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A molecular approach to 4′,6-diamidine-2-phenylindole (DAPI) photophysical behaviour at different pH values

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The photophysical mechanisms which determine the spectral properties and decay rates of 4′,6-diamidine-2-phenylindole (DAPI) in solution and in association with nucleic acids have not yet been fully elucidated. We have performed steady-state and time-resolved fluorescence experiments on DAPI in a wide pH range to investigate the hypothesis that different ground-state conformations are responsible for the photophysical properties of the probe. Several excited-state mechanisms are investigated and it is concluded that among the proposed models, the hypothesis of ground-state heterogeneity with rapid interconversion among conformations is the only one consistent with the experiments in the entire pH range investigated.

1. Introduction

The fluorescence of 4′,6-diamidine-2-phenylindole (DAPI), is enhanced by specific interaction with AT, AU, and IC double helical clusters [1]. DAPI is used in light microscopy to visualize AT-rich domains of DNA and in molecular biology investigations to electively modify transcribing DNA sequences [2–7]. The fluorescence decay of DAPI in different solvents and bound to DNA has been recently investigated using both time-domain and frequency-domain techniques [8–15]. It has been shown that the emission spectrum at pH 7 can be time-resolved in two bands: a blue-shifted band with a maximum at 445 nm and a decay time of approximately 2.8 ns and a red-shifted band with a maximum at about 480 nm with a characteristic decay time of approximately 0.19 ns. Due to the prevalence of the red-shifted band at low pH, it has been suggested that the short lifetime value arises from an excited-state proton-transfer mechanism involving the indole NH [15]. However, our previous DAPI fluorescence decay results suggested that the lifetime heterogeneity can also originate from ground-state molecular conformers arising either from a rotamer at C_6 of the indole ring or at the C_2 [16]. The dependence of the shape and maximum emission spectrum on the excitation wavelength at neutral pH is evidence of the coexistence of at least two ground-state molecular species [15]. Differential ground-state solvation of the conformers can be pH dependent. pH changes can also directly modify the degree of protonation of the indole and can influence the degree of efficiency of the proton-transfer process. Therefore, the putative proton-transfer process can be dependent on the conformer, the differential hydration, and the degree

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of protonation. The purpose of the present work is to further investigate the various mechanisms that can affect the lifetime of the excited state. In particular, the proton transfer hypothesis will be tested. The possibility of fast interconversion between the various solvation states will be evaluated at different pH values. Furthermore, since the processes which can affect the decay rate can be influenced by the different conformers of the DAPI molecule and the possible ground-state conformers for the molecule can be a large number, the rates can also be distributed. The conventional analytical approach previously used to resolve the DAPI decay process in terms of exponential components (decay rates, and of pre-exponential factors associated with two populations of well defined conformation) is revisited. A different analysis of the DAPI decay, based on continuous distribution of lifetime values, is tested. A similar distributional approach has been used to analyze the decay of tryptophan residues in proteins [17] and it is more adequate to investigate the multitude of rate constants that can be present in a molecule such as DAPI in buffer when many different conformers can affect the decay.

2. Materials and methods

4′,6-diamidine-2-phenylindole dihydrochloride was obtained from Boehringer Mannheim and checked for purity by thin layer chromatography (solvent mixture: 60% water, 20% acetic acid, 20% n-butanol). Sodium chloride, sodium hydroxide, TRIZMA (tris [hydroxymethyl] aminomethane hydrochloride), and TRIZMA BASE (tris [hydroxymethyl] amino methane) were from Sigma Chemical Company, St. Louis, MO. All other reagents were of the highest purity available. All solutions were prepared using double glass-distilled deionized water (Barnstead Nanopure II), and bubbled with nitrogen for several minutes before the measurements. The temperature of the sample was thermostated at 20°C using a circulating hot water bath. Fluorescence measurements were carried out at the Laboratory for Fluorescence Dynamics at the University of Illinois at Urbana-Champaign, IL, using a multifrequency phase and modulation fluorometer similar to that described by Gratton and Limkeman [18]. The excitation source was a continuous wave helium–cadmium laser, emitting at 325 nm. The laser intensity was modulated either using a Pockels cell modulator or an acousto-optic modulator. The frequency range was from 4 MHz to 200 MHz. Data were collected with a standard deviation of the phase and modulation, below 0.2° and 0.004°, respectively. Data were analyzed using the Globals Unlimited software package. The value of the reduced χ^2 was used as a comparison for the various models.

3. Results

3.1 Empirical analysis using discrete and distributed lifetime components

Fluorescence lifetime measurements of DAPI in tris buffer solution were performed at various pH values from 1.0 to 12.4. Both a nonlinear least-squares analysis using a sum of exponential components and an algorithm for the analysis of the decay using continuous lifetime distributions were used [14,19,20]. The Lorentzian shape always gave better fits than the uniform and the Gaussian distributions. The two analysis methods are compared using the value of the reduced χ^2. The parameters of the fit for the double exponential analysis and the Lorentzian continuous distribution analysis are reported in Table 1. At least two exponential components were necessary to describe the decay from pH 1.0 to pH 12.4, and the superposition of one Lorentzian distribution and one exponential were used for the distributional analysis. In the low pH range (< 8), the values of the exponential components and relative fractions were almost identical to those obtained using the continuous lifetime distribution approach and the width of the distribution was very narrow (~ 0.1 ns), indicating that, in this pH range, a distribution approach is unnecessary. However, by increasing the pH value in the range above 8.2, the lifetime distribution analysis gives a lower χ^2 values. The distribution still remained bimodal but

the shorter lifetime component was broad. At pH 9.0, the fluorescence fractional intensities were approximately of equal amplitude. By increasing the pH to 9.6, the distributional pattern changed significantly. The fractional fluorescence intensities were inverted with respect to the behavior at neutral pH, the long decay component giving the larger contribution. In the pH range from 10.0 to 11.5, the decay behavior is again biexponential, but the lifetime, as well as the center values of the distribution, were different with respect to lower pH. The more relevant features of the distributional pattern in this pH range are the following: the long lifetime component shifts to lower values, and the short one also shifts to lower value. There is a marked decrease of the width of the short lifetime component at lower pH values.

The results reported in Table 1 demonstrate that the fits are relatively good over the entire pH range. The analysis on the same set of data using distributions of lifetime values gives a marginal improvement of the χ^2 value, except in the pH region between 9.0 and 11.0. Close inspection of Table 1 reveals also that there is a large variation of the parameters of the fit only in the region between pH 9.0 and 12.4. In particular the value of the long lifetime component (τ_L) is essentially constant, about 2.65 ns, up to pH 9.6 and then decreases at higher pH values. The value of the short lifetime component (τ_S) varies gradually up to pH 9.6. As the pH increases above pH 9.6, there is a rapid increase in the lifetime value and then a decrease above pH 11.0. The fractional intensity of the short component gradually decreases up to pH 9.6 where there is a sudden variation (rise) and then continues to decrease at higher pH values.

4. Discussion

Three different hypotheses are discussed to explain the origin of the two observed lifetime values, viz. (1) excited-state proton transfer, (2) ground-state heterogeneity without and (3) with interconversion. Also the behaviour at pH > 12 will be briefly discussed.

4.1 Excited-state proton transfer mechanism

This process should correspond to the scheme shown in Fig. 1a. In this scheme, the decay associ-

Table 1

pH	Double exponential				Lorentzian and exponential				
	SAS_S	τ_S (ns)	τ_L (ns)	χ^2	SAS_S	C_S	W_S	τ_L (ns)	χ^2
1	0.86	0.15	2.61	1.27	0.90	0.12	0.15	2.92	1.11
2	0.80	0.18	2.56	1.88	0.81	0.18	0.05	2.61	1.53
3	0.72	0.19	2.67	0.75	0.75	0.11	0.14	2.75	0.43
4	0.71	0.22	2.68	0.53	0.73	0.21	0.05	2.71	0.45
5.5	0.71	0.22	2.66	1.51	0.72	0.21	0.05	2.69	1.62
6.5	0.70	0.23	2.43	2.94	0.72	0.22	0.12	2.46	2.53
7.5	0.75	0.24	2.75	4.09	0.77	0.24	0.10	2.83	2.63
8.2	0.73	0.17	2.65	0.58	0.74	0.16	0.05	2.69	0.45
9.05	0.61	0.19	2.78	1.376	0.64	0.15	0.18	2.80	0.76
9.6	0.23	0.18	2.63	0.78	0.25	0.10	0.26	2.64	0.62
10.0	0.46	0.33	2.36	3.404	0.52	0.33	0.34	2.38	0.29
11.0	0.43	0.77	2.49	4.555	0.37	0.78	0.77	2.00	2.32
11.5	0.15	0.14	1.44	13.766	0.17	0.05	0.26	1.43	2.83
12.4	0.42	0.43	1.04	4.171	0.19	0.01	0.58	0.83	1.50

SAS_S = Species Associated Spectral contour to the short decay component; τ_S = lifetime of the short decay component in nanoseconds; τ_L = lifetime of the long decay component in nanoseconds; C_S = center of the short decay component distribution, W_S = width, as FWHM, of the short decay component distribution; and χ^2 = the reduced χ^2.

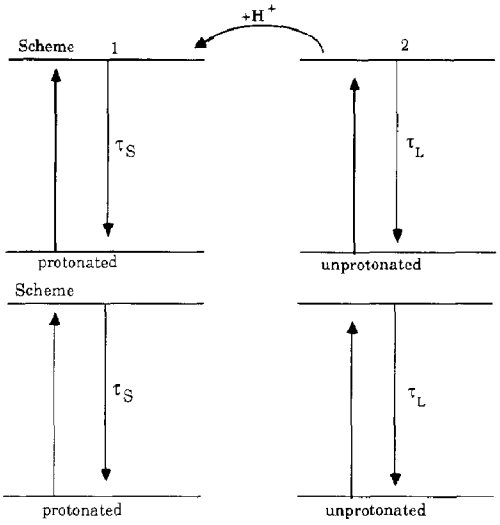

Fig. 1(a). Proton transfer mechanism. Requirement: τ_S, τ_L and SAS1 are constant, k_{21} dependent on proton concentration (bound dependent or ground-state equilibrium) Result: To keep τ_S and τ_L constant, a rate k_{21} must be added. The analysis shows that k_{21} is not a simple function of $[H^+]$. Fig. 1(b). Ground-state heterogeneity (pH dependent) with no interconversion. Requirement: τ_S and τ_L are constant, and SAS1 reflects the protonation–deprotonation equilibrium. Result: this fit gives very poor χ^2.

ated with the short lifetime component should represent the molecular form in which proton transfers occur, given the prevalence of the short lifetime component at low pH. It is well known that, for the scheme presented in Figure 1a, the decay time associated with the shorter lifetime value (τ_S) must be higher than the highest short lifetime component obtained using a double exponential decay (Table 1). The same line of reasoning applies to the value of the long lifetime component (τ_L). From Table 1, for the scheme of Fig. 1(a) to be valid, both lifetime components, τ_S and τ_L, must satisfy the following relationship: $\tau_S > 0.77$ ns and $\tau_L > 2.78$ ns. However, since the lifetime of the long component recovered using the double exponential analysis is essentially constant from pH 1.0 to pH 9.0, this implies that the hypothetical proton transfer is independent of the external proton concentration. Then only an intramolecular proton transfer process can be responsible for this effect, but this hypothesis will also be disregarded. Furthermore, τ_L decreases at higher pH, a result which is incompatible with the idea that proton transfer is the only mechanism responsible for the lifetime effects. Without success, we have also attempted to measure the rise time of the short component in the red part of the spectrum which should result from the excited state reaction, given the widely different spectra of the two lifetime components. This latter observation should also rule out the possibility of intramolecular proton transfer.

4.2 Ground-state heterogeneity without interconversion

This process corresponds to the scheme depicted in Fig. 1(b). Clearly, this scheme should again give two exponential decays. The ground-state conformers cannot be due to different protonated forms of the DAPI molecule, since two lifetime components coexist from pH 1.0 to pH 12.0. Instead, the ground-state heterogeneity must be related to different conformers whose ground-state equilibrium is marginally affected by pH, except perhaps in the region from pH 9.0 to pH 11.0. The results of the data analysis using this model is reported in Table 1. An attempt to perform a global fit maintaining the two lifetime components constant and allowing the fractional contributions to vary gives very large reduced χ^2. The relative population of the conformers should be proportional to the value of SAS1 (Species Associated Spectral contour) as reported in Table 1. In order to explain the changes of the value of τ_S, τ_L, and SAS1 as a function of pH (Table 1), we must assume that the efficiency of the quenching mechanisms, which generates the short lifetime component is very weakly pH-dependent from 1.0 to 9.0. However, in this region, the relative population of the long-lived conformer increases as the pH is increased. From pH 9.0 to 11.0, the quenching process, which gives rise to the short lifetime component, becomes much less efficient. Above pH 9.0, however, there is a quenching of the long-lived species instead of the short-lived component. This series of *ad hoc* hypotheses, although not impossible, renders this second mechanism unsatisfactory.

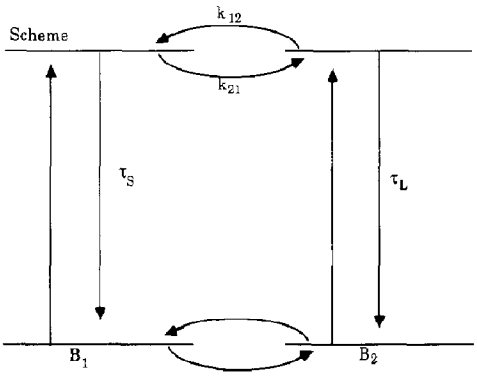

Fig. 2. Ground-state heterogeneity with fast interconversion between two forms. The ground-state equilibrium is the same as the excited-state equilibrium. There is also a protonation equilibrium. Requirement: τ_S and τ_L are constant, while the ground state equilibrium depends on k_{12}/k_{21}. B_1 and B_2 denote the initial fraction population of state 1 and 2, respec - tively.

4.3 Ground-state heterogeneity with interconversion

In this section, we explore the possibility of interconversion between conformers during the lifetime of the excited-state. The corresponding decay scheme is shown in Fig. 2. Since we have not observed a rise time of the short (or long) component, we must assume that, if there is interconversion, the excited-state equilibrium is the same as that of the ground-state and is not affected by the excitation. If the two characteristic decay times have a unique value, independent of pH, as shown from the data reported in Table 1, following the same argument given for the excited-state proton-transfer mechanism, we must assume that the two components have characteristic decay times of $\tau_S \geq 0.78$ ns and $\tau_L \geq 2.78$ ns, respectively. Of course, in this case the variation of the observed decay times (which, at pH 9.0, are 0.78 and 2.60, respectively) must be attributed to a change in the interconversion rate between conformers, rather than to a change of the efficiency of the quenching process. Also, the ratio of the interconversion rates must be related to the ground-state conformer equilibrium, i.e., to the relative excitation of the short and long component. However, this hypothesis cannot be rigorously tested since the spectral changes, both in absorption (and in emission), are related to the protonation of the indole rather than to direct changes in the relative population of the conformers.

Under the ground-state fast interconversion hypothesis (Table 2), we obtain a relatively fast interconversion from the short to the long component of about 3 to 5×10^9 s^{-1} from pH 1.0 to about pH 8.0. At pH 10.0, this rate decreases and the apparent lifetime of the short component in-

Table 2

pH	SAS	τ_S (ns)	τ_L (ns)	B_1	B_2	K_{21}	K_{12}	χ^2
1	0.879	0.8	4.0	0.5	0.07	4.56	0.07	1.09
2	0.879	0.8	4.0	0.5	0.08	3.61	0.08	0.55
3	0.879	0.8	4.0	0.5	0.33	5.02	0.84	0.68
4	0.879	0.8	4.0	0.5	0.39	2.95	0.54	0.44
5.5	0.879	0.8	4.0	0.5	0.39	2.92	0.55	1.46
6.5	0.879	0.8	4.0	0.5	0.295	2.62	0.66	2.55
7.5	0.879	0.8	4.0	0.5	0.36	2.50	0.41	2.63
8.2	0.879	0.8	4.0	0.5	0.31	4.04	0.71	0.55
9.05	0.879	0.8	4.0	0.5	0.80	3.51	0.54	0.84
9.6	0.879	0.8	4.0	0.5	2.12	3.73	0.69	0.64
10.0	0.879	0.8	4.0	0.5	0.91	1.43	0.46	0.95
11.0	0.879	0.8	4.0	0.5	1.34	0.04	0.16	4.42
11.5	0.879	0.8	4.0	0.5	0.44	3.23	3.03	2.63
12.4	0.879	0.8	4.0	0.5	0.002	0.29	1.44	2.06

SAS_S = Species Associated Spectral contour to the short decay component. τ_S = lifetime of the short decay component in nanoseconds; τ_L = lifetime of the long decay component in nanoseconds; B_1 = initial fraction population of state 1; B_2 = initial fraction population of state 2; χ^2 = the reduced χ^2. The first four parameters are linked during the analysis procedure.

creases. At pH 11.0 and higher, the rate of interconversion from the long-lived state to the shorter state increases substantially, reaching a value of about 10^9 s^{-1} at higher pH. The good fit shown in Table 2 (the values of the χ^2 for each file must be identical to the values reported in Table 1 for the two-exponential analysis) does not necessarily prove this model because the number of parameters have increased, the two fits are equivalent and the two models are not uniquely identifiable [21].

The hypothesis of fast interconversion should be supported by other measurements, for example, by temperature studies and by a global analysis of the values of the rate of interconversion. It is known that for tryptophan, the interconversion between rotamers is relatively slow [22–24], compared to the lifetime of the excited-state. The absolute values of the rates of interconversion obtained for this model contains an arbitrary scaling constant. In fact, the variation (as a function of pH) of the decay rates of the two ground-state species as obtained by the double exponential analysis is entirely attributed to interconversion between the two species rather than to other molecular mechanisms.

4.4 Region of high pH(> 12)

Although the effects in this pH region are irreversible, the structural modifications of the DAPI molecule do not involve the indole moiety but other parts of the molecule as demonstrated by ^{13}C NMR (data not shown). Therefore, the basic proposal for the decay mechanisms is not changed (since the chromophore is unchanged) but the pathways for quenching are varied. As a consequence of the chemical modifications, the ground state hydration equilibrium is different and this is reflected in the change at extreme pH of the fractional population (B_2) shown in Table 2. The chemically modified molecule maintain the single exponential decay upon return to pH 7 and also it retains the characteristic blue-shifted spectrum. This experiment provides further conformation of the two-state model since when the ground state population is affected by chemical modification, only one exponential term appears in the decay. Therefore, the effect of the polar expansions of DAPI in determining the decay modality is definitively shown.

Conclusions

The decay of DAPI as a function of pH can be interpreted on the basis of two (or more) ground-state conformers. The relative population of the two conformers is a slow function of pH from pH 1.0 to pH 9.0. It is likely that in one of the two conformers, the lifetime quenching mechanism can indeed involve the proton at the NH position of the indole ring. In fact, an intramolecular proton transfer mechanism can be responsible for the short lifetime species. However, given the stereochemistry of the molecule, the intramolecular proton transfer cannot be direct, but must involve a shell of hydration, perhaps forming a water bridge with the NH_2 of the diamidine part of the molecule. Above pH 9.0, the indole NH group is deprotonated and the quenching mechanisms essentially disappear, pointing again to the importance of the indole NH relative to the quenching process. Above pH 9.0, a different mechanism starts to operate which involves the quenching of the long lifetime component. A similar effect has been observed for N-acetyl tryptophanamide and tryptophan in this pH range [25]. Due to the protonation equilibrium in the pH region (9.0 to 11.0), several different ground-state species with different hydration shells can be present. Consequently, more configurations are possible which cause quenching. This effect appears in the fluorescence decay as a distribution of lifetime values in this pH range. Within the present instrument capabilities, it is impossible to distinguish between a large number of discrete lifetime components in a given lifetime range or a continuous lifetime distribution. The analysis using the latter approach is easier and uses less variable parameters. The fact that this form of analysis gives a better representation of the decay simply means that, in that pH range, there are more than two ground-state conformers.

One of the major motivations for this work was to obtain a better understanding of the spectroscopic properties of DAPI when it was interacting

with DNA. From the results reported here, it appears that the hydration shell around the DAPI molecule plays a major role in determining the fluorescence properties of DAPI. When DAPI is in the minor groove of the DNA molecule, the fluorescence lifetime is relatively long and close to 4 ns. However, it appears that DAPI can also interact with the phosphate group of DNA. In this case, there is no fluorescence enhancement and the lifetime is quite short. We believe that this behavior is due to the larger hydration of the DAPI molecule in this condition.

Our results show that a simple model can explain the complexity of the fluorescence decay of DAPI over a broad pH range. In particular the diamidine moiety is essential in determining the decay behavior. The knowledge of the decay mechanism is also required to analyze fluorescence depolarization data which can provide direct information on the nucleic acid dynamics at a molecular level. These studies are now in progress in our laboratory.

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References

1 J. Kapuscinski and W. Szer, Nucl. Acid Res. 6 (1979) 3519–3534.
2 D. Lehweizer, Chromosoma 58 (1984) 307–423.
3 A.W. Coleman, Exp. Cell Res. 152 (1984) 528–540.
4 E.M. Bühler and N.J. Malik, Cytogenet. Cell Genet. 47 (1988) 104–105.
5 D.C. Straney and D.M. Crothers, Biochemistry 26 (1987) 1987–1995.
6 G. Palú, S. Valisena, M.L. Barcellona, L. Masotti and G.A. Meloni, Biochem. Biophys. Res. Commun. 145 (1987) 40–45.
7 A. Montecucco, S. Spadovi, F. Foetrer, A. Pedrali-Noy, C. Sala, G. Palú and G. Ciarrocchi, Biochem. Pharmac. 37 (1988) 1853–1954.
8 L. Masotti, M.L. Barcellona, J. vonBerger, and M. Avitabile, Biosci. Rep. 1 (1981) 701–707.
9 G. Manzini, M.L. Barcellona, M. Avitabile and F. Quadrifoglio Nucl. Acid Res. 11 (1983) 8861–9976.
10 G. Manzini, L. Xodo, M.L. Barcellona and F. Quadrifoglio, Nucl. Acid Res. 13 (1985) 8955–8967.
11 P. Cavatorta, L. Masotti and A.G. Szabo, Biophys. Chem. 22 (1985) 11–16.
12 M.L. Barcellona, R. Favilla, J. vonBerger, M. Avitabile, N. Ragusa and L. Masotti, Arch. Biochem. Biophys. 250 (1986) 48–53.
13 A.G. Szabo, D.T. Krajcarski, P. Cavatorta, L. Masotti and M.L. Barcellona, Photochem. Photobiol. 44 (1986) 143–150.
14 M.L. Barcellona and E. Gratton, Biochem. Biophys. Acta 993 (1989) 174–178.
15 M.L. Barcellona and E. Gratton, Eur. Biophys. J. 17 (1990) 315–323.
16 G. Cardiel, M.L. Barcellona and E. Gratton, Ital. J. Biochem. 38 (1989) 125–129.
17 R.J. Alcala, E. Gratton and F.G. Prendergast, Biophys. J. 51 (1987) 925–936.
18 E. Gratton and M. Limkeman, Rev. Sci. Instrum. 54 (1983) 294–299.
19 J.R. Lakowicz, G. Laczko, H. Cherek, E. Gratton and M. Limkeman, Biophys. J. 46 (1984) 463–477.
20 E. Gratton, M. Limkeman, J.R. Lakowicz, G. Laczko and H. Cherek Biophys. J. 46 (1984) 478–486.
21 J.M. Beechem, E. Gratton, M.A. Ameloot, J.R. Knutson and L. Brand, Fluorescence Spectroscopy, Vol. I, Principles and Techniques, ed. J.R. Lakowicz (Plenum Press, New York, NY, 1989) 66 pp.
22 A.G. Szabo and D.M. Rainer, J. Am. Chem. Soc. 102 (1980) 554–563.
23 D. Creed, Photochem. Photobiol. 39 (1984) 537–562.
24 R.A. Engh, L. Chen and G.R. Fleming, Chem. Phys. Lett. 126 (1986) 365–372.
25 E. Vander Donckt, Prog. React. Kinet. 5 (1970) 273–299.