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A TIME-RESOLVED FLUORESCENCE STUDY OF 4′,6′-DIAMIDINE-2-PHENYLINDOLE DIHYDROCHLORIDE BINDING TO POLYNUCLEOTIDES

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At phosphate/dye (P/D) ratios greater than 30 the quantum yield of 4′,6′-diamidine-2-phenylindole dihydrochloride (DAPI)-DNA and DAPI-poly(d(A-T)) complexes was found to be 0.62 and 0.66, respectively. Contrary to earlier reports a fluorescence enhancement of DAPI-poly(d(G-C)) complexes was observed with a quantum yield of 0.22. Time-resolved fluorescence measurements of complexes with a P/D ratio of 150:1 indicate that there were three fluorescent components in DAPI-DNA complexes with lifetimes of 3.86, 1.79 and 0.13 ns. In DAPI-poly(d(A-T)) complexes the lifetimes were 3.91, 1.20 and 0.11 ns. Also, three components with lifetimes of 3.98, 0.87 and 0.12 ns were found in DAPI-poly(d(G-C)) complexes. At low P/D ratios (< 5) another binding form of DAPI was observed which was assigned to the interaction of one or more molecules of DAPI with one previously bound to DNA. It is concluded that DAPI does not exhibit A-T binding specificity and that at high P/D ratios there are two types of binding having similar binding constants.

1. Introduction

The fluorescent dye 4′-6-diamidine-2-phenylindole dihydrochloride (DAPI) has previously been shown to bind to DNA by absorption, circular dichroism, and fluorescence techniques [1–4]. DAPI has therefore been used as a probe of nucleic acid structure a well as in cytological applications [5–7]. The molecular nature of its interaction with DNA has not been rigorously established. It is not clear whether DAPI intercalates between base-pairs in DNA, or binds with strong electrostatic interactions to the phosphate backbone of DNA because of its dicationic nature, or interacts with the macromolecule by another form of binding. Recent studies have investigated the spectroscopic properties and binding parameters of free and complexed DAPI. It has been shown that the fluorescence of DAPI is markedly enhanced on binding to linear DNA and poly(d(A-T)) and other polynucleotides with the exception of polynucleotides containing guanine and cytosine [2]. In the latter polynucleotides, circular dichroism experiments suggest that DAPI forms complexes but these complexes do not result in a fluorescence enhancement.

In this work we report steady-state and time-resolved fluorescence experiments which were undertaken to characterize further the nature of the interaction of DAPI with different DNAs and to investigate the reported specificity of nucleic acid composition on the fluorescence enhancement.

2. Materials and methods

Steady-state fluorescence measurements were performed on a Perkin Elmer MPF44A spec-

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trophotofluorimeter. Spectra were corrected using a DCSU 2 spectral correction unit. The instrument was in turn interfaced with a Commodore PET minicomputer through an A/D converter so that spectra could be digitized and stored on floppy discs. Blank subtraction and integration for quantum yield calculations were thereby facilitated. Spectra were recorded using an excitation and emission bandpass of 3 nm each. Absorbances at the excitation wavelength (360 nm) were 0.05. Absorption spectral measurements were performed on a Cary 219 spectrophotometer. Dilute solutions of quinine sulfate in 1 M H_2SO_4 were used as a quantum yield standard [8].

All solutions were 0.01 M cacodylate buffer, pH 7, unless otherwise stated. Calf thymus DNA was obtained from Sigma. Synthetic polynucleotides were purchased from PL Biochemicals and were stored at −5°C prior to use. Water was glass distilled. DAPI was purchased from Serva and used without further purification.

Solutions of different polynucleotides were prepared in buffer and the concentrations determined from their absorbance at 260 nm. A stock solution of DAPI in buffer was prepared and a small aliquot added to the polynucleotide sample to give the desired phosphate/dye (P/D) ratio.

Time-resolved fluorescence measurements were conducted using a Spectra Physics mode-locked synchronously pumped and cavity dumped dye-laser system whose output was frequency doubled with a KDP angle-tuned crystal to give excitation pulses with a pulse width of 20 ps at a repetition rate of 825 kHz. The fluorescence was detected using a Hamamatsu 928 photomultiplier tube after passing through a Jobin Yvon H10 monochromator (4 nm bandpass). The electronic components were those usually employed in time-correlated single-photon counting fluorescence decay instrumentation [9]. Typically, experiments were performed using a resolution of 43 ps/channel and 1024 channels in the multichannel analyser. Usually at least 60 000 counts were accumulated in the maximum channel. The fluorescence decay parameters were obtained after convolution analysis using the Marquardt algorithm and the delta function convolution method [9] and the fluorescence decay of a reference material whose lifetime was accurately known. The adequacy of the fit to the decay data was determined by the inspection of the weighted residual plots, the autocorrelation of residuals and the root mean sum of weighted squares of residuals (RSMR) [10].

3. Results

Fig. 1 shows fluorescence spectra of free DAPI as well as that of the drug bound to poly(d(A-T)), poly(d(G-C)), at a P/D ratio of 150 : 1. The fluorescence spectrum of DAPI bound to calf thymus DNA was nearly identical to that of DAPI-poly(d(A-T)). As shown in Fig. 1, the fluorescence of DAPI bound to poly(d(G-C)) was enhanced by a factor of 6 when compared to that of DAPI alone. The observation of this fluorescence enhancement was completely reproducible with different samples of poly(d(G-C)). We are not able to offer any explanation of the discrepancy between these results and those reported earlier [2]. The emission maximum of DAPI was, in all cases, positioned near 460 nm.

In table 1 the quantum yields of these complexes are reported. The quantum yields of DAPI

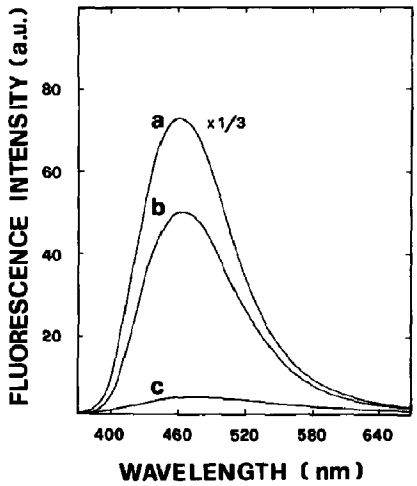

Fig. 1. Corrected fluorescence spectrum (λ_{ex} = 360 nm, excitation and emission bandpass, 3 nm) in solutions of 0.01 M cacodylate buffer, pH 7, of (a) DAPI-poly(d(A-T)), P/D = 150; (b) DAPI-poly(d(G-C)), P/D = 150; (c) DAPI. The intensity of spectrum a is reduced by 1/3.

Table 1

Fluorescence quantum yields and decay parameters for DAPI-polynucleotide complexes

Sample	Quantum yield [a]	t_1 (ns)	t_2 (ns)	t_3 (ns)	a_1 [b]	a_2	a_3	F_1 [c]	F_2	F_3
DAPI	0.035	2.77	0.16	–	0.09	0.91	–	68	32	–
DAPI/DNA										
$P/D = 150$	0.62	3.86	1.79	0.13	0.46	0.14	0.40	85	12	3
$P/D = 2.5$	–	10.8	2.72	0.23	0.15	0.11	0.74	77	14	8
$P/D = 2.5 + KCl$	–	4.20	1.70	0.12	0.03	0.06	0.91	33	31	31
DAPI/d(A-T)										
$P/D = 150$	0.66	3.94	1.31	0.12	0.44	0.08	0.49	92	5	3
$P/D = 2.5$	–	12.2	2.66	0.31	0.12	0.39	0.50	54	40	6
$P/D = 2.5 + KCl$	–	4.40	0.11	–	0.50	0.50	–	97	3	–
DAPI/d(G-C)										
$P/D = 150$	0.22	3.96	0.87	0.12	0.30	0.05	0.65	94	2	4
$P/D = 3.3$	–	8.87	2.84	0.18	0.05	0.07	0.88	53	27	20
$P/D = 3.3 + KCl$	–	2.76	0.13	–	0.02	0.98	–	72	28	–

[a] Quinine sulfate in 1 M H_2SO_4 was taken as a standard (quantum yield = 0.546).

[b] a_1, a_2 and a_3 represent normalized pre-exponential terms for each component: $a_1 + a_2 + a_3 = 1$.

[c] F_1, F_2 and F_3 represent the percentage of the total fluorescence of each component according to $F_i = a_i t_i / \sum_i^n a_i t_i$.

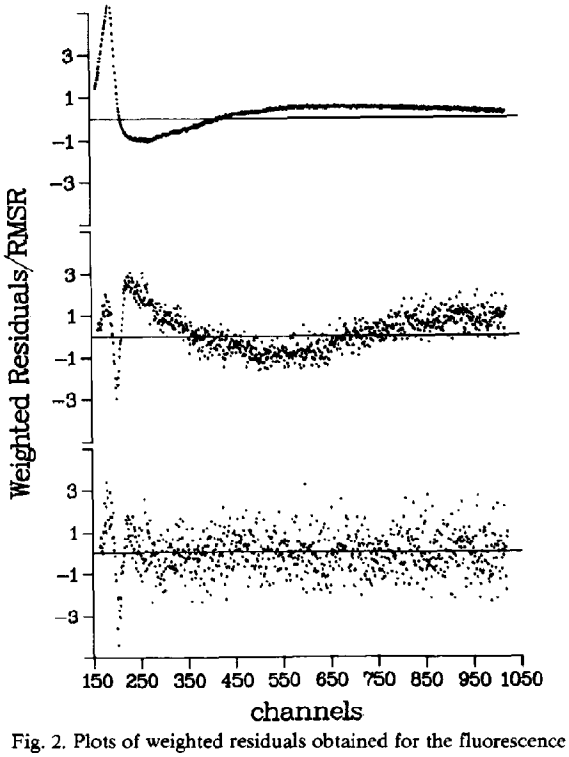

Fig. 2. Plots of weighted residuals obtained for the fluorescence decay data of DAPI-DNA, $\lambda_{em} = 430$ nm, $P/D = 150$ after convolution analysis for (a) single exponential, (b) double exponential, (c) triple exponential decay functions.

bound to poly(d(A-T)) and calf thymus DNA were 0.66 and 0.62, respectively, and that for the DAPI-poly(d(G-C)) complex was 0.22. This compares with that of DAPI alone at pH 7, which was 0.035.

The fluorescence decay of DAPI in each complex was measured at an emission wavelength of 430 nm and fig. 2 is an example of a plot of the weighted residuals for DAPI-DNA ($P/D = 150:1$) after convolution of the data with single, double and triple exponential decay functions.

It was clear that for all DAPI complexes the residuals were not randomly distributed after fitting to single and double exponential decays, indicating an inadequacy of these models in describing the fluorescence decay behavior. Other statistical parameters such as RSMR required a similar conclusion. Only when the data were fitted to a function of three exponentials for DAPI-DNA, with lifetimes of 3.86, 1.79 and 0.13 ns, and for DAPI-poly(d(A-T)) complexes with lifetimes of 3.91, 1.20 and 0.11 ns, was an adequate fit obtained. In the case of DAPI-poly(d(G-C)) complexes the data adequately fit a triple exponential decay function, with lifetimes of 3.98, 0.87 and 0.12 ns. The fractional contribution to the fluorescence of each decay component is also given in table 1.

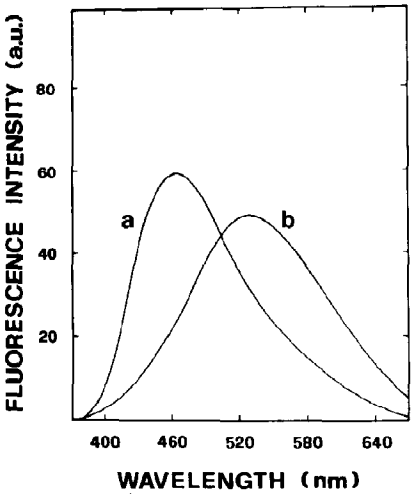

Fig. 3. Same as fig. 1 except spectra for DAPI-DNA, (a) $P/D = 10$, (b) $P/D = 2.5$.

Typical fluorescence spectra obtained in all the complexes at $P/D = 10$ are reported in fig. 3. It can be observed that at $P/D = 10$ there is a noticeable broadening of the spectrum compared to that of fig. 1. At $P/D = 2.5$ ($P/D = 3.3$ for poly(d(G-C))) the fluorescence maximum has shifted to 540 nm. Fluorescence decay measurements carried out at 530 nm at these P/D values again include three components with values of 10.8, 2.72 and 0.23 ns for DAPI-DNA; 12.2, 2.70 and 0.31 ns for DAPI-poly(d(A-T)); and 8.87, 2.84 and 0.18 ns in DAPI-poly(d(G-C)).

As reported previously [11], the fluorescence band centered at 540 nm is lost in the presence of 0.4 M KCl and accordingly the fluorescence lifetimes are modified.

4. Discussion

In this work we have sought to characterize the fluorescence properties of DAPI-polynucleotide complexes owing to the high degree of sensitivity which fluorescence allows in studies of nucleic acid structure and intercalative complexes. Our results clearly show that there was a marked fluorescence enhancement of DAPI when the drug is bound to polynucleotides. Even in the case of the DAPI-poly(d(G-C)) complex at high P/D ratios there was a 6-fold increase in fluorescence relative to the fluorescence of free DAPI. We must conclude, contrary to earlier reports [2], that DAPI binding and fluorescence enhancement is not specific for A-T base-pairs. Indeed, Chandra and Mildner [12] using absorbance methods reported that DAPI did form complexes with both poly(d(G-C)) and poly(d(A-T)) with comparable binding constants except at very low P/D.

Based on experiments performed with standard substances which have short fluorescence decay times we are confident that the 120 ps component was not due to an instrumental or sample artifact. The pre-exponential term for this short lifetime showed that it was an important decay component. However, the integrated intensity of this component, F_3, was low in all complexes, being between 3 and 6%. Two of the decay components (3.9 and 0.12 ns) were common to all DAPI complexes at $P/D = 150$. Their fractional contribution to the total fluorescence, F_1 and F_2, does vary from complex to complex. On the other hand, the intermediate lifetime component has different values in each complex, being 1.79 ns in DNA, 1.31 ns in poly(d(A-T)) and 0.87 ns in poly(d(G-C)). Furthermore, its fractional fluorescence also varies with polynucleotide.

In another work (Cavatorta, Masotti and Szabo, unpublished results) we have shown that the 2.77 ns component in free DAPI is the DAPI dication fluorescence. The 0.16 ns component results from a fast intramolecular proton transfer to the indole ring of DAPI followed by fluorescence. Although the 120 ps component has a value similar to one of the components of DAPI in buffer, it is not representative of a free DAPI component. Firstly, the quantum yield of DAPI-DNA is 18-times greater than that of DAPI alone. Secondly, the majority of DAPI in these complexes was bound ($K_{ap} = 2 \times 10^6$) [2,12]. Therefore, we would not be able to detect any fluorescence from the small amount of free DAPI present at $P/D = 150$.

It was previously reported [12] that at high P/D ratios there were two types of complexes. In all DAPI-polynucleotide complexes at $P/D = 150$ three exponential decay components were observed. These fluorescence decay results suggests

that there may be as many as three types of binding of DAPI to polynucleotides and DNA at high P/D ratios. Alternatively, it may be that the 3.9 and 0.12 ns components are characteristic of one binding form of DAPI while the intermediate decay components represents a second type of binding. We are not able to distinguish between these possibilities at this time.

The fractional fluorescence values show that the proportion of F_1 and F_2 varies with polynucleotide implying that the relative amounts of two of the possible three bound forms is dependent on nucleic acid composition.

Two of the decay components have similar values in the different polynucleotides. If they were characteristic of an intercalative complex it may be expected that they would be affected by the nucleic acid composition. The lack of this effect suggests that it may be reasonable to attribute them to a complex or complexes resulting from strong electrostatic interactions between the DAPI dication and the phosphate backbone. The observation of the same three lifetimes and fractional fluorescence in these complexes in the presence of 0.4 M KCl does not preclude the assignment of electrostatic binding to these components. At the higher ionic strength there would be a reduction in the binding constant values of the electrostatic complexes but any free DAPI released would not make an appreciable contribution to the total fluorescence.

On the other hand, the intermediate component does have some base specificity, being longer in A-T polynucleotides (1.31 ns) than in G-C polymers (0.87 ns). This suggests a fluorescence quenching effect depending on the nucleic acid composition, i.e., quenching in G-C relative to A-T. This might indicate that there is an intercalative binding mode or at least a complex in which there is a close approach to the nucleic acid bases. Alternate assignments are possible such as the 0.12 ns component being a quenched intercalative complex and the others representing binding in major and/or minor grooves. A definite assignment of the origin of each of the decay components will require further experiments.

The results at low P/D ratios confirmed the recent report by some of us [11] that a new fluorescent species was evident when $P/D = 10$. This new fluorescence spectrum with a λ_{max} at 540 nm was only observed in DAPI-polynucleotide complexes and not in concentrated solutions of free DAPI. Therefore we assign this fluorescence band to the binding of molecules of DAPI to sites in the polynucleotides which are very close to a previously bound DAPI molecule. The electronic interaction between these proximate DAPI molecules results in the new fluorescence band. The long fluorescence lifetime component in complexes (8.7–12 ns) with $P/D = 2.5$ (for DNA and A-T) or $P/D = 3.3$ (G-C) is assigned to this complex. The remaining lifetime components have values similar to free DAPI.

The latter type of complex was markedly affected by the presence of 0.4 M KCl as the fluorescence maximum at 540 nm and the long lifetime components are no longer observed. Obviously, the latter complex must be the result of weak electrostatic interactions.

In summary, we have shown that DAPI binding to polynucleotides containing either A-T or G-C base-pairs results in a fluorescence enhancement in each case. The lifetime results suggest there may be as many as three binding complexes present at high P/D of which at least one is electrostatic and the other may be intercalative. At low P/D another binding form which is characterized by DAPI-DAPI interactions is observed.

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References

1 P. Chandra, B. Mildner, O. Dann and A. Metz, Mol. Cell. Biochem. 18 (1977) 81.
2 J. Kapuschinski and W. Szer, Nucleic Acids Res. 6 (1979) 3519.

3 L. Masotti, P. Cavatorta, M. Avitabile, M.L. Barcellona, J. Von Berger and N. Ragusa. Ital. J. Biochem. 31 (1982) 90.
4 J. Kapuschinski and B. Skoczylas, Nucleic Acids Res. 5 (1978) 3775.
5 W.C. Russell, C. Newman and D.H. Williamson, Nature 253 (1975) 461.
6 D.H. Williamson and F.J. Fennel, Methods Cell Biol. 12 (1975) 335.
7 W. Schnedl, U. Roscher, M. van der Ploeg and O. Dann, Cytobiologie 15 (1978) 357.
8 J.N. Miller, in: Standards in fluorescence spectrometry (Chapman & Hall, London, 1981) p. 75.
9 M. Zuker, A.G. Szabo, L. Bramall, D.T. Krajcarski and B. Selinger, Rev. Sci. Instrum. 56 (1985) 14.
10 A.E. McKinnon, A.G. Szabo and D.R. Miller, J. Phys. Chem. 81 (1977) 1564.
11 L. Masotti, P. Cavatorta, R. Favilla, M.L. Barcellona, M. Avitabile, N. Ragusa and J. Von Berger, IRCS Med. Sci. 10 (1982) 957.
12 P. Chandra and B. Mildner, Cell Mol. Biol. 25 (1979) 137.