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Fluorescence Decay Studies of 9-Aminoacridine Bound to Polyriboadenylic Acid

Yukio KUBOTA* and Yuko MOTODA

Department of Chemistry, Faculty of Science, Yamaguchi University, Yamaguchi 753

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The interaction of the mutagenic dye 9-aminoacridine (9AA) with polyriboadenylic acid (poly rA) was studied by both steady-state and transient fluorescence measurements. It was found that the fluorescence decay of 9AA bound to poly d(A-T) follows a single-exponential decay law but that the decay data of 9AA bound to poly rA can be well described as a sum of two-exponential functions. The fact that nanosecond time-resolved fluorescence spectra are independent of time suggests that the double-exponential decay may be attributed to different microenvironments at the binding sites. The decay parameters, fluorescence emission maxima, and fluorescence quantum yields obtained at acid pH differ from those obtained at neutral pH. The fluorescence behavior of 9AA is discussed in connection with the conformational change of poly rA.

The dye 9-aminoacridine (9AA) is one of a number of acridines that are mutagenic[1] and that interact with DNA *in vitro*.[2] In previous work,[3,4] we have reported that guanine–cytosine base pairs completely quench the fluorescence of 9AA, upon binding to DNA, while adenine–thymine base pairs are responsible for its fluorescence and that the emitting sites of 9AA on DNA exhibit fluorescence decay curves which can be resolved into three-exponential components. The result has been attributed to the heterogeneity of the emitting sites.[4]

Studies on binding interactions between the dye and DNA are considerably complicated due to the content and the stereochemical arrangement of DNA bases.[2] Two approaches have been shown to be very useful in understanding the nature of interactions between the dye and DNA; one is to study using synthetic polynucleotides which contain simple types of binding sites, compared with DNA[3-6] and the other is to study complex formation between the dye and mononucleotides.[7-9] We have found that, upon binding to poly d(A-T) which contains only one type of site, the fluorescence quantum yield of 9AA is not substantially altered and its fluorescence decay follows a single-exponential decay law.[4] We have also found that the fluorescence of 9AA is markedly quenched by purine mononucleotides and that the weak fluorescence arises from 9AA complexed with adenosine 5′-monophosphate (AMP).[7,9] These findings suggest that the fluorescence behavior of 9AA may depend on whether the 6-amino group of adenine is hydrogen-bonded or free.

It is well established that the conformation of polyriboadenylic acid (poly rA) in solution changes from the hydrogen-bonded double-strand helix at low pH to the ordered single-strand structure of stacked bases at neutral pH.[10] Therefore, it is of great interest to study the fluorescence behavior of 9AA bound to poly rA in connection with its conformational change. Such a study may give a clue to the nature of interactions between 9AA and adenine residues.

In the present study, we investigated the fluorescence of 9AA bound to poly rA at various pH's by both steady-state and transient decay measurements. The decay behavior was found to be rather complex and possible interpretations are discussed.

Experimental

Materials. All chemicals were of reagent-grade purity or better. Poly rA was obtained from Miles, and, according to the manufacturer, had a molecular weight greater than 10^5. Poly d(A-T) was purchased from Miles. 9AA was the same sample as used before.[3,4] Poly rA solutions were prepared by first dissolving the required amounts of poly rA in 0.005 M† phosphate buffer (pH=6.9) and then dialyzing against the buffer solution to yield the desired pH. Concentrations of poly rA solutions were determined spectrophotometrically.[10] All measurements were carried out in 0.005 M phosphate buffer (pH=6.9) or 0.01 M acetate buffer (pH=4.1−5.9) solutions at (25±0.1)°C.

Absorption and Flow Dichroism Measurements. Absorption spectra were recorded using a Shimadzu UV-200S spectrophotometer. Flow dichroism measurements were made as described elsewhere.[11]

Steady-state Fluorescence Measurements. Steady-state fluorescence spectra were measured with a Hitachi MPF-2A spectrophotofluorometer. Correction was applied for the unequal quantum response of the detector system. The fluorescence quantum yield of the poly rA–9AA complex was determined by comparing the area under the fluorescence spectrum of the complex with the corresponding area of 9AA and by taking the quantum yield of 9AA to be 0.96.[3] For quantum yield measurements, the excitation monochromator was set at 408 nm with a 2—3 nm band pass, and the emission was recorded with a 2 nm band pass.

Transient Fluorescence Measurements. Transient fluorescence decay curves and time-resolved fluorescence spectra were measured with an Ortec nanosecond spectrophotofluorometer.[4,8,9] All decay measurements were performed under single photon counting conditions. The flash lamp was thyratron-triggered, air-filled (0.5 atm), and operated at 5.5 kV with a repetition rate of 25 kHz. The excitation wavelength at 375 nm was selected with an interference filter (Japan Vacuum Optics). The emission was observed by an RCA 8850 photomultiplier tube through a grating monochromator (Applied Photophysics Ltd.) whose bandpasses were varied from 2 to 10 nm according to the fluorescence intensity of the sample. Care was taken to eliminate anisotropic contributions to the observed decay according to the method described elsewhere.[4]

The observed decay curve $i(t)$ is represented by the convolution integral:

† 1 M=1 mol dm^{-3}.

$$i(t) = \int_0^t g(u)I(t-u)\mathrm{d}u \qquad (1)$$

where $g(t)$ is the apparatus response function and $I(t)$ is the fluorescence decay which would be obtained with the δ-pulse excitation. It has been shown that the response function $g(t)$ depends on the energy of the photon impinging on the photocathode.[12] According to the method of Wahl *et al.*,[12] the true $g(t)$ was determined from the fluorescence decay curve of 1,1,4,4-tetraphenyl-1,3-butadiene in deaerated cyclohexane ($\tau = 1.72 \pm 0.02$ ns at 25 °C).

Deconvolution of Eq. 1 was achieved by the methods of Laplace transformation[13] and nonlinear least-squares[14] on the assumption that the fluorescence decay $I(t)$ is given by a sum of exponential functions:

$$I(t) = \sum_{i=1}^{n} \alpha_i \exp(-t/\tau_i) \qquad (2)$$

where the α_i's and τ_i's are the amplitudes and lifetimes, respectively, of the ith component. Both methods yielded very similar results. The parameters obtained by analyses were convolved with the apparatus response function $g(t)$ and goodness of the fit between the observed and calculated decay curves was judged by inspection of the reduced χ^2, the weighed residuals, and the autocorrelation function of the residuals.[13,14] Data analysis was accomplished using a PDP 11/04 minicomputer (Digital Equipment Corp.) interfaced with an Ortec 6240B multichannel analyzer.

Results

Absorption and Steady-state Fluorescence Spectra. It is well established that the conformation of poly rA in solution changes from the double-strand helix at acid pH to the ordered single-strand structure of stacked bases at neutral pH.[10] The conformational transition depends on pH, temperature, and ionic strength.[10,15] Under the present conditions (ionic strength of 0.01 and 25 °C), the transition pH was found to be 6.0 in agreement with the previous finding.[10,15]

Since little is known about the optical properties of acridine dyes bound to poly rA,[16,17] absorption and steady-state fluorescence spectra at various pH's were first examined in order to obtain information on the

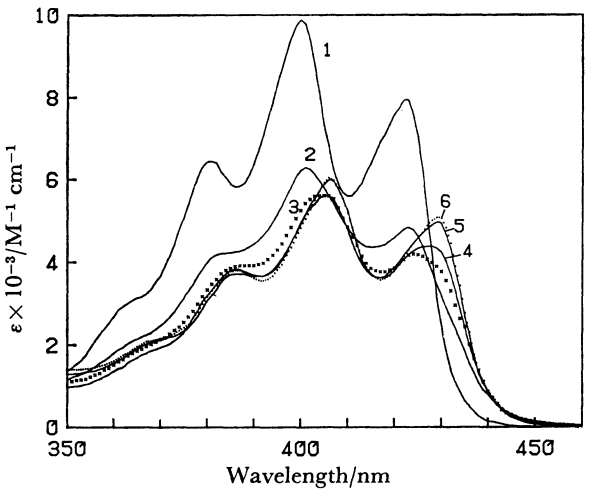

Fig. 1. Absorption spectra of poly rA–9AA complexes in 0.01 M acetate buffer (pH=4.9). 9AA: 3.8×10^{-5} M. *P/D*: (1) 0; (2) 4; (3) 10; (4) 20; (5) 150; (6) 193.

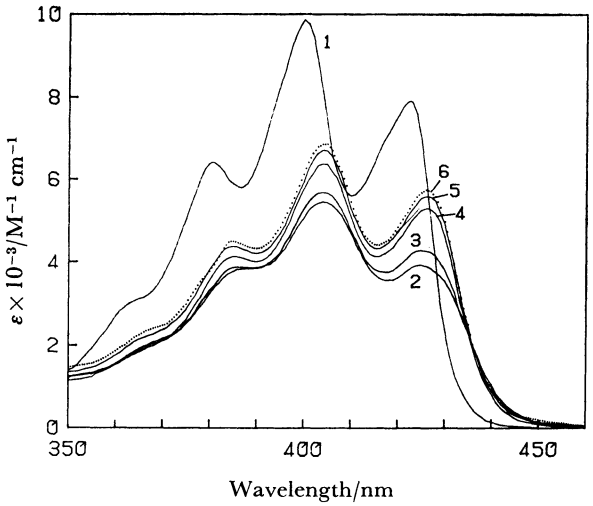

Fig. 2. Absorption spectra of poly rA–9AA complexes in 0.005 M phosphate buffer (pH=6.9). 9AA: 3.8×10^{-5} M. *P/D*: (1) 0; (2) 4; (3) 10; (4) 50; (5) 150; (6) 200.

binding state of 9AA. Figures 1 and 2 show absorption spectra of 9AA in the presence of poly rA, respectively, at pH=4.9 and pH=6.9. Absorption changes at low *P/D*** values (*P/D*<4), although spectra are not shown in Figs. 1 and 2 to avoid the complication due to overlaping, were found to result from the contributions of the bound and free 9AA. With increasing *P/D*, isosbestic points (425 nm at pH=4.9 (*P/D*>20) and 435 nm at pH=6.9 (*P/D*>4)) appear in the absorption spectra, and absorption bands can be seen to shift progressively toward a limit at high *P/D* values (*P/D*>200) (Figs. 1 and 2.) This finding suggests that the bound 9AA is transformed gradually from one type of complex (complex I) to a different type of complex (complex II). The limiting spectra at pH=4.9 and pH=6.9 (*P/D*> 200), respectively, resemble closely the spectra of 9AA intercalated into DNA[4] and of 9AA complexed with AMP;[7] both spectra show hypochromicity and red shift, compared with the spectrum of the free dye. As Dourlent *et al.*[17] first inferred from absorption spectra of proflavine bound to poly rA and their similarity to its spectra upon binding to DNA, complex I may be attributed to electrostatic interactions of the 9AA cation with the phosphate group, followed by cooperative binding along the phosphate backbone of poly rA, whereas complex II may be ascribed to stacking interactions between 9AA and adenine residues.

In order to avoid the complication due to the presence of different binding processes, the following fluorescence studies were performed at sufficiently high *P/D* values (*P/D*>400) where complex II predominates and the concentration of the free dye is negligible.

Figure 3 shows steady-state fluorescence spectra of the poly rA–9AA complexes obtained at acid and neutral pH's and also shows their comparison with spectra of 9AA complexed with AMP or poly d(A-T). As can be seen from Fig. 3, the spectrum at pH=6.9 is very similar to that of the AMP–9AA complex, and the

** Molar ratio of DNA Phosphate to Dye.

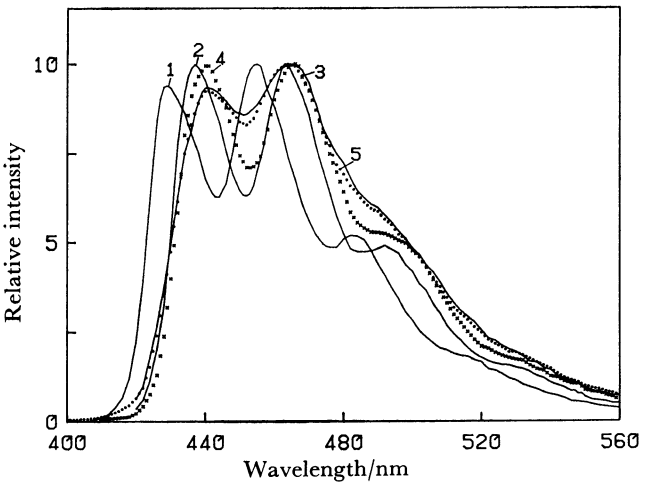

Fig. 3. Normalized fluorescence quantum spectra of 9AA in: (1) 0.005 M phoshpate buffer (pH=6.9); (2) poly rA (P/D=502, pH=4.9); (3) poly rA (P/D=503, pH=6.9); (4) poly d(A-T) (P/D=132, pH=4.9); (5) AMP (0.1 M, pH=6.9). The excitation wavelength was 390 nm, and the dye concentration was 7.4—12$\times 10^{-6}$ M.

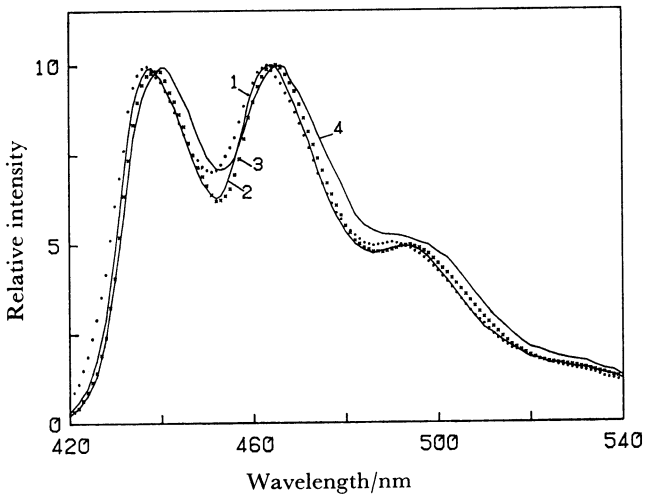

Fig. 4. Normalized fluorescence quantum spectra of poly rA–9AA complexes as a function of pH: (1) P/D=512, pH=4.1; (2) P/D=524, pH=5.3; (3) P/D=533, pH=5.9; (4) poly d(A-T)–9AA complex (P/D=132, pH=4.9). The excitation wavelength was 408 nm, and dye concentration was 1.2$\times 10^{-5}$ M.

spectra at pH=4.9 and of the poly d(A-T)–9AA complex are similar in shapes except that the fluorescence bands of the poly d(A-T)–9AA complex are shifted toward longer wavelengths compared to those of the poly rA–9AA complex. Figure 4 indicates fluorescence spectra obtained at other acid pH's; a gradual red shift of the spectra can be seen upon going from pH=4.1 to pH=5.9. On the other hand, the fluorescence quantum yield (Φ_F) in this pH region (pH=4.1—5.9) was almost constant, while the Φ_F value at neutral pH was smaller than that at acid pH (Table 1).

Flow Dichroism. The poly rA–9AA complexes at acid pH showed negative flow dichroism in both the visible and ultraviolet regions; the reduced flow dichroism at a velocity gradient of 3200 s^{-1} was found to be −0.23 at 260 nm and −0.18 at 400 nm under conditions of P/D=20, [9AA]=5$\times 10^{-5}$ M, and pH=5.0. This finding suggests that the acridine ring is oriented rather perpendicularly to the helix axis of poly rA like adenine residues.[11,18]

Fluorescence Decay Curves. Since fluorescence spectra of 9AA bound to poly rA depended on pH, fluorescence decay studies were undertaken to investigate further the nature of interactions between the dye and the binding sites. Decay curve measurements were made at high P/D values (P/D>400) and at low ionic strength (0.01). Under these conditions, the contribution of the free dye and energy transfer between bound dye molecules were negligibly small.

Figures 5 and 6 display typical decay data obtained at pH=4.9 and pH=6.9, respectively; the fit between the observed and theoretical convolved decay curves for a double-exponential decay law can be seen to be excellent. Sets of decay parameters obtained at various pH's are summarized in Table 1. In all cases, the χ^2, the weighed

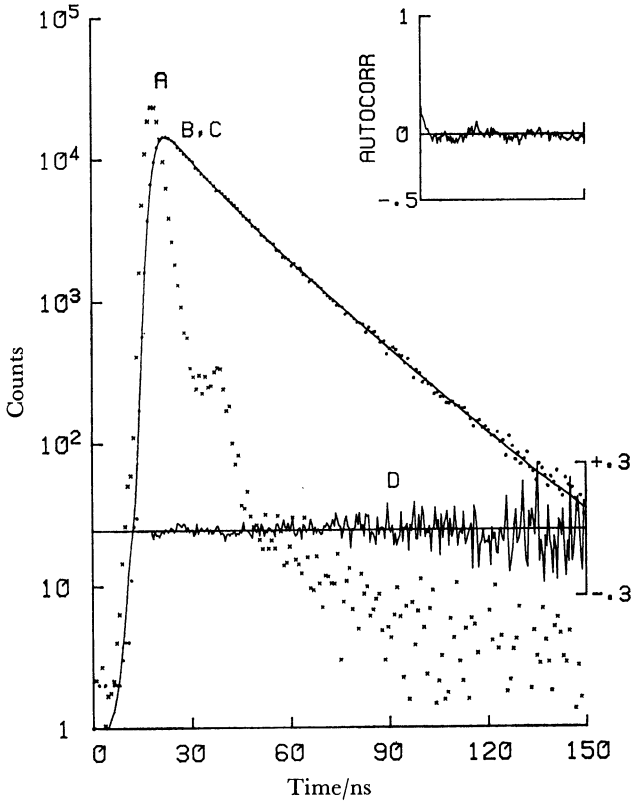

Fig. 5. Experimental decay curves and the results of a two-component analysis for poly rA–9AA complex (P/D=502) in 0.01 M acetate buffer (pH=4.9). 9AA: 7.4$\times 10^{-6}$ M. The excitation and emission wavelengths were 375 and 455 nm, respectively. A: Apparatus response function. B: Observed decay curve. C: Best theoretical decay curve (smooth curve). D: Weighed residuals. The inset is the autocorrelation function of the residuals. Parameters obtained: τ_1=21.2 ns, τ_2=8.4 ns, α_1=0.088, α_2=0.077, and χ^2=1.71. The amplitudes normalized to unity are α_1=0.54 and α_2=0.46.

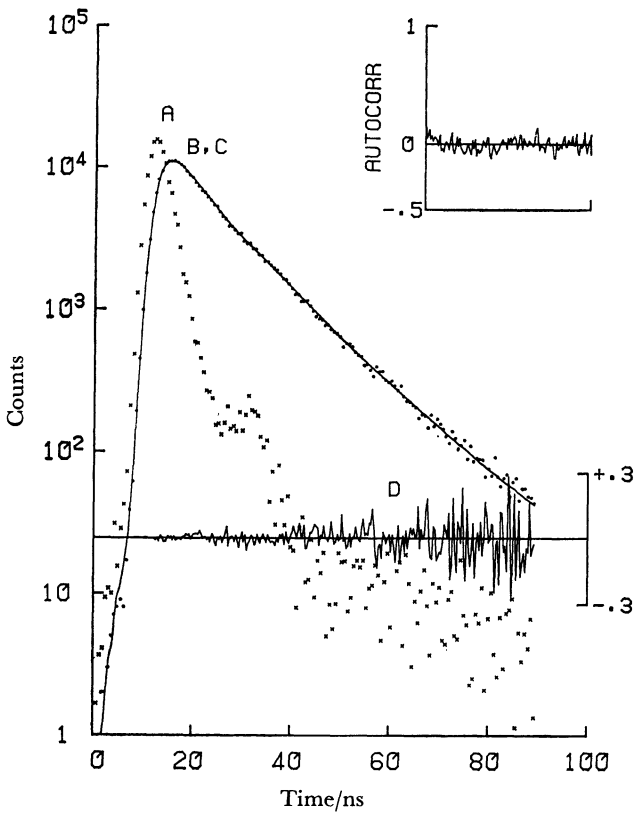

Fig. 6. Experimental decay curves and the results of a two-component analysis for poly rA–9AA complex (P/D=503) in 0.005 M phosphate buffer (pH=6.9). 9AA: 7.4×10^{-6} M. The excitation and emission wavelengths were 375 and 460 nm, respectively. Legends to each curve are as described for Fig. 5. The theoretical curve is based on the following parameters: τ_1=12.9 ns, τ_2=5.1 ns, α_1=0.111, α_2=0.123, and χ^2=1.19. The amplitudes normalized to unity are α_1=0.47 and α_2=0.53.

residuals, and the autocorrelation function of the residuals indicated that the data cannot be explained in terms of a single-exponential decay law but can be explained in terms of a double-exponential decay law. However, it must be emphasized that other more complex decay laws are not precluded because it is hard to analyze decay data in which two decay times are very close or one of amplitudes is very small.[13,19]

The decay parameters at acid pH differ from those at neutral pH, reflecting the differences in fluorescence spectra (Fig. 3) and quantum yields (Table 1). It should be noted that, at acid pH (pH=4.1—5.9), the value of τ_1 (long lifetime) increases with increasing pH, whereas that of τ_2 (short lifetime) is nearly constant in this pH range (Table 1). The implications of this finding are discussed below.

In contrast, the fluorescence decay of 9AA upon binding to poly d(A-T) at both acid and neutral pH's followed a single-exponential decay law (Table 1) in agreement with our previous finding.[4]

Nanosecond Time-resolved Fluorescence Spectra. Time-resolved fluorescence spectra in the nanosecond scale were measured in order to study any time-dependent interactions between the dye and its environments at the binding sites. The spectra were taken at time intervals ranging from 0 to 70 ns after the peak of exciting pulse. It was found that spectra showed no time dependence and were nearly identical with the steady-state fluorescence spectra. In agreement with this finding, there were no significant changes in the decay parameters when the decay was observed at emission wavelengths ranging from 430 to 510 nm.

Discussion

It is found in the present study that, at both acid and neutral pH's, the fluorescence decay of 9AA bound to poly d(A-T) obeys a single-exponential decay law, while that of 9AA bound to poly rA does not. The fluorescence decay curves of 9AA upon binding to poly rA can be well resolved into two-exponential components corresponding to the long (τ_1) and short (τ_2) lifetimes (Table 1).

Energy transfer between bound dye molecules might be possible as an explanation for the deviation of decay curves from single exponentiality.[6] On the basis of the Förster critical transfer distance (23±1 Å),[20] however, this appears unlikely at high P/D values. It is well

Table 1. Fluorescence decay parameters and quantum yields for 9AA bound to poly rA and poly d(A-T)[a]

System	pH	P/D	τ_1	α_1	τ_2	α_2	χ^2	Φ_F
Poly rA–9AA	4.10	512	20.2	0.63	9.0	0.37	1.11	0.47
	4.90	400	20.9	0.54	8.7	0.46	1.65	0.42
	4.90	502	21.2	0.54	8.4	0.46	1.71	0.42
	5.05	524	21.5	0.54	8.4	0.46	1.72	0.41
	5.30	524	22.4	0.54	8.3	0.46	1.50	0.41
	5.72	533	23.3	0.59	8.2	0.41	1.30	0.41
	5.90	533	23.8	0.58	8.5	0.42	1.31	0.43
	6.90	407	13.2	0.48	5.1	0.52	1.25	0.29
	6.90	503	12.9	0.47	5.1	0.53	1.19	0.28
Poly d(A-T)–9AA	4.90	132	30.3	1.00			1.38	0.71
	6.90	136	31.3	1.00			1.11	0.73

a) The dye concentration was $7.4—12\times10^{-6}$ M. The decay was observed at 455 or 460 nm; the results obtained here are not dependent on the emission wavelength. τ is given in nanoseconds, and the amplitudes (α's) are normalized to unity. Uncertainties in τ's and α's are within ±0.15 ns and ±1%, respectively.

established that the photophysics of excited-state reactions such as excimer or exciplex formation can easily lead to double-exponential decay kinetics.[21] If we focus our attention on such an excited-state system, the double-exponential decay of the poly rA–9AA complexes might be attributed to two distinct emitting species. This is unlikely, however, since the decay parameters do not show a significant dependence on the emission wavelength. The decay parameters obtained at acid and neutral pH's are seen to be quite different (Table 1). A conformational change at the binding sites may play an important role in the decay behavior of the bound 9AA. We suggest here that two lifetimes are related to different microenvironments at the binding sites.

If we assume that bound 9AA molecules are all fluorescent and that the radiative lifetime of the bound dye (τ_r) is independent of the nature of the binding sites, we obtain

$$\Phi_F = \frac{1}{\tau_r}(\alpha_1\tau_1 + \alpha_2\tau_2). \quad (3)$$

We observe that the absorption and fluorescence spectral behavior of the poly rA–9AA complexes at pH=4.9 and pH=6.9, respectively, is very similar to that of the poly d(A-T)–9AA and AMP–9AA complexes (Fig. 3).[4,7] It therefore seems reasonable to assume that the τ_r value of the poly rA–9AA complex at pH=4.9 or pH=6.9 is approximately equal to that of the poly d(A-T)–9AA or AMP–9AA complex. Using the observed lifetimes and quantum yields for the poly d(A-T)–9AA (Table 1) and AMP–9AA complexes (τ=1.45 ns and Φ_F=0.058),[9] the τ_r values for the poly d(A-T)–9AA and AMP–9AA complexes are evaluated to be 42.7 and 25.0 ns, respectively. Substituting the decay parameters given in Table 1 into Eq. 3 yields the following Φ_F values; $\Phi_F \approx 0.36$ at pH=4.9 and $\Phi_F \approx 0.35$ at pH=6.9. Taking into account the experimental error, both observed and calculated Φ_F values seem to be in reasonable agreement. This result and the double-exponential decay imply that there are at least two different microenvironments at the binding sites.

From the absorption and fluorescence spectral behavior at neutral pH and its similarity to that of the AMP-9AA complex, it seems likely that complex II at pH=6.9 results from stacking interactions between 9AA and adenine residues in a way very similar to the partial intercalation which predominantly occurs in single-strand denatured DNA;[22] the positive ring nitrogen of the dye is closely associated with the polynucleotide phosphate group and the dye lies between successive nucleotide bases. In such binding, greater contact between the acridine and adenine rings is expected to cause more effective quenching interactions.[9] Thus we propose that it is the mutual orientation or the extent of overlap between the dye and adenine residue which is responsible for the decay behavior. We attribute the component τ_1 (long lifetime) or τ_2 (short lifetime) to stacking interactions resulting in less or greater contact between the dye and adenine rings. It should be noted, however, that the actual decay law might be more complex since there would be many possibilities for the geometry of the bound dye; unfortunately more detailed decay analysis is beyond the present method.[13,19]

At acid pH, complex II shows the fluorescence spectrum similar to that of the poly d(A-T)–9AA complex and also shows negative flow dichroism. Furthermore, we observed that the decay of fluorescence anisotropy of the poly rA–9AA complex is very similar to that of ethidium bromide[23] or acridine dyes[24] intercalated between adjacent DNA base pairs. From these results it may be reasonably thought that the bound 9AA lies between hydrogen-bonded adenine-adenine pairs in a way similar to the intercalation into double-helical DNA.[25] We postulate that the above binding is responsible for the component τ_1 (long lifetime). Due to hydrogen bonding between the 6-amino group of adenine and the phosphate group and due to the protonation of 1-nitrogen (N_1) of adenine,[10,18,26] some steric hindrance may occur for the intercalation of 9AA. It is likely that 9AA binds rather outside the helix. In such binding sites (less hydrophobic ones), a part of the acridine ring would be exposed to solvent molecules, and hence the possibility of radiationless transition would be increased by collisions with the surrounding solvent. This explains why the τ_1 value is smaller than the lifetime of the poly d(A-T)–9AA complex (Table 1). The fraction of protonated N_1 is gradually decreased with increasing pH.[26] This allows 9AA to bind more interior areas of the helix, that is, more hydrophobic areas. This interpretation is consistent with a red shift of the fluorescence spectra (Fig. 4) and the increase of τ_1 with an increase in pH (Table 1).

Surprisingly there is an additional component τ_2 (short lifetime), in contrast with the poly d(A-T)–9AA complex (Table 1). The results in Table 1 show that the values of τ_2 and α_2 are almost independent of pH (pH=4.1—5.9). This means that the protonation of N_1 may not be so significant for the component τ_2. One possible explanation would assume that this component results from 9AA bound in the vicinity of discontinuities or ends of the helix,[10] in which adenine residues free from hydrogen bonding may cause some quenching of the 9AA fluorescence. However, the proportion of this component (α_2) seems to be rather high (Table 1). It is unlikely that there are a degree of strand discontinuities. Perhaps both sites corresponding to the components τ_1 and τ_2 may possess unequal binding affinity for 9AA owing to a conformational difference.

In conclusion, the results obtained here indicate that the decay behavior of 9AA bound to poly rA is rather complex. It is apparent that the 6-amino group of adenine free from hydrogen bonding plays a role in the quenching of the 9AA fluorescence. At present, however, there is no convincing evidence for the quenching mechanism. Indeed the study of transient intermediates by laser photolysis may answer the problem.

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