

TIME-RESOLVED FLUORESCENCE OF DAPI IN
SOLUTION AND BOUND TO POLYDEOXYNUCLEOTIDES

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Fluorescence decay studies, obtained by multifrequency phase-modulation fluorometry, have been performed on DAPI in solution and complexed with natural and synthetic polydeoxynucleotides. DAPI decay at pH 7 was decomposed using two exponential components of 2.8 and 0.2 ns of lifetime values, respectively. The double exponential character of the decay was maintained over a large pH range. Phase- and modulation-resolved spectra, collected between 420 and 550 nm, have indicated at least two spectral components associated with the two lifetime values. This, plus the observation of the dependence of the emission spectrum on the excitation wavelength, suggests a lifetime heterogeneity originating from ground-state molecular conformers, partially affected by pH changes. DAPI complexed with natural polydeoxynucleotides retained most of the features of DAPI decay in solution, except for the value of the long lifetime component that was longer (~4 ns) and the relative fractional fluorescence intensities of the two components that were inverted. AT polymers/DAPI complexes show single exponential decay. Solvent shielding when DAPI is bound to DNA changes the indole ring solvation and stabilizes the longer lifetime decay

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component. For polyd(GC)/DAPI complex, the decay was similar to that of free DAPI in solution, proving the dependence on the polydeoxynucleotides sequence of the different types of binding and the reliability of the fluorescence method to solve them.

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DAPI (4' 6 diamidino 2 phenylindole. H Cl) is known to interact reversibly with double stranded deoxyribonucleic acids and various synthetic analogs of different base content and sequence exhibiting a noticeable increase in fluorescence quantum yield (1-3), see Figure 1.

Previous studies have shown that only the binding between AT, AU, and IC clusters is responsible for the fluorescent complex formation (4). 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 some human chromosomes, as well as to probe the molecular environment of restriction endonuclease cleavage sites, the effectiveness in inhibiting initiation at the *lac* UV5 promoter by E.coli RNA polymerase, and to act as a possible modulator of a genetic expression (5-20).

The strong fluorescence enhancement following DNA binding has been interpreted in terms of two different modes of binding: (1) a highly energetic (-8 Kcal/mol of DAPI) and intercalative type of interaction (1,4), characterized by a high value of the affinity constant and a high specificity for AT, AU, IC clusters; and (2) a weaker one, nonspecific, with a lower affinity constant and no larger fluorescence enhancement (2,3,5,6).

Spectroscopic investigations, together with sedimentation, viscometric and calorimetric measurements, ruled out the intercalation as a molecular mechanism of the specific strong binding and suggested a model of interaction in which there is formation in the narrow helical groove of two hydrogen bonds between donor groups of the drug and N₃ and O₂ atoms of adjacent adenine and thymine rings, with electrostatic interactions. This model is also supported by the molecular structure of DAPI and by the lack of coplanarity of the rings in the molecule (7,9).

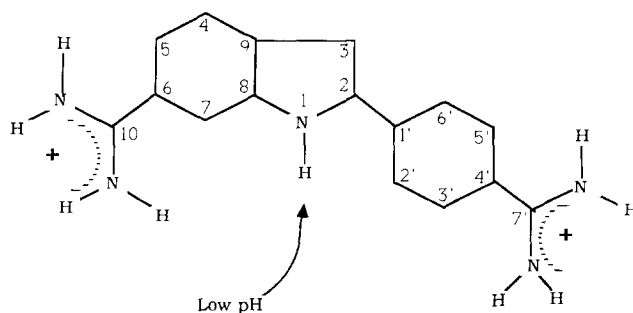


Fig. 1. DAPI numbered atoms and structural formula.

In this study, we investigated the fluorescence decay of DAPI in pure solvents and bound to natural and synthetic purine-pyrimidine alternating polydeoxynucleotides using multifrequency phase fluorometry, which is particularly suitable to resolve multiexponential decays rapidly and accurately. Furthermore, the technique of phase- and modulation-resolved spectra (21) allowed the direct detection of various components of the emission spectra.

Materials and Methods

Calf thymus DNA, Col E1 plasmid DNA, poly d(AT), poly d(A)•poly d(T), and poly d(GC) were obtained from Boehringer Mannheim Biochemicals. DNA molecules were checked for protein contamination either by the value of the optical density ratio, $A_{260}/A_{280} > 1.8$, or by Cs Cl-Eb buoyant density gradient centrifugation. The two fractions obtained by centrifugation, lower and upper band, corresponding to cc and linear DNA molecules, respectively, were collected and the intercalator removed (22). DAPI was purchased from Serva Biochemicals. The solution concentration was determined using the following molar extinction coefficients: calf thymus DNA (6600); Col E1 plasmid DNA (6600); poly d(AT) (6600); poly d(A)•poly d(T) (6000) and poly d(GC) (8400). A molar extinction coefficient of $23000 \text{ M}^{-1} \text{ cm}^{-1}$ at 342 nm was used to determine the concentration of DAPI solutions. Depending on the final concentration of the polydeoxynucleotides, different ratios as moles of phosphate (P) to dye (D) were obtained. In the measurements pertaining to solvent and to pH variation effects, typically a small amount of a concentrated DAPI aqueous solution was diluted to

the desired concentration with the appropriate solvent. In the polydeoxynucleotide/DAPI complexes experiments, the final concentration to obtain the suitable P/D ratio was achieved by adding increasing amounts of the polymer. Steady-state spectrofluorometric experiments were carried out with the microprocessor-controlled photon counting apparatus described by Gratton and Limkeman (23). Lifetime measurements were performed on the multifrequency phase and modulation fluorometer described by Gratton and Limkeman (21), which was equipped with an ISS1ADC interface (ISS, Inc., Champaign, Illinois) for data acquisition and analysis. The light source was a HeCd laser emitting at 325 nm. Phase and modulation data were analyzed using a sum of exponentials by a nonlinear least-squares routine (24). Phase-resolved spectra were obtained using the software method described by Gratton and Jameson (25). A solution of POPOP in ethyl alcohol with a lifetime value of 1.35 ns was used as a reference. The integration time was 2 sec per point and data were collected each 5 nm.

Results

The fluorescence decay of an aqueous solution of DAPI at pH 7 was measured at several different modulation frequencies in the range between 10 and 200 MHz. Phase and modulation values were fit using two exponential components with lifetimes of 2.8 and 0.19 nsec. Using the same lifetime values, phase-resolved spectra were obtained on a similar sample in the region between 420 and 550 nm. At 550 nm the fluorescence decay of DAPI was essentially single-exponential with a decay time shorter than 0.3 nsec. Phase- and modulation-resolved spectra were virtually identical, confirming that only two major spectral components associated with the two lifetime values were present: the long one, with an emission maximum near 445 nm and the short one, with a broad maximum centered at 482 nm and with a long red tail (Fig. 2).

The analysis of the DAPI fluorescence decay collected over the large pH range from pH 2 to pH 10 using two exponential components (Table 1) showed a progressive lengthening of both lifetime values from 2.0 to 3.3 nsec for the long component and from 0.06 to 1.40 nsec for the short one. At extreme pH values, the

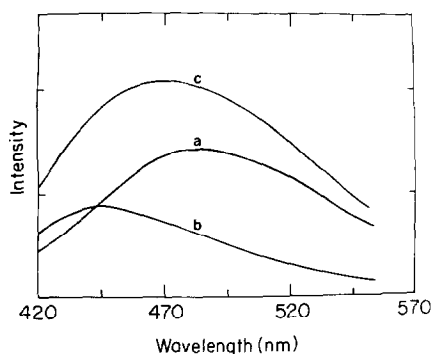


Fig. 2. Phase- and modulation-resolved spectra of DAPI at pH 7.1, excitation at 325 nm, modulation frequency at 100 MHz, spectra at (a) 0.15 ns and (b) 2.5 ns and (c) total intensity.

decay kinetic could be fit with a single exponential decay. In ethyl alcohol solution, the decay behavior was fit with a single exponential of 2.6 nsec.

Steady-state fluorescence measurements of DAPI bound to natural and synthetic polydeoxynucleotides showed a marked increase in fluorescence quantum yield (>20 with respect to DAPI in aqueous solution) and a blue shift of

Table 1. Fluorescence Decay Parameters of DAPI at different pH values

pH	T ₁ (nsec)	T ₂ (nsec)	F ₁	F ₂
0.5		0.40±0.01		1.00
1.75	2.01±0.43	0.06±0.07	0.30	0.70
3.0	2.09±0.30	0.11±0.03	0.32	0.68
4.0	1.90±0.15	0.10±0.02	0.33	0.67
7.1	2.80±0.25	0.19±0.02	0.27	0.73
10.0	3.37±0.70	1.40±0.03	0.12	0.88
12.0	---	1.50±0.05	---	1.00
13.5	---	0.16±0.01	---	1.00

F₁ and F₂ are fractional fluorescence intensities.

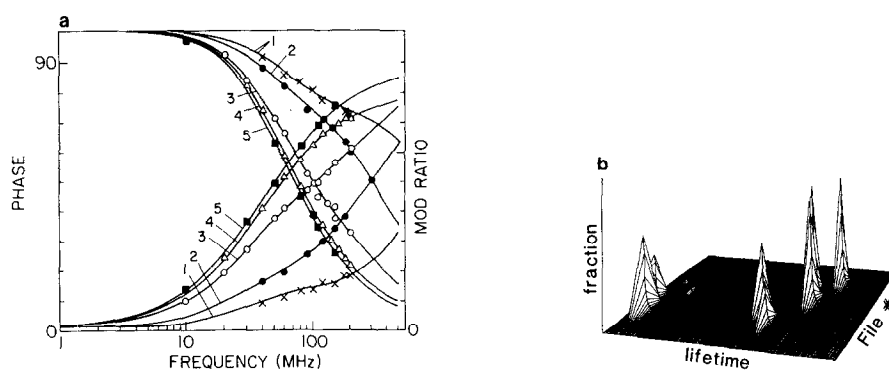


Fig. 3 a). Phase and modulation ratio values of DAPI at (1) pH 7.1 and complexed with (2) polyd(GC), (3) linear DNA, (4) circular closed DNA, and (5) AT polymers. The solid lines correspond to the best fit using two exponential decays. The parameters of the fits are in Table 1.

Fig. 3 b). Three-dimensional representation of the five fits of fluorescence decay parameters.

Table 2. Fluorescence decay parameters of DAPI in buffered solution and complexed with different natural and synthetic polydeoxynucleotides, analyzed by using two-exponential components

Fits	T ₁ (ns)	F ₁	T ₂ (ns)	χ^2
(1) DAPI in solution	2.74	0.40	0.17	0.748
(2) polyd(GC)/DAPI complexes	3.06	0.35	0.24	0.734
(3) linear DNA/DAPI complexes	3.48	0.97	0.36	0.936
(4) circular closed DNA/DAPI complexes	3.64	0.96	0.74	0.844
(5) poly(dA)•poly(dT) or polyd(AT)/DAPI complexes	3.97	0.99	0.07	0.451

T₁ = lifetime of the long decay component.

F₁ = fractional fluorescence intensity of the long lifetime component.

T₂ = lifetime of the short decay component.

the emission maximum of about 10 nm, with respect to the free DAPI. The poly d(GC)/DAPI complex, instead, did not exhibit a significant fluorescence enhancement, even at a high phosphate/dye ratio (P/D).

The fluorescence decay of linear (l) or covalently closed (cc) DNA and polydeoxynucleotide/DAPI complexes at high P/D ratios is shown in Fig. 3a and Table 2. The value of the longer component was about 3.6 nsec and gave the major contribution to the decay. The short lifetime component had a larger value than that found for DAPI in aqueous solution and its fractional contribution was strongly reduced. At low P/D ratio (about 1.0, data not shown), the decay was adequately described using two exponentials and both lifetimes had lower values

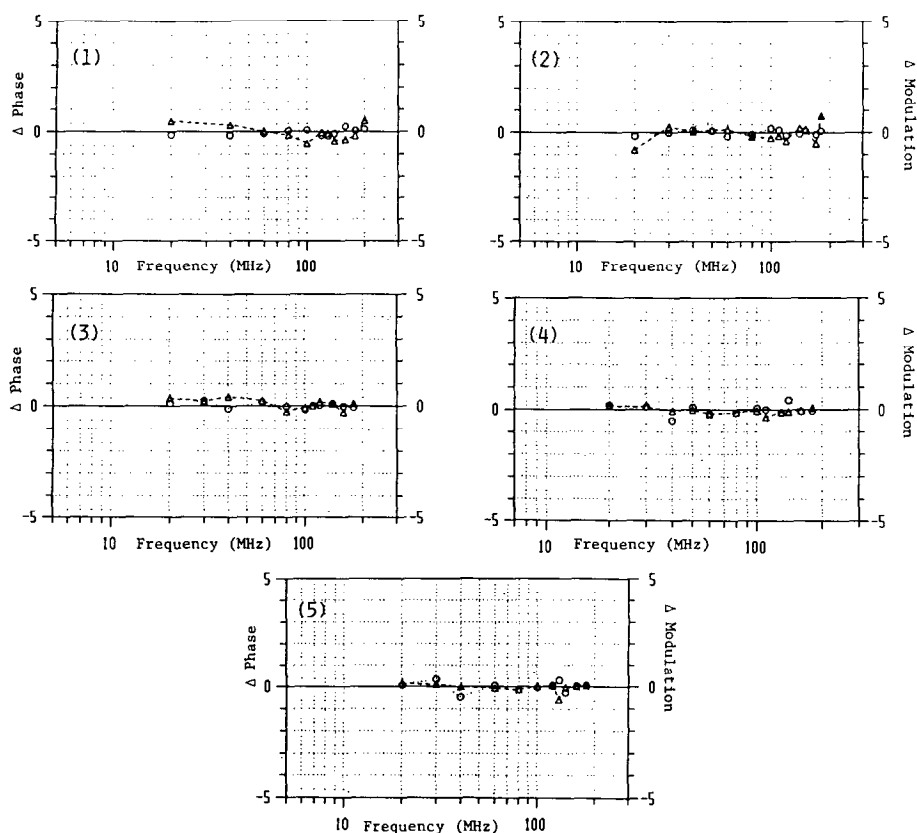


Fig. 4. Phase and modulation residuals for the double exponential analysis of (1) DAPI in solution; and complexed with (2) poly d(GC); (3) linear DNA; (4) circular closed DNA; and (5) fully AT polymers. Average deviation are 0.1° of phase and 0.002 in modulation.

(2.50 and 0.19 nsec) with respect to those observed at higher P/D ratios. The decay of fully AT containing polymers/DAPI complexes, either alternating copolymer or homopolymer, was fit using a single exponential with a lifetime of 3.97 nsec. The fluorescence decay of poly d(GC)/DAPI complex, contrary to the other polymers assayed, showed a behavior almost identical to that of free DAPI in solution, further supporting the previously reported conclusion that strong binding in these complexes does not take place. A three-dimensional representation of the fluorescence decay parameters for the five data sets is shown in Figure 3b. Phase and modulation residuals for the same data set (Fig. 4) show small deviations between the calculated and measured values.

Discussion

Lifetime measurements and phase- and modulation-resolved spectra of DAPI in aqueous solution show two distinct spectral components, each with a characteristic decay time. To examine whether these two components corresponded to two different protonated forms of DAPI, the fluorescence spectra of DAPI was measured at two extreme pH values: pH 0.5 and pH 12. The spectrum at pH 12 had a maximum at 445 nm and was similar to that of the long lifetime component found using the phase-resolved spectra technique. The spectrum of the acid solution had low intensity and a maximum at 482 nm, similar to the phase-resolved spectrum associated with the short lifetime component. Such a similarity suggests that the two spectral components correspond to different protonated forms of DAPI. The dependence of the emission spectra on the excitation wavelength indicates that these forms are both present as ground-state conformers. The short lifetime component can arise from a molecular structure in which the indole ring of DAPI is protonated as a consequence of preferential solvation in the ground-state, which allows proton transfer in the excited-state. The long lifetime component should originate from a conformation in which protonation of the indole ring does not take place. The fluorescence decay of DAPI in alcohol solution, which exhibits only a long exponential component, supports this interpretation and suggests that water is involved in the proton transfer process. The efficiency of such a process can

depend on 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 4'-amidiniumphenyl substituent.

The decay behavior of DAPI complexed with natural and synthetic polydeoxynucleotides, being double exponential except for AT polymers, reflects the binding site's heterogeneity and the high variety of combinations in the base's sequence of a natural DNA. Moreover, even a visual inspection of Figure 3a and b is compelling evidence of the effectiveness of the decay behavior analysis to monitor structural differences in the DNA molecules examined. We suggest that DAPI binding to nucleic acids changes the degree of solvation of the indole ring, enhancing or reducing the relative contribution of the two lifetimes. The strong binding favors the unprotonated form, probably due to a better shielding from the solvent; whereas for the weak binding, the DAPI molecule is essentially solvent exposed. The preferential binding to AT (AU, IC) clusters as an insertion of the drug in the narrow groove of the double helix (7,9) releases the solvation water molecules, both from DAPI and from the minor groove, accounting for the large increase in fluorescence intensity and quantum yield. The binding's stability is obtained through the formation of hydrogen bonds between the AT base's acceptor groups, namely: N₃, on the purine ring; and O₂, on the pyrimidine ring and the DAPI indole ring. This model is supported by the lengthening of the long decay component lifetime value at alkaline pH, where the proton transfer process is unlikely. DAPI's position in the narrow groove should be such that hydrogen atoms belonging to one side of the molecule will span three base pairs. Additional interactions can occur between O'₁ deoxyribose oxygens of the polynucleotide opposite strand and the hydrogen atoms on the other side of the DAPI molecule. Recent results of a theoretical study on the interaction of DAPI with double stranded oligonucleotides support and confirm this mechanism of DNA/DAPI interaction (28).

In conclusion, our time-resolved fluorescence studies have shown the existence of various types of DAPI binding with various polydeoxynucleotides. Lifetime values were different in the protonated and unprotonated form of the DAPI molecule. When the dye was complexed with double-stranded nucleic

acids, the protonation mechanism was partially inhibited, causing large variations of the lifetime values. These results show that time-resolved fluorescence decay analysis is an alternative method to elucidate the photophysical origin of the spectroscopic properties of DAPI bound to nucleic acids and can be used to discriminate among the different types of binding.

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