Eur Biophys J (1990) 17:315–323

European Biophysics Journal
© Springer-Verlag 1990

The fluorescence properties of a DNA probe *

4′-6-Diamidino-2-phenylindole (DAPI)

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Received January 27, 1989/Accepted in revised form September 30, 1989

Abstract. Steady-state and dynamic fluorescence measurements have been performed on DAPI in solution and in complexes formed with a number of synthetic and natural polydeoxynucleotides. The decay of DAPI in buffer at pH 7 was decomposed using two exponentials having lifetime values of approximately 2.8 ns and 0.2 ns. The double exponential character of the decay was maintained over a large pH range from 3 to 9. At pH 1 the short component dominated, whereas at pH 12, only the long component was detectable. Two distinct spectra were associated with the two lifetime components; the short component was shifted to the red. The short lifetime component occurs in the presence of water. In water the excitation spectra depended on the emission wavelength and there was no viscosity dependence of the two forms. To explain these results we propose that there is a ground state conformer in which preferential solvation of the indole ring allows proton transfer in the excited state. DAPI complexed with polydeoxynucleotides retained most of the features of the decay of DAPI in solution. However, the complexes with fully AT-containing polymers stabilized the longer lifetime form of DAPI because the stronger binding enhanced solvent shielding. A gradual increase of the short lifetime component, which monitors dye solvent exposure, was obtained as the AT content was decreased. For polyd(GC) the decay was similar to that of free DAPI.

Key words: Fluorescence, DAPI, DNA

Abbreviations: DAPI: 4′-6-diamidino-2-phenylindole; POPOP: 1,4-bis(5-phenyl-2-oxazolyl)-benzene; 2,2′-p-phenylene-bis(5-phenyloxazole)

* Financial support for this work was provided by a M.P.I. grant 1984, Roma, Italy for M.L.B. and NSF-PCM 84-03107 and PHS-IP41RR03155 for E.G.

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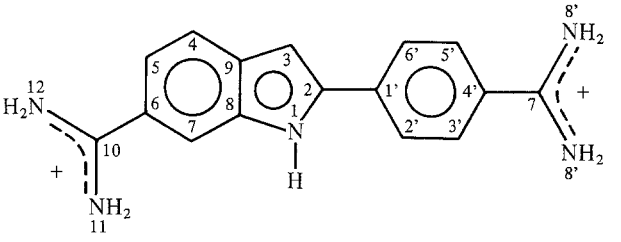
Introduction

DAPI, synthesized as an analogue of diarylamidine (berenyl) (Dann et al. 1971), is known to bind reversibly with double stranded deoxyribonucleic acids and with various synthetic analogs of different base content and sequence. DAPI exhibits a remarkable increase in fluorescence quantum yield upon binding (Russell et al. 1975; Kapuscinski and Skoczylas 1978; Masotti et al. 1982).



Scheme 1. Structure of 4′-6-diamidino-2-phenylindole (DAPI)

Previous studies have shown that only the binding with AT, AU or IC clusters produces a fluorescent complex (Kapuscinski and Szer 1979). Because of this property, DAPI is currently used in a number of biochemical and cytochemical investigations including a staining procedure for the selective visualization of the paracentromeric constrictions of human chromosomes 1, 9 and 16 and *Q*-banding-like pattern of the short arm of chromosome 15 and the distal part of the *Y* and for the assessment of all chromosomal DNA in flow systems (Avitabile et al. 1985; Coleman 1984; Manzini et al. 1983; Masotti et al. 1981; Schweizer 1976; Shinsichi and Setsuya 1983; Rocchi et al. 1980). DAPI also probes the molecular environment of restriction endonuclease cleavage sites (Kania and Fannnig 1976; Palú et al. 1987).

The spectroscopic properties of DAPI are greatly modified as a consequence of its interaction with nucleic acids. The strong fluorescence enhancement, the

induced CD spectrum and the hypochromism in the ultraviolet absorption following binding to DNA have been interpreted in terms of two different modes of binding by Kapuscinski and Skoczylas (1978). The first mode, in their view, corresponds to a highly energetic and intercalative type of interaction, characterized by a high value of the affinity constant and a high specificity for AT, AU and IC cluster. The second mode, which corresponds to a lower affinity constant, is non-specific, mainly electrostatic in character and shows no increase in fluorescence intensity. However, recent spectroscopic, sedimentation equilibrium, viscometric and calorimetric investigations, have ruled out intercalation as the molecular mechanism for the specific, strong binding and suggested a new model for the interaction (Manzini et al. 1983). This new model assumes the formation, in the narrow helical groove, of two hydrogen bonds between the amidino groups of the drug and the adjacent acceptor groups of AT base pairs, in addition to electrostatic interactions of the dye's positively charged ends with the phosphate groups of the polynucleotide backbone. A similar mode of interaction has been proposed and verified for netropsin and distamycin A, two well known antibiotics, non-intercalating and strongly interacting with DNA (Dattagupta et al. 1980; Patel 1982; Gupta et al. 1984). This model of interaction is also supported by the molecular structure of DAPI, which is characterized by three hydrogen donor groups, the two amidino moieties and the indole NH, which, together with the total positive charge of the molecule, provide a strong driving force for the interaction with DNA.

In this work, we have investigated the fluorescence properties of DAPI in solvents and in complexes with natural and synthetic polydeoxynucleotides with the purpose of studying the origins of the photophysical behavior of DAPI and the molecular details of its interaction with nucleic acids. To better understand the different aspects of these interactions, we approached the characterization of the fluorescence of the polydeoxynucleotide-DAPI complexes using frequency domain fluorometry, which has proven to be suitable for studying complex emitting systems (Gratton et al. 1984a and b; Jameson et al. 1984; Lakowicz et al. 1984). The use of frequency domain fluorometry is particularly useful in this case, since this technique has the capability to resolve multiexponential decays rapidly and accurately. An immediate test for lifetime heterogeneity is provided, during the measurement, by the comparison of the apparent phase and modulation lifetime values. The resolution of the spectral emission into two or three components can be obtained in a few minutes, using the method of phase and modulation resolved spectra (Gratton and Jameson 1985). These features have been a determinant factor for studying the spectral properties of DAPI and for recognizing specific molecular mechanisms of interaction with polydeoxynucleotides. Recently Szabo et al. (1986) have performed an accurate study of the decay of DAPI in solutions at different pH values and in different solvents, using correlated single photon counting techniques. Our experimental data are in substantial agreement with these studies, but we propose a molecular mechanism involving solvation to explain the origin of the species observed in the lifetime measurements.

Materials and methods

Calf thymus DNA (Type I, Sigma, St. Louis, Missouri) and Col E1 plasmid DNA (Boehringer, Indianapolis, Indiana) containing no more than 0.5% protein contamination, were used without further purification. pUC_8 DNA was a kind gift of Prof. G. Palù (Microbiologia, Padova, Italy). Polyd(AT), polyd(A)-polyd(T), and polyd(GC) were from Boehringer. DAPI was purchased from Serva Biochemicals (Heidelberg, FRG) and checked for purity by thin layer chromatography. All organic solvents were of spectroscopic grade 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 extinction coefficients: calf thymus DNA (6,600), Col E1 DNA (6,600), pUC_8 (6,600), polyd(AT) (6,600), polyd (GC) (8,400), polyd(A)-polyd(T) (6,000). A molar extinction coefficient of 23,000 $M^{-1}\,cm^{-1}$ at 342 nm was used to determine the concentration of DAPI solutions. All polynucleotides 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. All the measurements were performed at 24 °C, unless otherwise specified. In the measurements pertaining to pH variation effects, a small amount of a concentrated aqueous solution of DAPI was diluted to the desired concentration. For the solvent studies, DAPI powder was directly dissolved in the solvent. For the polydeoxynucleotide/DAPI binding 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. In all experiments each sample was allowed to equilibrate at room temperature for at least 5 min before measurement.

Steady-state spectrofluorometric experiments were carried out with the microprocessor-controlled photon-counting apparatus described by Gratton and Limkeman (1983). Lifetime measurements were performed on the multifrequency phase and modulation fluorometer described by Gratton and Limkeman (1984) equipped with an ISS1ADC interface (ISS, Champaign, Illinois) for data acquisition and analysis. The exciting source was a HeCd laser (Liconix model 4240N; Sunnyvale, California) emitting at 325 nm. In each experiment a set of eight to ten different modulation frequencies were employed in the range 10 to 200 MHz. The emission was observed using a RG370 band-pass filter (Janos Technology, Townshend, Vermont). 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 (Lakowicz et al. 1984; Gratton et al. 1984a). Phase resolved spectra were obtained using the software method described by Gratton and Jameson (1985). An emission monochromator (Model UV10, Jobin-Yvon) was used with 8-nm bandpass. The reference phase and modulation readings were obtained using a glycogen solution. The integration time was 2 s per point and data were collected at 5-nm intervals.

Results

A. Steady-state measurements

pH Dependence of the absorption and fluorescence spectra. The absorption spectrum of DAPI in buffer *A* is shown in Fig. 1. The position of the maximum, which was constant from pH 3 to pH 9, was shifted to the blue by about 9 nm at pH 12. At pH values below one the maximum was shifted by 8 nm to longer wavelengths ($\lambda_{max} = 350$ nm). The corrected emission spectra displayed a similar pattern, i.e., no variation in the pH range from 3 to 9. By lowering the pH to 1.75 a red shift was observed. Below pH 1, an additional red shift and a quenching of the fluorescence intensity was obtained. In contrast, at high pH values, the fluorescence maxima shifted to shorter wavelengths (Fig. 2) and there was a noticeable fluorescence enhancement (not shown, spectra in Fig. 2 are normalized). The fluorescence emission spectrum of DAPI at pH 7.1 was dependent on the excitation wavelength. For excitation at 380 nm, there was a red shift of the maximum and a consistent difference in the red tail with respect to 320 nm excitation (Fig. 3). A dependence of the excitation spectra on the emission wavelength was also observed, except for the maximum intensity which was positioned at the same wavelength (344 nm), regardless of the emission wavelength.

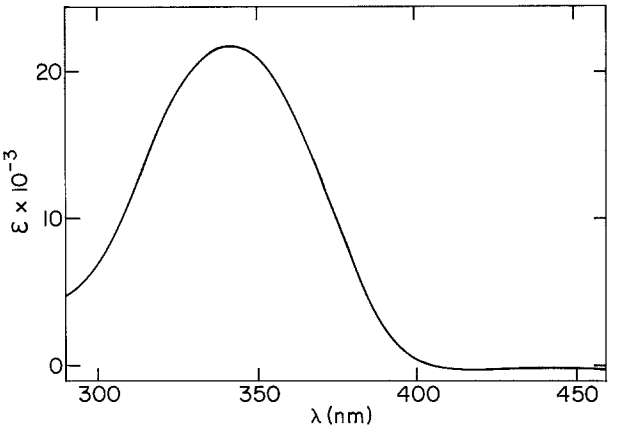


Fig. 1. Absorption spectrum of DAPI in buffer at pH 7.1, 20 °C

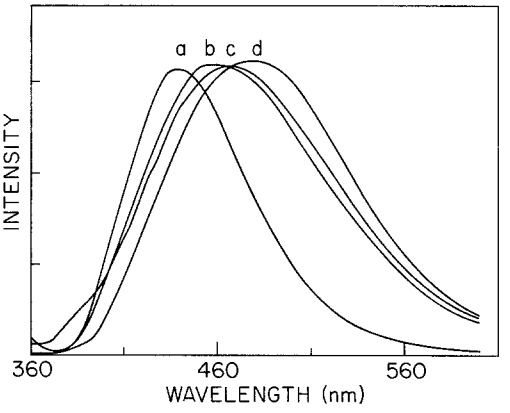


Fig. 2. Corrected emission spectra of DAPI at different pH values (excitation at 340 nm) (*a*) pH = 12, (*b*) pH = 7.1, (*c*) pH = 1.75, (*d*) pH = 0.5. All spectra are normalized to the same total intensity

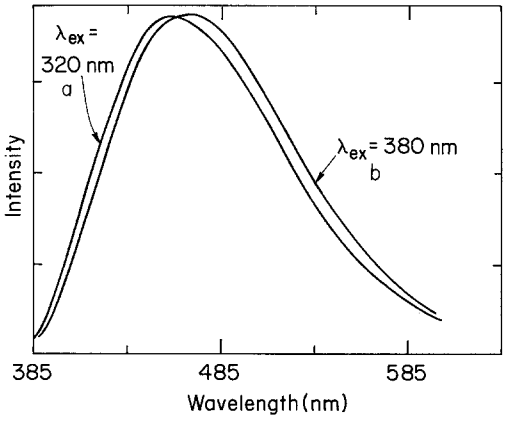


Fig. 3. Normalized emission spectra of DAPI in 0.01 *M* NaCl, 0.1 *M TRIS*, pH 7.2 at two different excitation wavelengths (*a*) $\lambda_{ex} = 320$ nm, (*b*) $\lambda_{ex} = 380$ nm

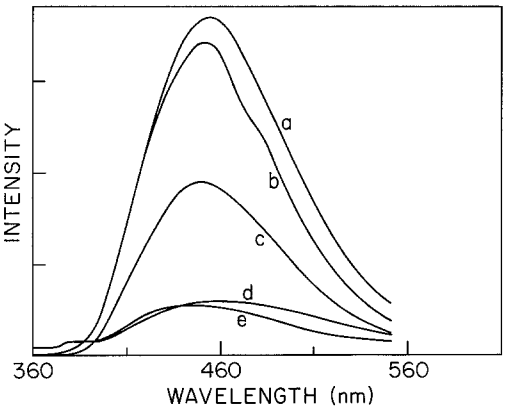


Fig. 4. Emission spectra of free DAPI (*d*, ×20) and bound to linear DNA (*b*), covalently closed DNA (*c*, ×10), polyd(AT) (*a*), polyd(GC) (*e*, ×20) at *P/D* ratio (≅60) excitation was at 340 nm

Solvent effects on the excitation and emission spectra. The maximum of the absorption spectrum was progressively red shifted from 349 nm in methanol, to 353 nm in ethanol and to 356 nm in butanol with respect to that in water (342 nm). In parallel with the absorption spectrum, the maxima of the excitation spectra were also red shifted as the polarity of the solvent decreased. Conversely, the corrected emission maxima were progressively blue shifted. The decrease of the Stokes shift demonstrated that, in the ground state, DAPI interacts progressively less with solvents less polar than water.

Natural and synthetic polydeoxynucleotides/DAPI complexes. Steady-state fluorescence measurements of DAPI bound to natural (calf thymus DNA, bacteriophage DNA, pUC_8 DNA), and synthetic polydeoxynucleotides (polyd(AT), polyd(A)-polyd(T)) showed a high degree of fluorescence enhancement (Fig. 4). At variance with other literature reports (Szabo et al. 1986), the fluorescence intensity was not increased when DAPI was complexed with polyd(GC) even at *P/D* ratios as high as 64.

Irrespective of the base content and sequence, the emission spectrum of polydeoxynucleotides/DAPI complexes at high and stoichiometric *P/D* ratios showed a blue shift of the emission maximum of about 10 nm relative to free DAPI and a decrease of the spectral half-widths from 110 nm to 90 nm in the nucleotide complexes. In the case of the poyd(GC)-DAPI complex, we attributed the lack of fluorescence enhancement to a binding characterized by a loose fit and by a less hydrophobic environment than experienced on binding to polyd(AT).

Steady-state anisotropy. Fluorescence anisotropy measurements of DAPI in propylene glycol at low temperature and complexed with calf thymus DNA, polyd (AT) and polyd(GC) at high *P/D* ratios were performed

Table 1. The steady-state fluorescence anisotropy, average lifetime and apparent rotational correlation time of DAPI in solution and in polydeoxynucleotide complexes

Sample	*A* [ns]	$\langle\tau\rangle$ [ns]	ϕ [ns]
Free DAPI (pH 7.1, 20 °C)	0.125	0.29	0.14
Polyd(GC)	0.200	0.79	0.90
DNA (linear)	0.270	2.07	5.3
Polyd(AT)	0.271	3.9	9.9
DAPI in propylene glycol (−20 °C)	0.376		

λ_{ex} = 325 nm
λ_{em} = RG370 filter
A = Anisotropy
$\langle\tau\rangle$ = Average lifetime calculated using $\langle\tau\rangle = a_1\tau_1 + a_2\tau_2$, where $a_{1,2}$ are preexponential factors, $a_1 = f_1\tau_1/(f_1\tau_1 + f_2\tau_2)$ etc.
ϕ = Rotational correlation time calculated using the Perrin equation in the form: $1/A = 1/A_0\left(1 + \frac{\langle\tau\rangle}{\phi}\right)$; $A_0 = 0.376$ see above

to evaluate the motional freedom of the ligand. Although the large size of the DNA molecule and the relatively short lifetime of DAPI does not provide information on the rotational mobility of the whole DNA molecule, anisotropy values can provide a measure of the flexibility of the microdomain of the bound dye. Anisotropy values and average lifetimes were used to calculate approximate rotational correlation times (ϕ) using the Perrin equation (Table 1). The value increased with respect to the value measured for DNA/DAPI complex when DAPI was bound to fully AT-containing polymers in homopolynucleotides, as well as in alternating sequences. The value of the anisotropy obtained for the AT-polymers and DNA complexes, which was lower than the limiting value of the anisotropy in propylene glycol at low temperature, indicated either a local flexibility of the AT clusters involved in the binding or some degree of motional freedom of the complex.

B. Lifetime measurements

pH Dependence of lifetime values. The fluorescence decay of an aqueous solution of DAPI at pH 7.0 was measured at several different modulation frequencies in the range of 10 MHz to 200 MHz. The phase and modulation values, analyzed with a non-linear least-squares routine, gave a satisfactory fit (based on the chisquare value) using two exponential components having lifetimes of 2.8 ns and 0.19 ns and fractional fluorescence intensity of 0.27 and 0.73, respectively, integrated over the emission band observed using an RG370 filter. Phase resolved spectra were obtained on a similar sample as a function of emission wavelength in the region between 420 nm and 550 nm at a fixed modulation frequency of 100 MHz. At all wavelengths

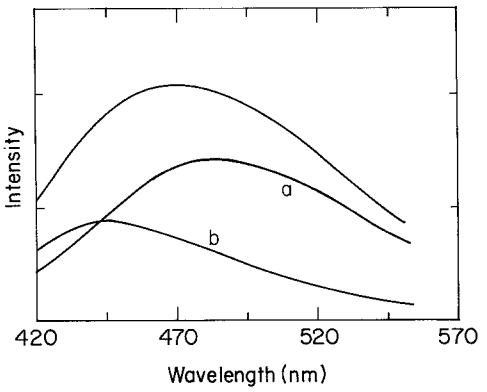


Fig. 5. Phase and modulation resolved spectra of DAPI at pH = 7.1. Excitation at 325 nm; modulation frequency 100 MHz (*a*) Spectrum at 0.15 ns and (*b*) at 2.5 ns

the values of τ^P and τ^M were different indicating that at least two components were present. Also, τ^P was smaller than τ^M and the lifetime values were between 1.9 ns and 0.29 ns (Table 2). In a multiexponential system, in which the decay is given by independent species, the measured phase and modulation lifetime values must always be lower than the largest lifetime and higher than the smallest lifetime present in the mixture. At 550 nm the fluorescence decay of DAPI was essentially a single exponential with a decay time shorter than 0.3 ns. Phase resolved spectra were obtained using two components of value 2.8 ns and 0.19 ns, respectively (Fig. 5). The modulation resolved spectra obtained using the same lifetime values were virtually coincident with the phase spectra. The similarity of the two solutions (using phase and modulation data) suggested that only two components were present (Gratton and Jameson 1985). The phase resolved spectra showed a clear separation of two spectral components associated with the two lifetime values. The long lifetime component had a maximum near 445 nm and the short component had a broad maximum centered near 480 nm and a very long red tail.

A set of phase and modulation data were collected at different pH values. The analysis of the fluorescence decay results at pH values between 1.75 and 9, using two exponential components, showed that both the long and the short lifetime values remained essentially constant at 2.8 ns and 0.2 ns and the fractional intensities maintained the same distribution (Table 3). The use of three exponentials did not improve the fit.

Decay in other solvents. In ethanol and methanol solutions, the decay was fitted with a single exponential of 2.6 ns. The emission of DAPI in propylene glycol was resolved using two components. The lifetime values were 3.1 ns and 1.2 ns, with fractional amplitudes of 0.83 and 0.17, respectively. In a 50% propylene glycol/water solution, two components were still observed.

Table 2. Phase and modulation lifetime values obtained from the phase and modulation resolved spectra of DAPI at pH 7 using 100 MHz modulation frequency

λ [nm]	τ^P [ns]	τ^M [ns]	I [a.u.]	f_1
420	0.71	1.91	53.0	0.50
425	0.79	1.80	61.4	0.55
430	0.64	1.81	70.9	0.46
435	0.69	1.81	78.3	0.49
440	0.67	1.70	85.4	0.48
445	0.57	1.71	91.2	0.41
450	0.63	1.61	95.6	0.45
455	0.50	1.48	98.9	0.35
460	0.45	1.44	102.7	0.31
465	0.50	1.33	104.2	0.35
470	0.47	1.24	104.3	0.33
475	0.42	1.24	104.1	0.28
480	0.42	1.19	103.0	0.28
485	0.43	1.15	101.2	0.29
490	0.42	1.03	98.3	0.28
495	0.36	1.01	94.6	0.22
500	0.38	0.92	90.7	0.24
505	0.41	0.95	87.2	0.27
510	0.37	0.92	82.4	0.23
515	0.35	0.90	78.6	0.21
520	0.27	0.97	73.1	0.11
525	0.31	0.95	68.4	0.16
530	0.32	0.86	63.3	0.17
535	0.35	0.79	58.1	0.21
540	0.36	0.90	52.9	0.22
545	0.29	0.88	48.2	0.14
550	0.31	0.78	43.5	0.16

τ^P was calculated from the phase values using $\tau^P = \tan\phi/\omega$;
τ^M was calculated from the modulation value using $\tau^M = (1/M^2 - 1)^{1/2}/\omega$;
I is the fluorescence intensity in arbitrary units ($\lambda_{ex} = 325$ nm).
f_1 is the fractional intensity of the 2.8 ns component obtained using the phase data at a given emission wavelength assuming that the decay is composed of two components of 2.8 ns and 0.19 ns, respectively

The value of the longer lifetime was similar, but its fractional amplitude was reduced to 0.14. The value of the shorter lifetime component was 0.6 ns.

Decay of polydeoxynucleotides/DAPI complexes. Fluorescence lifetime measurements of DAPI complexed with linear DNA, covalently-closed DNA (cc-DNA), polyd(AT), polyd(A)-polyd(T) and polyd(GC) were performed at different P/D ratios (Fig. 6). The fluorescence decay of linear or cc-DNA/DAPI complexes was doubly exponential (Table 4). At very high P/D ratios, the value of the longer component ($\sim$3.8 ns) was common to all DAPI complexes and the longer component, which gave a minor contribution in free DAPI, was the major contributor to the decay. The short lifetime component had a larger value ($\sim$0.5 ns) than that found for DAPI in aqueous solution, and its fractional contribution was reduced to 0.2 for linear-DNA/DAPI complexes and almost negligible for cc-

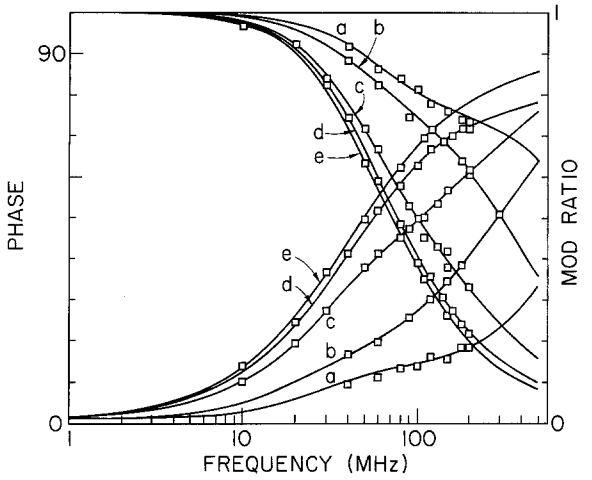


Fig. 6. Phase and modulation ratio values of DAPI at pH 7.1 (*a*) and complexed with polyd(GC) (*b*), linear DNA (*c*), circular DNA (*d*) and AT-polymers (*e*). The *solid lines* correspond to the best fits using a bi-exponential decay model. Excitation, 325 nm; Emission (RG370 filter) $\lambda > 370$ nm

Table 3. Fluorescence decay parameters for DAPI at different pH values

pH	τ_1 [ns]	τ_2 [ns]	f_1	f_2
1.00	2.80±0.50	0.15±0.01	0.14	0.86
1.75	2.55±0.43	0.19±0.07	0.20	0.80
3.0	2.60±0.30	0.19±0.03	0.28	0.72
4.0	2.60±0.15	0.20±0.02	0.29	0.71
7.1	2.80±0.25	0.19±0.02	0.27	0.73
10.0	2.60±0.70	0.40±0.03	0.70	0.30
12.0	1.50±0.05		1.00	
13.5	0.16±0.01		1.00	

f_1 and f_2 are fractional fluorescence intensities, as in Table 1
λ_{ex} = 325 nm
λ_{em} = RG370 filter

Table 4. Fluorescence decay parameters for different polydeoxynucleotide/DAPI complexes

	P/D	τ_1 [ns]	τ_2 [ns]	f_1	f_2
1-DNA	60	3.8±0.1	0.5 ±0.1	0.80	0.20
	7	2.9±0.1	0.4 ±0.1	0.75	0.25
	0.4	2.5±0.2	0.19±0.03	0.25	0.75
cc-DNA	60	3.8±0.1	0.4 ±0.2	0.95	0.05
Polyd(AT)	60	3.9±0.1		1.00	
	7	3.9±0.1		1.00	
	1	3.9±0.1	0.2 ±0.1	0.50	0.50
Polyd(A)-polyd(T)	60	3.9±0.1		1.00	
	7	3.9±0.1		1.00	
	1	3.9±0.1	0.2 ±0.1	0.50	0.50
Polyd(GC)	60	2.9±0.1	0.6 ±0.1	0.29	0.71

f_1 and f_2 are fractional fluorescence intensities, as in Tables 1 and 3

DNA/DAPI complexes at all P/D ratios examined. By lowering the DNA/DAPI ratio to 7, the fluorescence decay still corresponded to two exponentials with lifetime values of 2.9 ns and 0.4 ns. According to the fluorimetric titration data on polyd(AT), this P/D ratio should be ideal for type I binding (Kapuscinski and Szer 1978). The reduction of the lifetime value of the long component may be due to the heterogeneity and high variety of sequence combinations of a DNA molecule from a mammalian source. At very low P/D ratios, the decay was adequately described using two exponentials and both lifetimes had lower values (2.5 ns and 0.19 ns), relative to those observed at higher and stoichiometric P/D ratios. These values and the fractional intensities were similar to those obtained for DAPI in aqueous solution. The decay of polyd(A)-polyd(T), polyd(AT)/DAPI complexes, at high and at stoichiometric ratios, was well fitted using a single exponential with a lifetime of 3.9 ns. At P/D ratios of about one, a satisfactory fit was obtained using two components, having lifetimes of 3.9 ns and 0.2 ns, respectively, and fractional amplitudes of 0.5 indicating the occurrence of strong binding even in this quite unfavorable condition.

At a high P/D ratio, the fluorescence decay of polyd(GC)/DAPI complex, at variance with the behavior of fully AT-containing polymer/DAPI complexes, showed an inversion of the fractional intensities of the long and the short lifetime component whose relative amounts were 0.29 and 0.71, respectively. Furthermore, the value of the shorter decay time component was 0.6 ns.

Discussion

DAPI in solvents

The experimental data using multifrequency phase fluorometry obtained for DAPI in pure solvents are in good agreement with the data of Szabo et al. (1986) obtained using correlated single photon counting. However, we differ on the interpretation of the molecular origin of the effects observed. Time resolved fluorescence results of DAPI in aqueous solution have shown that there are two distinct spectral components, each with a characteristic decay time. To discuss the origin of the two components we must consider the possibility of excited state reactions and of ground state heterogeneity.

a. Excited state reactions. One possible mechanism by which two distinct lifetime components may arise is that of excimer formation, which can be excluded for at least three reasons. First, excimer decay kinetics, when resolved using two exponentials, should give a negative pre-exponential term at the emission wave-

length where excimer fluorescence is expected (Szabo et al. 1986). For DAPI, in aqueous solution, positive pre-exponential terms were found at all wavelengths examined. Secondly, by changing the concentration of DAPI there was no change in the decay behavior. Thirdly, at intermediate pH, DAPI carries two positive charges. It is difficult to imagine how the attractive forces of excimer formation can overcome the repulsive forces of the positive charges on the two molecules. A second possible mechanism can involve an exciplex of DAPI and water molecules. If a new transient equilibrium is being established for DAPI in the excited state, a double exponential decay can in principle be observed. The two species, the new one formed in the excited state and the one corresponding to the ground state equilibrium, should have characteristic spectra and different decay kinetics. In the spectral region corresponding to the fluorescence of the excited complex, an exponential decay with a negative pre-exponential factor should be observed due to the delay of the emission of the exciplex. The time resolution of our technique is about 5 ps (Gratton and Limkeman 1984). If the exciplex formation is faster than 5 ps, it will be undetected in our experiments, but in that case, both species will appear to decay with a common lifetime.

b. Ground state mechanisms. In our steady-state experiments, at variance with the results of Szabo et al. (1986), the emission spectrum was dependent on the excitation wavelength, which is strong evidence that ground state heterogeneity is responsible for the observed biexponential decay behavior of DAPI in aqueous solution. Such lifetime heterogeneity may originate from the presence of two different ground-state molecular conformers or rotamers (Szabo et al. 1986). One possibility is that, at pH 7, the two lifetimes values are associated with a mixture of two forms in which one or both amidinium groups are protonated. However, absorption and static fluorescence studies show that there are no large changes in the titration curve between pH 3 and pH 9. At pH 1.75, where only one protonated form should be present, a double exponential decay was still observed, though with shorter lifetime values than occur at neutral pH. Therefore, the explanation of lifetime heterogeneity based on different ionic forms, whose origin is attributable to the pH of the medium, can be ruled out. A second possibility, the one that we favor, is that preferential solvation of the indole ring in the ground state allows proton transfer in the excited state. Upon excitation, the pK of the indole ring should change to ~ 1 (Vander Donckt et al. 1969). At pH 0.5, where mainly a single component was found, the proton concentration is high enough to allow protonation by the solvent in the ground state. The long lifetime component of about 2.5 ns originates from a conformation of the DAPI in which proton transfer at the indole ring does not occur. The fluorescence decay of DAPI in ethanol solution, which has only a long exponential component, supports this interpretation, indicating that in this solvent there is no preferential solvation of the indole ring. Water must be involved in the proton transfer process in aqueous solution. The crucial effect of water is also shown in the propylene glycol/water mixture experiments in which an increase of the water content causes an inversion of the contribution of the long and short lifetime components. The comparison between the lifetime values in ethanol and propylene glycol shows that the viscosity of the medium is not a factor in determining the lifetime values. Szabo et al. (1986) showed that the value of the component lifetimes are longer in D_2O than in H_2O.

c. Mechanisms for preferential solvation. We propose that preferential solvation is the determinant factor for the proton transfer process and that at neutral pH, at least two different solvated forms coexist and that proton transfer in the excited state can occur in only one of the two forms. At sufficiently extreme pH conditions we should obtain either one or the other of these two forms. To examine whether the two components obtained by the phase resolved spectra corresponded to two different protonated forms of DAPI, the fluorescence spectra of DAPI at two extreme pH values, 1 and 12 was measured (Fig. 2). The spectrum at pH 12 had a maximum at 445 nm and was very similar to the spectrum of the long lifetime component found at neutral pH using the phase-resolved spectra technique. The spectrum of the strongly acidic solution had low intensity and a maximum at 482 nm with a long tail extending past 600 nm similar to the phase resolved spectrum associated with the short lifetime component. The similarity of the shape and maxima of the extreme pH steady-state spectra with the time resolved spectra and the similar value of the component lifetimes compared to those found at the extreme pH values allows us to identify the nature of the complex which gives rise to lifetime heterogeneity. The dependence of the excitation spectra on the emission wavelength indicated that these forms are both present in the ground state. The two different solvated species can be more or less stable, depending upon the different conformers that can arise either from rotamers at C6 of the indole ring, involving the 6-amidinium group, or rotamers at the C2 involving the 4′-amidiniumphenyl substituent. For the C2 substituent two different conformations can occur: one in which the phenyl ring and the indole ring are coplanar and another in which they are not. In the planar configuration, the 4′-amidiniumphenyl substituent is conjugated with the indole ring, increasing the basic character of this latter form with respect to the twisted configura-

tion, and therefore also its proton transfer capability. For the C6 rotamer, different stabilization of the solvation shell can occur depending on the orientation of the amidinium group.

DNA-DAPI complexes

A marked increase of fluorescence intensity was associated with the binding of DAPI only with polymers containing clusters of AT (AU, IC) base pairs, contrary to results reported earlier (Cavatorta et al. 1985). These authors also attributed a fluorescence enhancement to polyd(GC)/DAPI complex formation. The modification of the fluorescence spectrum of DAPI bound to polyd(GC) suggests that the binding is not specific for clusters of AT base pairs, in accordance with the appearance of an extrinsic CD band under the same experimental conditions (Manzini et al. 1983). The alteration of the long wavelength portion of the polyd (GC)/DAPI spectrum at high *P/D* ratios can be due to electrostatic interaction of the positively charged ends of the drug with the phosphate group of the polymer. This assignment is supported by the disappearance of the spectral change by increasing the ionic strength of the medium or by reducing the *P/D* ratio. Therefore any spectroscopic method based on a differential behavior of DAPI when bound to DNA, AT-containing polydeoxynucleotides and polyd(GC) should be considered with some caution with respect to the technique employed and the region of the spectrum examined (Barcellona et al. 1986).

Since the 3.8 ns and 0.4 ns components are always present despite the differences in sequence and composition of the DNA molecule examined (but their relative contribution is different), it is suggested that the binding of DAPI to nucleic acids changes the degree of solvation of the indole ring enhancing or reducing the relative contribution of the two lifetime components, which are hydration dependent. Binding to AT clusters reduces the proton transfer process probably by shielding the fluorophore from solvent, whereas for the GC binding case the DAPI molecule is largely exposed to the solvent, thereby facilitating proton transfer. For the polyd(GC)/DAPI complexes, the increased value of the short lifetime component and its fractional contribution, show that the relative amount and therefore the two types of binding are strongly dependent on the polydeoxynucleotide composition. Irrespective of the model proposed and of the molecular interpretation of the observed features, the data reported in Fig. 6 are compelling evidence of a progressive change of the spectroscopic properties from polyd(GC) to fully AT-containing polymers.

Binding mechanisms

Recent results from a theoretical study of the interaction of DAPI with double stranded oligonucleotides (Gresh 1985), support and confirm the mechanism of DNA/DAPI interaction as an insertion of the drug in the narrow groove of the double helix (Manzini et al. 1983). The difference between GC and AT base pairs is the presence of the amino group in position 2 of the purine ring. This difference may be the basis for the preferential binding of DAPI to AT (AU, IC) clusters. The binding can be obtained through the formation of hydrogen bonds with AT base acceptor groups, namely N_3 on the purine ring and O_2 in the pyrimidine ring (accessibility forbidden in the GC pairs). The length of the DAPI molecule is such that it cannot form hydrogen bonds within the same AT pair without a severe distortion of the polymer structure. The preferential binding configuration is such that hydrogen atoms belonging to one side of the molecule, assuming that the molecular conformation is planar, will span three base pairs, O_2 of thymine, N_3 of adenine and again O_2 of the next thymine. Additional interactions can occur between O_1', deoxyribose oxygens of the opposite strand of the polynucleotide and the hydrogen atoms of the other side of the DAPI molecule. Such a model is in agreement with previous studies concerning the binding of non-intercalating ligands to B-DNA (Gresh and Pullman 1984a and b). There is also an electrostatic contribution to the overall energy of interaction: it derives from the bond(s) that one (or both) amidino groups can form with phosphate groups or even with the negative surface of AT sequences in the minor groove, as a reflection of its strong attractive molecular electrostatic potential (Pullman and Pullman 1981; Pullman et al. 1983). One may speculate that electrostatic binding (usually a second order process: 10^6–$10^7\ M^{-1}\ s^{-1}$), is followed by isomerization of this external complex to another form, exhibiting type I binding, in which the dye molecule is located in the minor groove of the nucleic acid helix without intercalation.

The lower anisotropy of the DNA/DAPI complex, with respect to that observed for DAPI in propylene glycol at low temperature, may be due to torsional motions of the DNA molecule which occurs in the nanosecond time scale (Millar et al. 1980).

In conclusion, our steady-state and time resolved fluorescence studies have shown the existence of different spectroscopic forms of non-intercalatively polydeoxynucleotide-bound DAPI. Lifetime values were different in the solvated and non-solvated form of the DAPI molecule. When the dye formed a complex with the polydeoxynucleotide the proton transfer mechanism was partially inhibited. Therefore the analysis of the fluorescence decay using two exponentials discrim-

inates and quantitates the two spectroscopic forms, provided that the polymer is homogeneous and the saturation condition is satisfied.

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