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FLUORESCENCE DECAY STUDIES OF POLY(RIBOADENYLIC ACID) CONTAINING 1-N^6-ETHENOADENOSINE

Yukio KUBOTA [a], Akira SANJOH [a], Yasuo FUJISAKI [a] and Robert F. STEINER [b]

[a] *Department of Chemistry, Faculty of Science, Yamaguchi University, Yamaguchi 753, Japan, and* [b] *Department of Chemistry, University of Maryland Baltimore County, Catonsville, MD 21228, U.S.A.*

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The fluorescence properties of the 1-N^6-etheno derivatives of poly(riboadenylic acid) (poly(rA,ϵrA)) have been examined. The fluorescence quantum yield of poly(rA,ϵrA) decreases with an increase in the degree of the ϵA substitution and is much smaller than that for ϵAMP even for low degrees of ϵA substitution. The nearest-neighbor interactions such as ϵ-adenine-adenine and ϵ-adenine-ϵ-adenine may be responsible for this behavior. It is found that the fluorescence decay kinetics obeys a three-exponential decay law for poly(rA,ϵrA), suggesting that there exist at least three different stacked conformational states.

1. Introduction

Because of the stability, high fluorescence yields and relatively long fluorescence lifetimes of 1-N^6-ethenoadenosine (ϵA) and its nucleotides [1–3], they may serve as excellent probes for studying the conformation of nucleic acids. It is well established that the conformation of poly(riboadenylic 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 [4]. Therefore, it is of great interest to study the fluorescence behavior of poly(rA) containing ϵA groups (poly(rA,ϵrA)) in connection with its conformational change. Steiner and coworkers [5] first investigated poly(rA,ϵrA) using fluorescence polarization techniques and showed that the intensity and polarization of fluorescence respond to helix–coil transitions.

In the preceding paper [6], we have reported that the fluorescence decay kinetics of dinucleoside monophosphates containing ϵA, which depends on both the kind of bases and the base sequence, obeys a three-exponential decay law; it has been proposed that there are three or more conformational states in ϵ-dinucleoside monophosphates. To elucidate further the base-stacking interactions in polynucleotides, we have reexamined the fluorescence of poly(rA,ϵrA) by steady-state and transient decay measurements. It is found in the present study that the fluorescence decay kinetics of poly(rA,ϵrA) also follows a three-exponential decay law at both acid (pH 5) and neutral pH (pH 7), suggesting that there exist at least three different stacked conformational states in poly(rA,ϵrA). Preliminary results have been recently reported [7].

2. Materials and methods

Poly(rA) was purchased from Seikagaku Kogyo and was used without further purification ($s^0_{20,w}$ = 10.7). ϵA groups were introduced into poly(rA) according to the procedure described by Steiner et al. [5]. The degree of the ϵA substitution was controlled by varying the reaction time. After completion of the reaction, the pH of the solution was adjusted to about 7, and then the solution was dialyzed against the desired buffer solution at 4°C. Approximate values of εAMP contents of the derivatives were determined from the absorption spectrum according to the method of Steiner et al. [5]. Glass-redistilled water and analytical grade reagents were used for the preparation of all solutions.

Absorption and steady-state fluorescence spectra were measured with a Shimadzu UV-200S spectrophotometer and a Hitachi MPF-2A spectrophotofluorometer, respectively. Observed fluorescence spectra were corrected for the unequal quantum response of the detector system which consists of lenses, a monochromator and an R106-UH photomultiplier tube (Hamamatsu TV). Fluorescence quantum yields of the εA derivatives were determined by comparing the area under the fluorescence spectrum with the corresponding area of εAMP and by taking 0.52 for the quantum yield of εAMP [8]. Transient fluorescence decay measurements and the analysis of data were described in the preceding paper [6].

All measurements were carried out using two solvent systems: 0.005 M sodium phosphate, 0.1 M NaCl (pH 7.0) and 0.01 M sodium acetate, 0.1 M NaCl (pH 5.0). The concentrations of poly(rA,εrA) were in the range 5.0×10^{-5}–10^{-4} M in monomer units.

3. Results

3.1. Absorption and fluorescence spectra

Fig. 1 shows absorption spectra for poly(rA) and poly(rA,εrA) at pH 7. The absorption spectra of poly(rA,εrA) resemble qualitatively the expected summation of AMP and εAMP contributions.

Fig. 2 summarizes the temperature dependence of absorbance at around 260 nm at pH 7. Poly(rA,εrA) derivatives show a decrease in hypochroism with increasing temperature. This behavior is similar to the thermally induced spectral change observed for the single-stranded helical form of poly(rA) itself [4]. In parallel to changes observed for poly(rA), the increase in absorbance is gradual and shows no qualitative indication of any cooperative sharpening. It should be noted that the hyperchromicity decreases with increasing degree of εA substitution (fig. 2). In contrast to the results at pH 7, the increase in absorbance at pH 5 indicates a cooperative sharpening (fig. 3). This behavior responds to helix–coil transitions [4]. As seen in fig. 3, both the transition tempera-

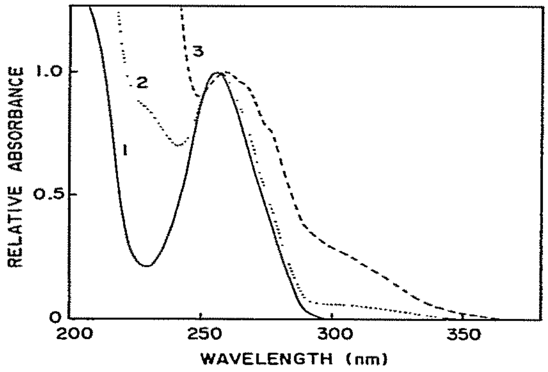

Fig. 1. Absorption spectra of poly(rA) and poly(rA,εrA) in 0.005 M sodium phosphate, 0.1 M NaCl, pH 7.0, 25°C: (1) poly(rA), (2) poly(rA$_{0.65}$,εrA$_{0.35}$), (3) poly(rA$_{0.20}$,εrA$_{0.80}$). The maximum of each spectrum (around 260 nm) is normalized to unity.

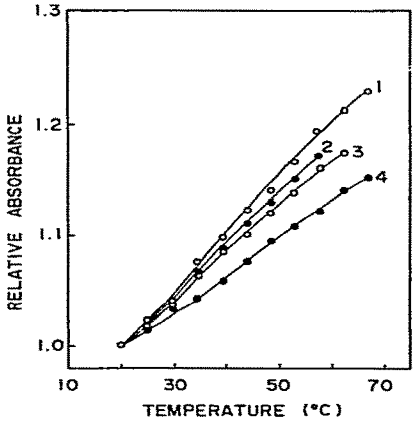

Fig. 2. Temperature dependence of absorbance for poly(rA) and poly(rA,εrA) in 0.005 M sodium phosphate, 0.1 M NaCl, pH 7.0: (1) poly(rA) (λ = 256 nm), (2) poly(rA$_{0.90}$,εrA$_{0.10}$) (λ = 256 nm), (3) poly(rA$_{0.65}$,εrA$_{0.35}$) (λ = 257 nm), (4) poly(rA$_{0.20}$,εrA$_{0.80}$) (λ = 258 nm).

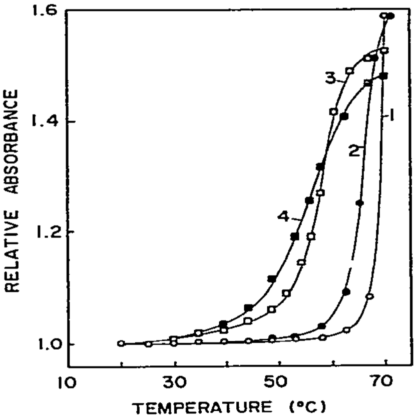

Fig. 3. Temperature dependence of absorbance for poly(rA) and poly(rA,εrA) in 0.01 M sodium acetate, 0.1 M NaCl, pH 5.0: (1) poly(rA) ($\lambda = 251$ nm), (2) poly($rA_{0.97}$,$\epsilon rA_{0.03}$) ($\lambda = 251$ nm), (3) poly($rA_{0.90}$,$\epsilon rA_{0.10}$) ($\lambda = 252$ nm), (4) poly($rA_{0.65}$,$\epsilon rA_{0.35}$) ($\lambda = 252$ nm).

ture and the hyperchromic change decrease with an increase in degree of εA substitution. In view of these facts, it is concluded that the introduction of εA may destabilize the ordered structure of poly(rA).

As depicted in fig. 4, the fluorescence spectrum of poly(rA,εrA) at both pH 5 and pH 7 shows a wavelength dependence similar to that of εAMP except that its maximum wavelength for low degrees of εA substitution (3–35%) slightly shifts to shorter wavelength. This means that εA groups are embedded into the hydrophobic region. The magnitude of the spectral shift was greater at pH 5 than at pH 7, suggesting that the microenvironment of εA is more hydrophobic at pH 5. On the other hand, the fluorescence spectrum for a high degree of εA substitution (80%) was very similar to that of εAMP itself, indicating that εA groups are considerably exposed to the surrounding solvent.

3.2. Fluorescence quantum yields

The fluorescence quantum yields (Φ_F) for poly(rA,εrA) markedly decreased with increasing degree of εA substitution (fig. 5). The Φ_F values for poly(rA,εrA) even for low degrees of substitution are much smaller than that for εAMP (fig. 5, and tables 1 and 2). In the preceding paper [6], it was also found that the Φ_F value for εApεA is much smaller than that for εAMP. In view of the fact that adenine residues can quench the fluorescence of εAMP [8], the nearest-neighbor interactions between ε-adenine and adenine or ε-adenine and ε-adenine may be responsible for fluorescence

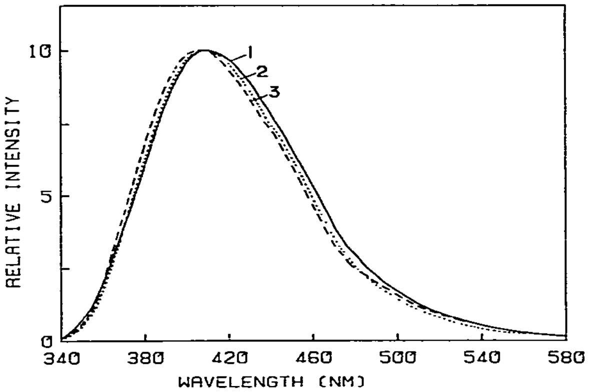

Fig. 4. Normalized fluorescence quantum spectra of εAMP and poly(rA,εrA) at 25°C: (1) εAMP (pH 5.0, pH 7.0), (2) poly($rA_{0.90}$,$\epsilon rA_{0.10}$) (pH 7.0), (3) poly($rA_{0.90}$,$\epsilon rA_{0.10}$) (pH 5.0).

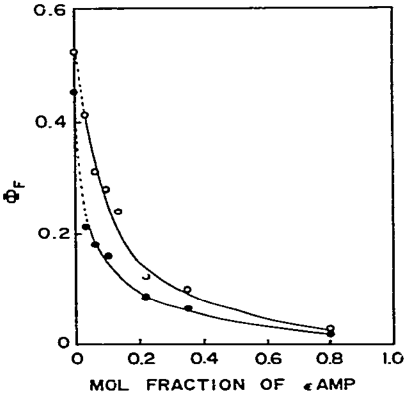

Fig. 5. A plot of fluorescence quantum yields (Φ_F) for poly(rA,εrA) against the degree of εA substitution: (○) 0.005 M sodium phosphate, 0.1 M NaCl, pH 7.0, 25°C; (●) 0.01 M sodium acetate, 0.1 M NaCl, pH 5.0, 25°C. The Φ_F values where the mole fraction of εAMP is zero are those for εAMP itself.

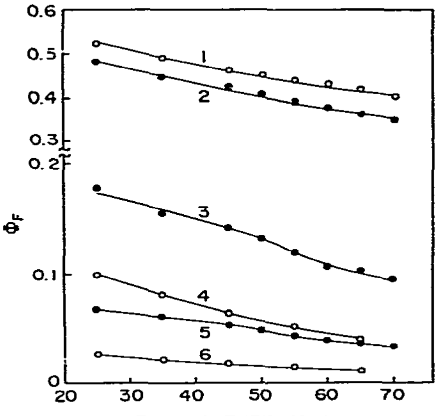

Fig. 6. Temperature dependence of fluorescence quantum yields (Φ_F) for εAMP and poly(rA,εrA): (1) εAMP (pH 7.0), (2) εAMP (pH 5.0), (3) poly($rA_{0.90}$,$\epsilon rA_{0.10}$) (pH 5.0), (4) poly($rA_{0.65}$,$\epsilon rA_{0.35}$) (pH 7.0), (5) poly($rA_{0.65}$,$\epsilon rA_{0.35}$) (pH 5.0), (6) poly($rA_{0.20}$,$\epsilon rA_{0.80}$) (pH 7.0).

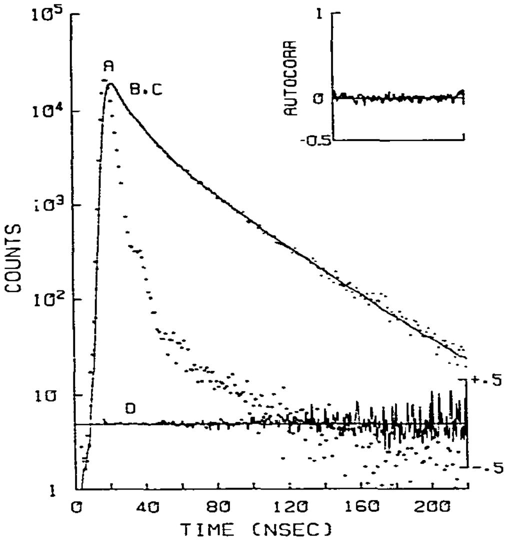

Fig. 7. Three-component analysis of the fluorescence decay of poly($rA_{0.65}$,$\epsilon rA_{0.35}$) in 0.005 M sodium phosphate, 0.1 M NaCl, pH 7.0, 25°C. Curve A is the apparatus response function. Curve B is the observed decay curve. The smooth curve C shows the theoretical decay curve. Curve D is the weighted residuals. The inset is the autocorrelation function of the residuals. Parameters obtained: $\tau_1 = 32.8$ ns, $\tau_2 = 11.6$ ns, $\tau_3 = 1.8$ ns, $\alpha_1 = 0.047$, $\alpha_2 = 0.093$, $\alpha_3 = 0.213$ and $\chi^2 = 1.29$. The amplitudes normalized to unity are $\alpha_1 = 0.13$, $\alpha_2 = 0.27$ and $\alpha_3 = 0.60$.

quenching. A marked reduction in Φ_F observed for highly substituted etheno derivatives poly($rA_{0.20}$,$\epsilon rA_{0.80}$)) or εApεA suggests that homologous interactions of ε-adenine are more efficient in producing quenching than interactions between ε-adenine and adenine.

In analogy with the change in absorbance, the fluorescence quantum yield was dependent upon temperature (fig. 6). εAMP itself showed no temperature dependence of absorbance, while it was found to show an important degree of quenching with increasing temperature. This may arise from the increased rates of the radiationless deactivation processes which compete with direct emission. The Φ_F value for poly(rA,εrA) at pH 7 is gradually decreased with a rise in temperature, whereas the Φ_F value at pH 5 shows a cooperative abrupt decrease at the transition temperature as well as the absorption change (cf. fig. 6 and figs. 2 and 3). In the present case, however, it should be noted that the effects of thermal destruction of the base

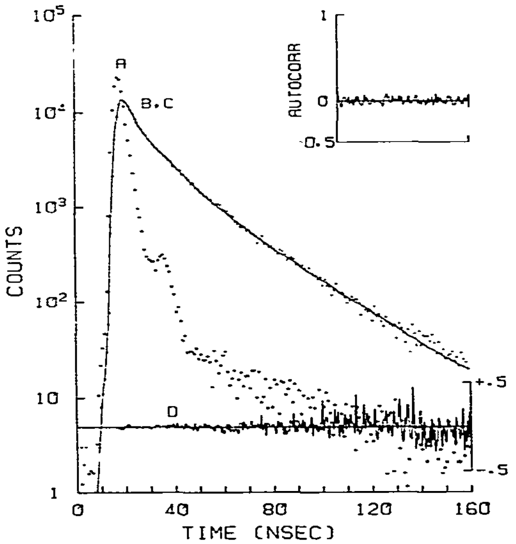

Fig. 8. Three-component analysis of the fluorescence decay of poly($rA_{0.65}$,$\epsilon rA_{0.35}$) in 0.01 M sodium acetate, 0.1 M NaCl, pH 5.0, 25°C. Legends to each curve are as described for fig. 7. The theoretical decay curve is based on the following parameters: $\tau_1 = 25.2$ ns, $\tau_2 = 10.5$ ns, $\tau_3 = 1.9$ ns, $\alpha_1 = 0.032$, $\alpha_2 = 0.077$, $\alpha_3 = 0.203$ and $\chi^2 = 1.38$. The amplitudes normalized to unity are $\alpha_1 = 0.10$, $\alpha_2 = 0.25$ and $\alpha_3 0.65$.

Table 1

Fluorescence decay parameters and quantum yields for poly(rA,ϵrA) derivatives in 0.005 M sodium phosphate, 0.1 M NaCl, pH 7.0

The excitation and emission wavelengths were 337 and 410 nm, respectively; the results obtained here are not dependent on the emission wavelength (cf. table 3). χ^2 values ranged from 1.2 to 1.9. The amplitudes (α) are normalized to unity.

Preparation	Temperature (°C)	τ_1 (ns)	α_1	τ_2 (ns)	α_2	τ_3 (ns)	α_3	Φ_F
ϵAMP	25	25.0	1.00					0.52
	45	22.6	1.00					0.46
	65	20.3	1.00					0.41
Poly(rA$_{0.97}$,ϵrA$_{0.03}$)	25	36.3	0.65	16.1	0.15	2.4	0.20	0.41
	45	29.9	0.42	12.8	0.24	2.0	0.34	0.27
	65	21.0	0.33	9.2	0.25	1.8	0.42	0.17
Poly(rA$_{0.94}$,ϵrA$_{0.06}$)	25	35.7	0.47	14.6	0.20	2.2	0.33	0.31
Poly(rA$_{0.90}$,ϵrA$_{0.10}$)	25	35.4	0.44	13.8	0.24	2.2	0.32	0.28
Poly(rA$_{0.87}$,ϵrA$_{0.13}$)	25	34.4	0.38	12.4	0.24	1.8	0.38	0.22
Poly(rA$_{0.78}$,ϵrA$_{0.22}$)	25	33.7	0.21	12.1	0.32	2.0	0.47	0.12
Poly(rA$_{0.65}$,ϵrA$_{0.35}$)	25	32.8	0.13	11.6	0.27	1.8	0.60	0.099
	45	27.3	0.08	8.5	0.28	1.4	0.64	0.064
	65	19.1	0.06	6.1	0.21	1.2	0.73	0.041
Poly(rA$_{0.20}$,ϵrA$_{0.80}$)	25	26.9	0.05	8.6	0.20	1.4	0.75	0.026
	45	21.0	0.02	6.5	0.20	1.3	0.78	0.018
	65	21.9	0.003	5.1	0.16	1.0	0.84	0.011

Table 2

Fluorescence decay parameters and quantum yields for poly(rA,ϵrA) derivatives in 0.01 M sodium acetate, 0.1 M NaCl, pH 5.0

The excitation and emission wavelengths were 337 and 410 nm, respectively; the results obtained here are not dependent on the emission wavelength (cf. table 3). χ^2 values ranged from 1.1 to 1.9. The amplitudes (α) are normalized to unity.

Preparation	Temperature (°C)	τ_1 (ns)	α_1	τ_2 (ns)	α_2	τ_3 (ns)	α_3	Φ_F
ϵAMP	25	20.7	1.00					0.45
	45	18.1	1.00					0.42
	65	16.5	1.00					0.36
Poly(rA$_{0.97}$,ϵrA$_{0.03}$)	25	27.5	0.24	11.2	0.27	2.3	0.49	0.21
	45	24.6	0.17	10.5	0.29	1.8	0.54	0.18
	65	19.8	0.15	9.2	0.33	1.9	0.52	0.13
Poly(rA$_{0.94}$,ϵrA$_{0.06}$)	25	28.3	0.23	12.0	0.33	2.8	0.44	0.18
Poly(rA$_{0.90}$,ϵrA$_{0.10}$)	25	28.2	0.24	12.2	0.29	2.5	0.47	0.16
	45	25.3	0.13	11.0	0.30	2.1	0.57	0.14
	65	23.6	0.06	10.3	0.35	2.2	0.59	0.10
Poly(rA$_{0.78}$,ϵrA$_{0.22}$)	25	26.6	0.13	11.3	0.27	2.1	0.60	0.084
Poly(rA$_{0.65}$,ϵrA$_{0.35}$)	25	25.2	0.10	10.5	0.25	1.9	0.65	0.067
	45	22.6	0.06	8.9	0.25	1.7	0.69	0.052
	65	19.8	0.03	6.4	0.21	1.1	0.76	0.037
Poly(rA$_{0.20}$,ϵrA$_{0.80}$)	25	20.5	0.04	7.4	0.14	1.1	0.82	0.024

stacking are superimposed upon those of enhanced thermal quenching of the excited state.

3.3. *Fluorescence decay curves*

It was found that the fluorescence decay kinetics of εAMP followed a single-exponential decay law at both pH 5 and pH 7, while the decay behavior for poly(rA,εrA), which depends upon temperature and pH, was rather complex. Typical decay curves obtained with poly($rA_{0.65}$,$\epsilon rA_{0.35}$) are shown in figs. 7 and 8. All decay curves clearly indicated the deviation from the single exponentiality. The reduced χ^2, the weighed residuals and the autocorrelation function of the residuals indicated that the decay data could not be explained in terms of a double-exponential decay law but could well be resolved into three-exponential components corresponding to the long (τ_1), medium (τ_2) and short (τ_3) lifetimes. Typical sets of decay parameters together with Φ_F are summarized in tables 1 and 2. The χ^2 values obtained were in the range 1.1–1.9.

Time-resolved fluorescence spectra on the nanosecond scale were measured in order to study any time-dependent interactions. The spectra were taken at time intervals ranging from 0 to 150 ns after the peak of the exciting pulse. It was found that spectra showed no time dependence and were nearly identically with the steady-state fluorescence spectra. In harmony with this finding, the decay parameters did not show any significant dependence on the emission wavelength (table 3).

4. Discussion

It is found in the present study that the fluorescence decay kinetics of poly(rA,εrA) follows a three-exponential decay law. As is the case with dinucleoside monophosphates containing εA [6], two explanations for this behavior are possible: one is a reversible excited-state reaction and the other is the existence of three different conformational states.

Considerable stacking interactions between two bases are necessary to produce efficient fluorescence quenching. Relaxation kinetic studies indicate that the stacking of bases in poly(rA) takes 10^{-6}–10^{-7} s, and double-stranded helix formation in oligomers ranges from 10^{-3} to 10^{-1} s [9–11]. Taking into account the restrictions of sugar puckering and backbone rotations in polynucleotide chains, it seems unlikely that a reversible reaction like unstacked $\rightleftarrows$ stacked occurs during the lifetime of the excited state. In fact, the finding that nanosecond time-resolved fluorescence spectra of poly(rA,εrA) are independent of time (cf. table 3) suggests that the environment of the εA group is not substantially altered during its lifetime.

We propose here another explanation which would assumed that the decay behavior has its

Table 3

Decay parameters obtained for poly($rA_{0.65}$,$\epsilon rA_{0.35}$) at 25°C

The excitation wavelength was 337 nm. χ^2 values ranged from 1.2 to 1.8. The amplitudes (α) are normalized to unity.

Solvent	Emission wavelength (nm)	τ_1 (ns)	α_1	τ_2 (ns)	α_2	τ_3 (ns)	α_3
pH 7 [a]	410	32.8	0.13	11.6	0.27	1.8	0.60
	440	33.1	0.13	11.9	0.26	1.6	0.61
	470	31.9	0.14	11.5	0.27	1.5	0.59
	500	32.2	0.13	11.4	0.27	1.5	0.60
pH 5 [b]	410	25.2	0.10	10.5	0.25	1.9	0.65
	440	25.7	0.10	10.3	0.26	1.9	0.64
	470	25.9	0.09	10.7	0.25	2.0	0.66

[a] 0.005 M sodium phosphate, 0.1 M NaCl.

[b] 0.01 M sodium acetate, 0.1 M NaCl.

origin in different conformational states, depending upon the stacking interaction between ϵ-adenine and its neighbors (ϵ-adenine or adenine). The present decay data are not compatible with the two-state model proposed for a conformational change in dinucleoside phosphates [12,13]. The three-exponential decay kinetics for poly(rA,ϵrA) suggests that the fluorescent states of ϵA groups consist of at least three different classes of conformations. Temperature-jump relaxation studies on single-stranded poly(rA) reveal that there are two relaxation times for its conformational change, demonstrating the existence of more than two independent conformational states [9,10]. Laser temperature-jump techniques also suggest that the relaxation kinetics of poly(rA), which depends upon ionic strength, is complicated and that there are more than two conformational states at high ionic strengths [14].

Now, the structure of these conformations disclosed by fluorescence decay measurements must be sought. It can be clearly seen in tables 1 and 2 that the amplitude α_1 decreases with increasing degree of ϵA substitution, whereas α_3 increases. It is proposed, therefore, that the long lifetime (τ_1) is ascribed to unstacked ϵA groups, while the short lifetime (τ_3) is a result of strong stacking interactions between ϵ-adenine and its neighbors (ϵ-adenine or adenine). The medium lifetime (τ_2) is presumably due to weak stacking interactions. The long lifetime (τ_1) for low degrees of ϵA substitution at 25°C is 34–36 ns at pH 7 and 27–28 ns at pH 5, being much larger than the corresponding lifetimes for ϵAMP. This increase in lifetime may be due, in part, to the immersion of ϵA groups in the hydrophobic region where they are protected against quenching by collisions with the surrounding solvent. The τ_1 value decreases with an increase in degree of ϵA substitution and is very close to that for ϵAMP itself for the highest degree of substitution (poly($rA_{0.20}$,$\epsilon rA_{0.80}$)) (tables 1 and 2). The τ_1 value at 65°C is also nearly equal to that of ϵAMP. It is expected that the ordered structure is considerably ruptured for a high degree of substitution or at high temperature. Under these conditions, ϵA groups are exposed to the surrounding solvent and hence, the lifetime becomes closer to that of ϵAMP. As seen in tables 1 and 2, the amplitude α_3 at pH 5 is larger than at pH 7. This demonstrates that there exist more efficient stacking interactions at pH 5 due to the ordered double-helical structure.

The three-exponential decay law is also found for ϵ-dinucleoside phosphates [6]. However, the decay behavior of poly(rA,ϵrA) differs from that of ϵ-dinucleoside phosphates. Especially, for low degrees of ϵA substitution, the amplitude α_1 and the lifetime τ_1 are considerable larger than those of ϵ-dinucleoside phosphates (tables 1 and 2; and table 1 in ref. 6). Because of the limitations of sugar puckering and backbone rotations in polynucleotide chains, it seems likely that although ϵA groups in poly(rA,ϵrA) are immersed in the hydrophobic region, all of them cannot orient with their neighbors in favorable geometries to produce efficient quenching.

In conclusion, the present decay data indicate that poly(rA,ϵrA) can be described as consisting of at least three different conformational states. It may be that fluorescence decay measurements can be used to reveal the multistate behavior in other polynucleotide systems

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