case only the heterogeneity of the binding sites can contribute to the spread of lifetime components observed in IDNA. The origin of the Gaussian shape for the lifetime distribution is likely to reside in the large number of different binding sites available for DAPI in the natural polydeoxynucleotides.

In conclusion, fluorescence lifetime determinations provide a rapid and accurate method to determine the heterogeneity of environment of the DAPI molecule when bound to DNA. Based on different lifetime values of different interacting species, the characteristics of the binding process has previously been characterized, and a model which postulates more than one binding form has been proposed [6,7]. The present fluorescence study supports the previous conclusions. The analysis using continuous lifetime distributions provides a new approach for the characterization of the microheterogeneity of the binding sites.

References

1 Kapuscinski, J. and Skoczylas B. (1976) Nucl. Acids Res. 5, 3775–3799.
2 Masotti, L., Barcellona, M.L., Von Berger, J. and Avitabile, M. (1981) Biosci. Rep. 1, 701–707.
3 Masotti, L., Cavatorta, P., Avitabile, M., Barcellona, M.L. and Ragusa, N. (1982) It. J. Biochem. 31, 90–99.
4 Kapuscinski, J. and Szer, W. (1979) Nucl. Acids Res. 6, 3519–3534.
5 Masotti, L., Cavatorta, P., Barcellona, M.L., Avitabile, J. and Ragusa, N. (1982) I.R.C.S. Med. Sci. Biochem. 10, 957–958.
6 Manzini, G., Bercellona, M.L., Avitabile, M. and Quadrifoglio, F. (1983) Nucl. Acids Res. 11, 8861–8875.
7 Barcellona, M.L., Manzini, G., Xodo, L., Ragusa, N., Avitabile, M. and Quadrifoglio, F. (1985) It. J. Biochem. 34, 467–473.
8 Manzini, G., Xodo, L., Barcellona, M.L. and Quadrifoglio, F. (1985) J. Biosci. 8, 699–711.
9 Barcellona, M.L., Avitabile, M., Ragusa, N., Cavatorta, P. and Masotti, L. (1983) It. J. Biochem. 32, 214–215.
10 Avitabile, M., Ragusa, N., Barcellona, M.L. and Masotti, L. (1983) It. J. Biochem. 34, 472–476.
11 Hajduk, S.L. (1976) Science 191, 858–859.
12 Schweizer, D. and Nagl, W. (1976) Exp. Cell Res. 98, 411–423.
13 Schweizer, D. (1976) Chromosoma 58, 307–324.
14 Schweizer, D., Ambros, O. and Andrle, M. (1978) Exp. Cell Res. 111, 327–332.
15 Rocchi, A., Di Castro, M. and Prantera, G. (1980) Cytogen. Cell Genet. 27, 70–72.
16 Yezierski, R.P. and Bowker, R.M. (1981) Neurosci. Methods 4, 53–62.
17 Coleman, A.W. (1984) Exp. Cell Res. 152, 528–540.
18 Shinsichi, H. and Setsuya, F. (1983) Acta Histochem. Cytochem. 16, 606–609.
19 Schmid, M. and Haaf, T. (1984) Cytogen. Cell Genet. 38, 192–199.
20 Wilkins, R.J. and Kearney, J.T. (1984) Anal. Biochem. 136, 301–308.
21 Coleman, A.W. and Goff, L.J. (1985) Stain Technol. 60, 145–154.
22 Otto, F. and Tsau, C. (1985) Stain Technol. 60, 7–11.
23 Kania, J. and Fanning, T.G. (1976) Eur. J. Biochem. 67, 367–371.
24 Palù, G., Valisena, S., Barcellona, M.L., Masotti, L. and Meloni, G.A. (1987) Biochim. Biophys. Res. Commun. 145, 40–45.
25 Barcellona, M.L., Favilla, R., Von Berger, J., Avitabile, M., Ragusa, N. and Masotti, L. (1986) Arch. Biochem. Biophys. 250, 48–55.
26 Cavatorta, P., Masotti, L. and Szabo, A.G. (1985) Biophys. Chem. 22, 11–16.
27 Szabo, A.G., Krajcarski, D.T., Cavatorta, P., Masotti, L. and Barcellona, M.L. (1986) Photochem. Photobiol. 44, 143–150.
28 Fiorini, R., Valentino, M., Wang, S., Glaser, M. and Gratton, E. (1987) Biochemistry 26, 3864–3870.
29 Parasassi, T., Conti, F., Gratton, E. and Sapora, O. (1987) Biochim. Biophys. Acta 898, 196–201.
30 Jameson, D.M., Gratton, E. and Eccleston, J. (1987) Biochemistry 26, 3894–3901.
31 Alcala, J.R., Gratton, E. and Prendergast, F.G. (1987) Biophys. J. 51, 925–936.
32 Rosato, N., Finazzi-Agrò, A., Gratton, E., Stefanini, S. and Chiancone, E. (1987) J. Biol. Chem. 262, 14487–14491.
33 Radloff, R., Bauer, W. and Vinograd, J. (1967) Proc. Natl. Acad. Sci. USA 57, 1514.
34 Gratton, E. and Limkeman, M. (1983) Rev. Sci. Instrum. 54, 294–299.
35 Gratton, E. and Limkeman, M. (1984) Biophys. J. 44, 315–324.
36 Lakowicz, J.R., Laczko, G., Cherek, H., Gratton, E. and Limkeman, M. (1984) Biophys. J. 46, 463–477.
37 Gratton, E., Limkeman, M., Lakowicz, J.R., Laczko, G. and Cherek, H. (1984) Biophys. J. 46, 478–486.
38 Beechem, J.M. and Gratton, E. (1988) in Time Resolved Laser Spectroscopy in Biochemistry, Proceedings of S.P.I.E., Vol. 909, pp. 70–81.
39 Alcala, J.R., Gratton, E. and Prendergast, F.G. (1987) Biophys. J. 51, 587–596.
40 Lakowicz, J.R., Cherek, H., Gryczynski, I., Joshi, N. and Johnson, M.L. (1987) Biophys. Chem. 28, 35–50.