Biophysical Chemistry 18 (1983) 225–232
Elsevier

BPC 00803

FLUORESCENCE DECAY STUDIES OF MODIFIED DINUCLEOSIDE MONOPHOSPHATES CONTAINING 1-N^6-ETHENOADENOSINE

Yukio KUBOTA [a], Yuko MOTODA [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.*

Received 21st March 1983
Accepted 7th June 1983

Key words: Fluorescent dinucleotide; Ethenoadenosine; Nanosecond pulse fluorometry; Fluorescence lifetime

Five dinucleoside monophosphates containing 1-N^6-ethenoadenosine (ϵA) have been studied using fluorescence measurements. The fluorescence spectra of these dinucleoside monophosphates are almost the same as the fluorescence spectrum of ϵAMP. Fluorescence quantum yields of these dimers are greatly reduced compared to that of ϵAMP. Intramolecular base-base interactions may be responsible for fluorescence quenching. It is found that the fluorescence decay kinetics does not obey a simple decay law but that the decay data can be well described as a sum of three exponentials. This implies that these dimers cannot be characterized as a two-state system, but can be described as systems consisting of three or more conformational states. Sequence effects upon the fluorescence behavior are observed. The fluorescence quenching and decay parameters of GpϵA and UpϵA indicate a higher degree of base-base interaction than in their ϵApG and ϵApU counterparts.

1. Introduction

Since dinucleoside monophosphates are the simplest repeating units of polynucleotides, they are taken as appropriate models for studying the nearest-neighbor interactions between nucleic acid bases and their stacking properties. One important model in interpreting the temperature dependence of optical properties of dinucleoside phosphates is a two-state model [1,2]. Stacked (ordered) and unstacked (disordered) configurations of the dimers are proposed to exist in equilibrium with each other. However, NMR [3,4] and spectroscopic data [5,6] have suggested that the two-state model for the stacking equilibrium is too simplistic. Theoretical calculations also indicate that there exist several possible conformational states for dinucleoside phosphates [7,8].

Of spectroscopic techniques, fluorescence techniques could be potentially useful in obtaining detailed information on the base-stacking interactions. Fluorescent modification of the base residues in nucleic acids is of much importance for structural and functional investigation of RNA and DNA, since naturally occurring nucleic acid bases exhibit almost no fluorescence at normal temperature. Barrio et al. [9] found that chloroacetaldehyde reacts with adenosine and cytidine derivatives to form highly fluorescent products. In particular, 1-N^6-ethenoadenosine (ϵA) and its nucleotides have the advantage of long fluorescence lifetimes and high quantum yields [9,10]. Therefore, it is expected that the chloroacetaldehyde-modified dimers can serve as an excellent probe to help determine the conformation of dinucleoside phosphates. Tolman et al. [11] have studied static and dynamic fluorescence quenching due to base stacking for various ϵ-dinucleoside phosphates using phase and modulation techniques. On the other hand, Baker et al. [5] have shown that fluorescence decay curves of ϵApϵA, which depend upon temperature and solvent, deviate from single exponentiality. They proposed a dynamic model in which relative fluorophore motion leads to deexcitation via intramolecular collision. Based on fluorescence-detected circular di-

chroism (FDCD) measurements, Reich and Tinoco [6] have suggested that ϵApU and ϵApϵC can be characterized as two-state systems but that ϵApϵA, a more complicated system, can be described as consisting of at least three states.

In this paper we have studied the fluorescence of ϵ-dinucleoside phosphates by steady-state and transient decay measurements. This technique may provide valuable informaton on the microenvironments of a fluorophore and the molecular dynamics on the nanosecond time scale. Five ϵ-dinucleoside phosphates, ϵApϵA, ϵApG, GpϵA, ϵApU and UpϵA, are examined. It is found that all fluorescence decay curves are complex and can be well described as a sum of three exponentials, and possible interpretations are discussed.

2. Materials and methods

ApA, ApG, GpA, ApU, UpA and 5'-AMP were purchased from Sigma Chemical Co. The ϵA derivatives were synthesized according to the method of Tolman et al. [11] and purified using thin-layer chromatography. All derivatives were pure as judged from thin-layer chromatography. Glass-redistilled water and analytical grade reagents were used for the preparation of all aqueous solutions. Glycerol, ethylene glycol and ethanol were of spectrophotometric grade.

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 ϵ-dinucleoside phosphates 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 [12].

Transient fluorescence decay curves were measured with an Ortec time-resolved emission spectrophotometer [13]. The 337- or 315-nm bands of an air-filled (0.5 atm) flashlamp were isolated using appropriate interference filters (Vacuum Optics Corp., Japan) and used for excitation. The emission was observed by an RCA 8850 photomultiplier tube through a grating monochromator (Applied Photophysics Ltd.), the half-bandwidth being 5–10 nm according to the fluorescence intensity of the sample. The observed fluorescence decay $i(t)$ is represented by the convolution integral:

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

where $g(t)$ is the apparatus response function and $I(t)$ the fluorescence decay which would have been obtained with the δ-pulse excitation.

The apparatus response function $g(t)$ was determined from the fluorescence decay curves of 1,4-bis(5-phenyloxazol-2-yl)benzene (POPOP) in deaerated cyclohexane which has a single exponential decay ($\tau = 1.10$ ns at 25°C) [14]. Deconvolution of eq. 1 was made with the aid of the method of nonlinear least squares [15] and/or the method of moments [16,17] by assuming that $I(t)$ is a sum of exponential functions:

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

where α_i and τ_i are the amplitude and lifetime, respectively, of the ith component. Goodness of the fit between observed and theoretical decay curves was judged by inspection of the reduced χ^2, the weighed residuals and the autocorrelation function of the residuals [15,18,19]. Data analysis was accomplished with a PDP 11/04 minicomputer (Digital Equipment Corp.) interfaced with an Ortec 6240B multichannel analyzer.

All instruments were equipped with a thermostatically controlled cell compartment; the temperature was controlled by circulating water and regulated to ±0.1°C. All measurements were carried out in 0.05 M phosphate buffer (pH 7.0) unless otherwise stated. The concentrations of ϵ-dinucleoside monophosphates were in the range 5.0×10^{-5}–1.4×10^{-4} M.

3. Results and discussion

As shown in fig. 1, the fluorescence spectrum for each ϵ-dinucleoside monophosphate was al-

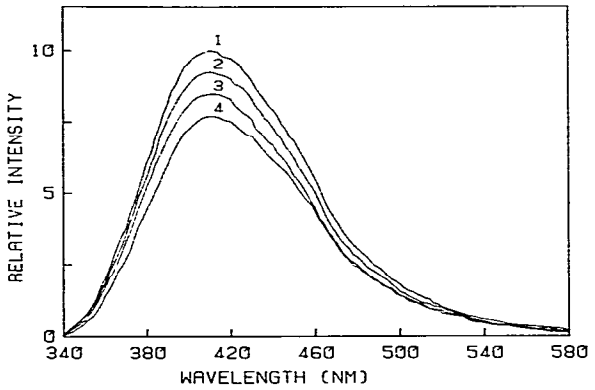


Fig. 1. Fluorescence quantum spectra of ϵAMP and ϵ-dinucleoside monophosphates in 0.05 M phosphate buffer (pH 7.0) at 25°C: (1) ϵAMP, (2) ϵApϵA, (3) ϵApG, (4) ϵApU. The excitation wavelength was 320 nm. The units of the ordinate are arbitrary; the maximum of each spectrum is properly reduced to avoid overlapping.

most the same as that of ϵAMP. However, the fluorescence quantum yields (Φ_F) for ϵ-dinucleoside monophosphates are greatly reduced compared to that of ϵAMP (table 1). In view of the fact that mononucleotides such as GMP quench the fluorescence of ϵAMP [12], it appears that there are strong intramolecular quenching interactions in ϵ-dinucleoside monophosphates.

The Φ_F values for ϵAMP and ϵ-dinucleoside monophosphates are dependent on temperature, showing a considerable quenching with increasing temperature (fig. 2). If only one temperature-dependent deactivation process is present, we have [20]

$$\Phi_F^{-1} - 1 = Ae^{-E/RT} \tag{3}$$

where E is the activation energy, R the gas constant and T the absolute temperature. In the present case, the effects of thermal disruption of base stacking may be superimposed on those of enhanced thermal quenching of the excited state. Thus, computation of E from a linear plot of $\ln(\Phi_F^{-1} - 1)$ vs. $1/T$ may result in an apparent value of E. The apparent value obtained on this basis ranges from 7.7 to 11.9 kJ mol^{-1}, in close agreement with the value (9.9 kJ mol^{-1}) for ϵAMP.

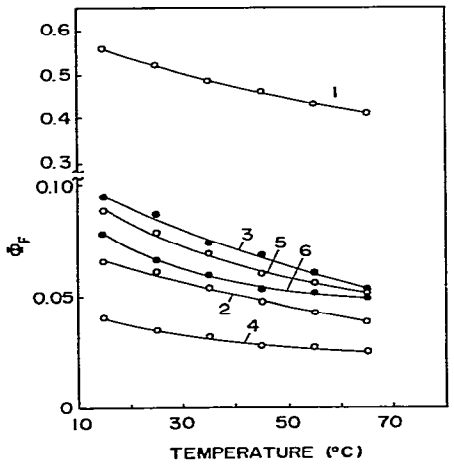


Fig. 2. Fluorescence quantum yields (Φ_F) of ϵAMP and ϵ-dinucleoside monophosphates as a function of temperature: (1) ϵAMP, (2) ϵApϵA, (3) ϵApG, (4) GpϵA, (5) ϵApU, (6) UpϵA.

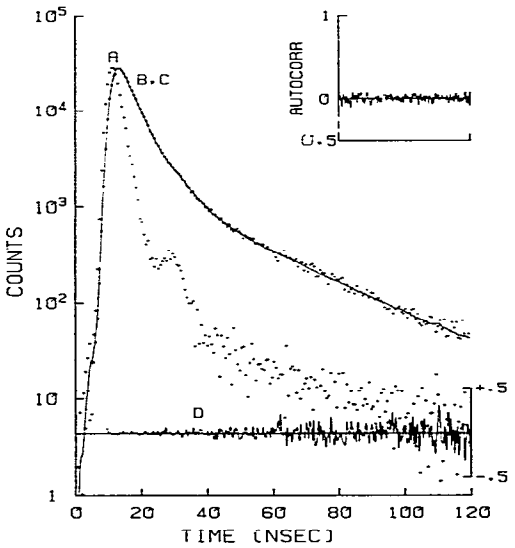


Fig. 3. Three-component analysis of the fluorescence decay of ϵApϵA (6.6×10^{-5} M) in 0.05 M phosphate buffer (pH 7.0) at 25°C. The excitation and emission wavelengths were 337 and 410 nm, respectively. Curve A is the apparatus response function. Curve B is the observed decay curve. The smooth curve C shows the best theoretical decay curve. Curve D is the weighed residuals. The inset is the autocorrelation function of the residuals. Parameters obtained are: $\tau_1 = 25.1$ ns, $\tau_2 = 5.0$ ns, $\tau_3 = 1.6$ ns, $\alpha_1 = 0.014$, $\alpha_2 = 0.230$, $\alpha_3 = 0.314$ and $\chi^2 = 1.68$.

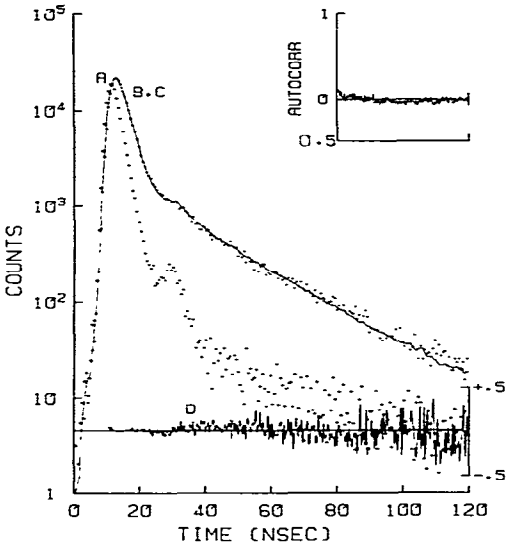


Fig. 4. Three-component analysis of the fluorescence decay of ϵApϵA (6.6×10^{-5} M) in 0.05 M phosphate buffer (pH 7.0) at 65°C. The excitation and emission wavelengths were 337 and 410 nm, respectively. Legends to each curve are as described for fig. 3. The theoretical decay curve is based on the following parameters: $\tau_1 = 20.1$ ns, $\tau_2 = 4.4$ ns, $\tau_3 = 1.7$ ns, $\alpha_1 = 0.020$, $\alpha_2 = 0.003$, $\alpha_3 = 0.805$ and $\chi^2 = 1.74$.

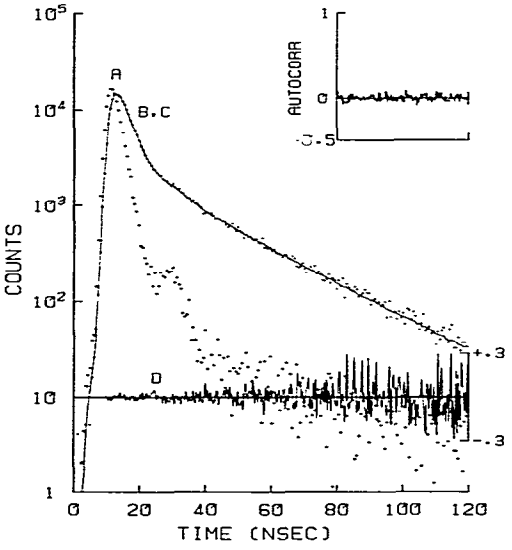


Fig. 5. Three-component analysis of the fluorescence decay of GpϵA (6.6×10^{-5} M) in 0.05 M phosphate buffer (pH 7.0) at 25°C. The excitation and emission wavelengths were 337 and 410 nm, respectively. Legends to each curve are as described for fig. 3. The theoretical decay curve is based on the following parameters: $\tau_1 = 22.8$ ns, $\tau_2 = 4.1$ ns, $\tau_3 = 1.3$ ns, $\alpha_1 = 0.030$, $\alpha_2 = 0.139$, $\alpha_3 = 0.456$ and $\chi^2 = 1.19$.

It was found that the fluorescence decay kinetics of ϵAMP followed a single-exponential decay law but that of ϵ-dinucleoside phosphates was complex. Typical decay curves obtained with ϵApϵA and GpϵA are shown in figs. 3–5. All decay data for ϵ-dinucleoside phosphates obeyed neither a single- nor double-exponential decay law. As illustrated in figs. 3–5, the reduced χ^2, the weighed residuals and the autocorrelation function of the residuals indicated that the data could be well explained in terms of a three-exponential decay law. Decay parameters thus obtained are summarized in tables 1 and 2. The χ^2 values were in the range 1.0–1.8. As seen in table 2, the decay parameters obtained here were not significantly dependent on the emission wavelength. It must be emphasized, however, that other more complex decay laws are not precluded because it is hard to analyze decay data in which two lifetimes are very close or one of the amplitudes is very small [18,21].

Fluorescence decay curves for ϵApϵA were very similar to those observed by Baker et al. [5]. In order to interpret the nonexponentiality of the decay, they proposed that the unstacking of ϵApϵA is a continuous process, which results from an intramolecular diffusional motion between two bases. However, this model does not predict such an initial fast decay that is displayed in figs. 3–5, as pointed out by Baker et al. [5]. It is also expected that there is a dramatic effect of solvent viscosity on fluorescence decay curves [5]. On the contrary, we observed very similar fluorescence decay curves in mixed solvents of buffer/ethanol, buffer/ethylene glycol and buffer/glycerol. These data could be well resolved into three-exponential components (table 1). Furthermore, Reich and Tinoco [6] showed that this continuous stacking model cannot explain the FDCD data for ϵApϵA.

Our present criteria (χ^2, the weighed residuals and the autocorrelation function of the residuals)

Table 1

Fluorescence decay parameters and quantum yields for ϵAMP and dinucleoside monophosphates containing ϵA

The excitation and emission wavelengths were 337 and 410 nm, respectively; the resuits obtained here are not dependent on the emission wavelength (cf. table 2). χ^2 values ranged from 1.0 to 1.8. The amplitudes (α) are normalized to unity. $\langle\Phi\rangle$ was calculated using eq. 4. Solvents: (a) 0.05 M phosphate buffer (pH 7.0); (b) 0.1 M acetate buffer (pH 5.0); (c) 0.05 M phosphate buffer/ethanol (1 : 1, v/v); (d) 0.05 M phosphate buffer/ethylene glycol (1 : 1, v/v); (e) 0.05 M phosphate buffer/glycerol (1 : 1, v/v).

Compound	Solvent	Temperature (°C)	τ_1 (ns)	α_1	τ_2 (ns)	α_2	τ_3 (ns)	α_3	Φ_F	$\langle\Phi\rangle$ [b]
ϵAMP	a	25	24.8	1.00					0.52	
	a	45	22.4	1.00					0.46	
	a	65	20.2	1.00					0.41	
	b	25	20.7	1.00					0.45	
	c	25	24.0	1.00					0.48	
	d	25	25.2	1.00					0.51	
	e	25	25.0	1.00					0.54	
ϵApϵA	a	25	25.1	0.03	5.0	0.41	1.6	0.56	0.061	0.077
	a	45	22.6	0.02	3.7	0.27	1.6	0.71	0.048	0.054
	a	65	20.1	0.02	4.4	0.004	1.7	0.98	0.038	0.043
	b	25	20.8	0.02	4.5	0.40	1.5	0.58		
	c	25	23.3	0.03	11.8	0.70	3.9	0.27	0.143	0.200
	d	25	26.4	0.02	11.8	0.70	3.8	0.28	0.142	0.197
	e	25	22.1	0.07	13.2	0.56	3.4	0.37	0.167	0.204
ϵApG	a	25	22.8	0.10	7.6	0.38	2.2	0.52	0.088	0.132
	a	45	22.7	0.07	7.2	0.28	2.1	0.65	0.069	0.104
	a	65	22.6	0.06	6.9	0.19	2.0	0.75	0.054	0.087
GpϵA	a	25	22.8	0.05	4.1	0.22	1.3	0.73	0.035	0.062
	a	45	22.0	0.02	6.3	0.06	1.2	0.92	0.028	0.040
	a	65	22.8	0.02	6.1	0.02	1.0	0.96	0.024	0.032
ϵApU	a	25	24.5	0.03	6.4	0.54	2.3	0.43	0.079	0.108
	a	45	23.2	0.03	4.4	0.26	2.2	0.71	0.060	0.071
	a	65	19.0	0.03	4.3	0.01	2.2	0.96	0.052	0.057
UpϵA	a	25	25.5	0.03	4.5	0.32	2.1	0.65	0.066	0.073
	a	45	23.3	0.02	4.3	0.12	1.8	0.86	0.053	0.053
	a	65	20.2	0.03	4.0	0.004	1.8	0.97	0.049	0.049

Table 2

Decay parameters obtained for ϵApϵA and ϵApG in 0.05 M phosphate buffer (pH 7.0) at 25°C

The excitation wavelength was 337 nm. χ^2 values ranged from 1.1 to 1.6. The amplitudes (α) are normalized to unity.

Compound	Emission wavelength (nm)	τ_1 (ns)	α_1	τ_2 (ns)	α_2	τ_3 (ns)	α_3
ϵApϵA	410	25.1	0.03	5.0	0.41	1.6	0.56
	440	25.2	0.03	5.1	0.40	1.7	0.57
	470	25.5	0.03	5.1	0.41	1.7	0.56
	500	25.7	0.03	5.3	0.42	2.1	0.55
ϵApG	410	22.8	0.10	7.6	0.38	2.2	0.52
	440	22.2	0.10	8.0	0.39	2.0	0.51
	470	22.4	0.10	8.4	0.38	2.5	0.52

clearly indicate that the fluorescence decay curves for ϵ-dinucleoside monophosphates can be well resolved into three-exponential components corresponding to the long (τ_1), medium (τ_2) and short (τ_3) lifetimes. As is the case with any kinetic experiment, this does not rule out other decay laws which might also be found to agree with the data.

Optical and NMR measurements for ApA and ϵApϵA [3,4,6] suggest that a discrete multistate hypothesis is most plausible for conformations of these dimers in solution. Therefore, it is proposed that the decay behavior has its origin in a discrete multistate equilibrium. Three-exponential decay kinetics means that there exist three fluorescing conformations in dinucleoside phosphates. As seen in table 1, the component τ_1 is almost the same as the fluorescence lifetime of ϵAMP itself. Accordingly, this finding may lead us to the conclusion that the component τ_1 is attributed to the unstacked conformation and the components τ_2 and τ_3 to the stacked conformations. This conclusion, however, suggests that the unstacked population (α_1) is too low and the stacked population (α_2 and α_3) remains almost constant irrespective of temperature (table 1).

If we assume that all conformations are responsible for fluorescence emission and that the radiative lifetime of ϵA (τ_r) is constant regardless of the kind of dinucleoside phosphates (i.e., equal to that of ϵAMP itself), we obtain

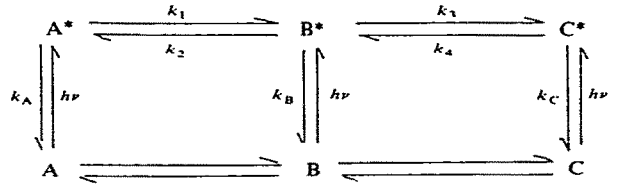
$$\langle \Phi \rangle = \frac{1}{\tau_r} \sum_{i=1}^{3} \alpha_i \tau_i \qquad (4)$$

Applying the relation $\tau_r = \tau / \Phi_F$ to data for ϵAMP (table 1), the τ_r value can be estimated to be 48 ns. The $\langle \Phi \rangle$ values calculated from eq. 4 are listed in table 1; it appears that the $\langle \Phi \rangle$ values are a little higher than the observed Φ_F values. From the studies on the fluorescence quenching between ϵAMP and mononucleotides such as AMP, it was found that mononucleotides almost completely quench the fluorescence of ϵAMP [12]. Therefore, it seems reasonable to conclude that the fully stacked conformation is almost nonfluorescent. This is the reason for the inconsistencies between Φ_F and $\langle \Phi \rangle$.

In view of these findings, we now propose that dinucleoside monophosphates in solution can be described as systems consisting of the following multiconformational states: the fully stacked conformation and three fluorescent conformational states. The fully stacked conformation may correspond to the well recognized right-handed stack [3], in which maximum π-electron overlap of stacked base rings and thus a strong quenching interaction is expected. According to the Φ_F and $\langle \Phi \rangle$ values in table 1, the fully stacked population can be approximately evaluated to be in the range 20–40% and it decreases with increasing temperature. The component τ_1 is ascribed to the extended conformation in which two bases are far apart and thus no interactions causing fluorescence quenching occur. The components τ_2 and τ_3 are attributed to loosely stacked conformations in which there are weak base-base interactions producing some fluorescence quenching. The possibility of such conformations was indicated on the basis of NMR measurements and theoretical calculations [3,4,7,8]. Since the amplitude α_2 markedly decreases with increasing temperature, the conformation corresponding to the component τ_2 may be thermally unstable. In contrast to the component τ_2, the conformation corresponding to the component τ_3 appears to be considerably stable even at high temperature. On the other hand, the amplitude α_1 is almost constant over the temperature range 25–65°C. This corresponds to a slight increase in the proportion of the component τ_1 at high temperature, since the proportion of the fully stacked conformation decreases with a rise in temperature. Nevertheless, the present decay data indicate that the proportion of the extended conformation is relatively small. The decay behavior observed in mixed solvents such as water/ethanol differs from that observed in water (table 1). This difference may be ascribed to reduction in base-stacking interactions because of the increased hydrophobicity in water/organic solvent mixtures.

It is also important to bear in mind that the dynamics of a reversible excited-state reaction can readily lead to multiexponential decay kinetics [22]. First, the excited-state proton transfer does not seem to occur, because the proportion of each amplitude at pH 5 is very similar to that at pH 7 (table 1). Since the decay parameters do not show any significant dependence on the emission wave-

length (table 2), the excimer or exciplex mechanism is also unlikely [22]. An alternative explanation would be a reversible excited-state reaction that transforms the fluorescent state (extended or loosely stacked state) to a nonfluorescent state (fully stacked state). The dynamic photophysics of a reversible excited-state system of this type can also lead to multiexponential decay kinetics [23]. One of such reversible excited-state processes may be described in the following scheme.



Here, A and B, respectively, denote the extended and loosely stacked states, while C is the fully stacked nonfluorescent state. The total fluorescence for this scheme can be shown to follow a three-exponential decay law.

$$I_F(t) = \alpha_1 e^{-t/\tau_1} + \alpha_2 e^{-t/\tau_2} + \alpha_3 e^{-t/\tau_3} \quad (5)$$

If we assume that k_1 is small compared to k_A (the sum of the rate constants for fluorescence and quenching of A*), we have

$$\tau_1^{-1} = k_A \quad (6)$$

$$\tau_2^{-1}, \tau_3^{-1} = \tfrac{1}{2}\left[(X+Y) \mp \{(Y-X)^2 + 4k_3k_4\}^{1/2}\right] \quad (7)$$

where $X = k_B + k_2 + k_3$ and $Y = k_C + k_4$.

In this scheme, τ_1 is expected to be nearly equal to the lifetime of ϵAMP itself. Temperature and solvent might be expected to affect the rate constants of the excited-state reaction and thus influence the decay parameters. This model also introduces a qualitative picture of observed decay curves, although we have not attempted to fit the decay profiles quantitatively.

The fluorescence quenching and decay parameters of GpϵA and UpϵA indicate a little higher degree of base-base interaction than in their ϵApG and ϵApU counterparts. This result is in agreement with that observed by Tolman et al. [11].

On the basis of the FDCD data, Reich and Tinoco [6] suggested that ϵApϵC and ϵApU can be described as two-state systems consisting of a fluorescent and a stacked, nonfluorescent species, while ϵApϵA can be described as consisting of at least three states. However, the fluorescence decay data presented in this paper are not consistent with the two-state hypothesis, but clearly indicate the multistate equilibrium for all ϵ-dinucleoside monophosphates examined. It should be noted that nanosecond fluorometry is a more direct and sensitive method for determining the microenvironments of a fluorophore and the molecular dynamics than steady-state measurements such as absorption and fluorescence spectra, fluorescence intensity and CD. In conclusion, the present data are understood to indicate that conformations of dinucleoside phosphates in solution might in fact be rather complex.

Acknowledgements

We wish to thank Misses Shoko Yasumi and Yuki Kuromi for their technical assistance.

References

1 K.E. van Holde, J. Brahms and A.M. Michelson, J. Mol. Biol. 12 (1965) 726.
2 J.T. Powell, E.G. Richards and W.B. Gratzer, Biopolymers 11 (1972) 235.
3 N.S. Kondo and S.S. Danyluk, Biochemistry 15 (1976) 756.
4 C.H. Lee and I. Tinoco, Jr, Biochemistry 16 (1977) 5403.
5 B.M. Baker, J. Vanderkooi and N.R. Kallenbach, Biopolymers 17 (1978) 1361.
6 C. Reich and I. Tinoco, Jr, Biopolymers 19 (1980) 833.
7 M.M. Dhingra, R.H. Sarma, C. Giessner-Prettre and B. Pullman, Biochemistry 17 (1978) 5815.
8 C.H. Lee, E. Charney and I. Tinoco, Jr, Biochemistry 18 (1979) 5636.
9 J.R. Barrio, J.A. Secrist, III and N.L. Leonaord, Biochem. Biophys. Res. Commun. 46 (1972) 597.
10 J.A. Secrist, III, J.R. Barrio, N.J. Leonard and G. Weber, Biochemistry 11 (1972) 3499.
11 G.L. Tolman, J.R. Barrio and N.J. Leonard, Biochemistry 13 (1974) 4869.
12 Y. Kubota, Y. Motoda and H. Nakamura, Biophys. Chem. 9 (1979) 105.
13 Y. Kubota, Y. Motoda, Biochemistry 19 (1980) 4189.
14 D.M. Rayner, A.E. McKinnon, A.G. Szabo and P.A. Hackett, Can. J. Chem. 54 (1976) 3246.
15 A. Grinvald and I.Z. Steinberg, Anal. Biochem. 59 (1974) 583.
16 I. Isenberg and R.D. Dyson, Biophys. J. 9 (1969) 1337.

17 I. Isenberg, R.D. Dyson and R. Hanson, Biophys. J. 13 (1973) 1090.
18 A. Gafni, R.L. Modlin and L. Brand, Biophys. J. 15 (1975) 263.
19 P.R. Bevington, Data reduction and error analysis for the physical sciences (McGraw-Hill, New York, 1969).
20 I. Weinryb and R.F. Steiner, in: Excited states of proteins and nucleic acids, eds. R.F. Steiner and I. Weinryb (Plenum, New York, 1971) p. 282.
21 I. Isenberg, J. Chem. Phys. 59 (1973) 5708.
22 J.B. Birks, Photophysics of aromatic molecules (Wiley-Interscience, New York, 1970).
23 A. Gafni and L. Brand, Biochemistry 15 (1976) 3165.