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Anisotropic Rotational Diffusion of the Adenine Moiety in 1,*N*⁶-Ethenoadenosine Triphosphate in Viscous Solvents Investigated by Time-Resolved Fluorescence Spectroscopy^{†,‡}

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Abstract: A recent ¹³C NMR study of the rotational dynamics of ATP in viscous solvents suggested that rotation of the adenine moiety about the glycosidic bond that attaches it to the ribose persists at high rates even at high viscosities [Rao, B. D. N.; Ray, B. D. *J. Am. Chem. Soc.* **1992**, *114*, 1566–1573]. This is a surprising finding, since it is generally accepted that the rotational diffusion of solutes is attenuated by viscous drag from solvent molecules (according to the classical Stokes–Einstein relationship). In the present study, we have carried out a detailed fluorescence spectroscopic investigation of the rotational diffusion of the adenine in ATP in aqueous solutions containing increasing concentrations of glycerol. For this purpose, we have utilized the well-characterized fluorescent ATP derivative, 1,*N*⁶-ethenoadenosine triphosphate (ϵ -ATP), in which the adenine moiety has been rendered fluorescent. Fluorescence lifetimes and anisotropy decay measurements of ϵ -ATP were performed in solutions presenting viscosities spanning 3 orders of magnitude (ranging from *ca.* 1.7 to 1310 cp). We have further explored the effect of viscosity on the rotations of the adenine moiety by performing fluorescence anisotropy measurements across the entire excitation band of ϵ -ATP. Selective excitation of ϵ -ATP at different relative orientations between the absorption and emission dipole moments enabled detection of anisotropic rotations of the molecule. Regardless of the direction of the axis selected for excitation of fluorescence, rotations of the adenine moiety in ϵ -ATP were found to be linearly attenuated by increasing solvent viscosity.

I. Introduction

The rotational mobility of ATP bound to ATP-utilizing enzymes or in viscous solvents has recently been investigated.¹ From measurements of ¹³C NMR line shapes of [2-¹³C]ATP, it was concluded that the rotational mobility of ATP is markedly reduced at the enzyme binding sites.¹ However, it was also reported that in highly viscous solvents, fast internal motions in the ATP molecule still persist. This led to the suggestion that these fast motions (namely rotation of the adenine moiety about the glycosidic bond that attaches it to the ribose) are independent of the viscosity of the medium.¹ This is against the widely accepted view that the rotational diffusion of solute molecules in liquids involves drag from the surrounding solvent.² Thus, a linear dependence of the rotational correlation time of the solute on viscosity is expected. Specifically with regard to the ATP molecule, rotation of the rather bulky adenine base about the glycosidic bond could be expected to involve displacement of solvation water molecules and thus should be dependent on viscosity.

Motivated by the unusual findings of Rao and Ray,¹ we have carried out a detailed fluorescence spectroscopic study of the rotational mobility of the adenine moiety of ATP in solution. We have utilized the well-characterized fluorescent ATP analog, 1,*N*⁶-

ethenoadenosine triphosphate (ϵ -ATP).³ In this analog, the adenine is rendered fluorescent by an etheno bridge between the heterocyclic N atom at position 1 and the NH₂ group attached to position 6 of the adenine in ATP³ (Figure 1). The fluorescence properties of ϵ -ATP depend on the physicochemical properties of the surrounding environment. Thus, motions or other factors affecting the adenine ring may be suitably followed with this fluorescent probe. The chemical modification of ATP to yield ϵ -ATP does not appear to have significant effects on the structure of the nucleotide, since it can still be effectively utilized instead of the natural substrate by a number of nucleotide-binding enzymes.⁴

In the present study, frequency-domain measurements of the decays of fluorescence intensity and anisotropy of ϵ -ATP were carried out in solutions containing increasing concentrations of glycerol to increase the viscosity of the medium. Fluorescence anisotropy measurements carried out across the entire excitation band of ϵ -ATP revealed markedly anisotropic rotations of the molecule. These results show that the adenine moiety rotates with different rates depending on the direction of the dipole transition selected. This is expected due to the anisotropic shape of the molecule. However, regardless of excitation wavelength (i.e., of the orientation of the electronic absorption dipole moment relative to the emission moment), our results show a linear decrease in mobility of the adenine moiety with increasing viscosity, as expected from the Stokes–Einstein relation.

II. Materials and Methods

Steady-State Fluorescence. Fluorescence excitation and emission spectra, as well as steady-state anisotropy measurements, were carried out on a photon-counting spectrofluorometer (ISS Inc., Champaign, IL)

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[‡] Abbreviations: ϵ -ATP, 1,*N*⁶-ethenoadenosine triphosphate; NMR, nuclear magnetic resonance; Tris, tris(hydroxymethyl)aminomethane.

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(1) Rao, B. D. N.; Ray, B. D. *J. Am. Chem. Soc.* **1992**, *114*, 1566–1573.
(2) See, for example, Mantulin, W. W.; Weber, G. *J. Chem. Phys.* **1977**, *66*, 4092–4099. Barkley, M. D.; Kowalczyk, A. A.; Brand, L. J. *J. Chem. Phys.* **1981**, *75*, 3581–3593. Weber, G.; Scarlata, S.; Rholam, M. *Biochemistry* **1984**, *23*, 6785–6788. Scarlata, S.; Rholam, M.; Weber, G. *Biochemistry* **1984**, *23*, 6789–6792. Rholam, M.; Scarlata, S.; Weber, G. *Biochemistry* **1984**, *23*, 6793–6796.

(3) Secrist, J. A., III; Barrio, J. R.; Leonard, N. J. *Science* **1972**, *175*, 646–647. Secrist, J. A., III; Barrio, J. R.; Leonard, N. J.; Weber, G. *Biochemistry* **1972**, *11*, 3499–3506. Spencer, R. D.; Weber, G.; Tolman, G. L.; Barrio, J. R.; Leonard, N. J. *Eur. J. Biochem.* **1974**, *45*, 425–429.

(4) For a review, see: Leonard, N. J. *CRC Crit. Rev. Biochem.* **1984**, *15*, 125–199.

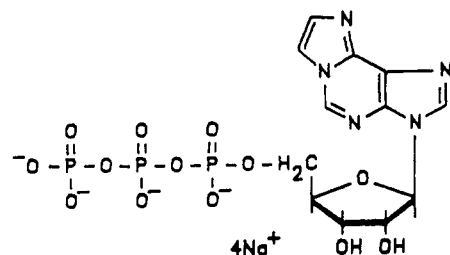


Figure 1. Structure of 1,*N*⁶-ethenoadenosine triphosphate (ϵ -ATP).

with a 4-nm band-pass for both excitation and emission. Temperature was controlled with a thermocouple in the thermostated cuvette holder. In addition to the excitation and emission monochromators, a Corning 7-54 filter in the excitation path and a Hoya Un-36 filter in the emission were used in anisotropy measurements; standard deviations in these measurements were ± 0.001 .

Time-Resolved Fluorescence. Fluorescence lifetimes were measured by frequency-domain fluorometry. Excitation at 290 nm was provided by a cavity-dumped, externally frequency-doubled Rhodamine 6G dye laser (Coherent, Model 700), pumped by a mode-locked frequency-doubled Nd:YAG laser (Coherent, Antares model). Magic angle excitation configuration was used to avoid polarization artifacts in lifetime measurements.⁵ Emission was collected through a Hoya UV-30 filter to eliminate scattered excitation light. Phase-modulation data were collected at 12 frequencies ranging from 1 to 30 MHz. Color errors in measurements were minimized by use of a reference standard solution of *p*-terphenyl (Eastman Kodak, Rochester, NY) in ethanol (lifetime = 1.05 ns). Temperature was maintained at 0.6 ± 0.1 °C and controlled with a thermocouple in the thermostated cuvette holder.

Fluorescence anisotropy decay measurements were performed on the same instrument, and differential phase angles and modulation ratios were measured at 14 frequencies from 1 to 30 MHz.

Data Analysis. Lifetime data were analyzed with one-exponential or a sum of exponential decays (see Results and Discussion) using the Globals Unlimited software.⁶ The analysis took into account standard deviations of $\pm 0.2^\circ$ and ± 0.004 for phase and modulation measurements, respectively, for minimization of reduced χ^2 for the fit as:

$$\chi^2 = \frac{1}{2n - f - 1} \sum \left[\frac{(\Phi_m - \Phi_c)^2}{(\sigma_\Phi)^2} + \frac{(M_m - M_c)^2}{(\sigma_M)^2} \right] \quad (1)$$

whom Φ_m and Φ_c are measured and calculated phase angles and M_m and M_c are measured and calculated modulation ratios, respectively; σ_Φ and σ_M are the standard deviations for phase and modulation, respectively; n is the number of frequencies of measurements; and f is the number of fitting parameters.

Fluorescence anisotropy decay analysis was performed with the same software and incorporated the independently measured lifetime parameters at each concentration of glycerol. The decay of the fluorescence anisotropy was assumed to originate from a single emitting species displaying a given set of lifetime parameters (shown in Table 1) and one or two rotational correlation times (as described in Results and Discussion).

Materials. ϵ -ATP was purchased from Molecular Probes Inc. (Eugene, OR). Ultrapure glycerol (USB Corp., Cleveland, OH) was redistilled prior to use to remove traces of fluorescent impurities and was a kind gift of Dr. N. Silva (Mayo Clinic, Rochester, MN). Samples containing 100 μ M ϵ -ATP and the indicated concentrations of glycerol were prepared in aqueous buffer containing 1 mM Tris-Cl, pH 7.0.

III. Results and Discussion

A detailed characterization of the fluorescence properties of ϵ -ATP has been presented.³ The ultraviolet absorption of ϵ -ATP is dominated by a strong band peaking at 275 nm; the major contributor to the fluorescence emission, however, is a weaker band centered at about 300 nm. The fluorescence emission presents a maximum at approximately 415 nm. The excitation anisotropy spectrum reaches the highest value at the longest

Table 1. Fluorescence Lifetime Analysis of ϵ -ATP at Different Glycerol Concentrations^a

[glycerol], % (v/v)	single-exponential fit		double-exponential fit ^b			
	τ (ns)	χ^2	τ_1 (ns)	τ_2 (ns)	f_1	χ^2
0	26.1 \pm 3.1	15	27.0 \pm 0.4	2.4 \pm 0.4	0.989	2.4
20	26.0 \pm 3.1	14	26.8 \pm 0.4	2.4 \pm 0.4	0.989	1.8
40	24.6 \pm 3.0	28	25.9 \pm 0.4	2.4 \pm 0.4	0.983	2.0
60	23.5 \pm 2.8	19	24.4 \pm 0.4	2.4 \pm 0.4	0.986	2.4
80	20.4 \pm 2.4	201	23.8 \pm 0.4	2.4 \pm 0.4	0.945	1.9
90	15.5 \pm 1.8	1095	24.0 \pm 0.7	2.4 \pm 0.4	0.842	4.6

^a Phase-modulation lifetime measurements were carried out as described in Materials and Methods, and data were analyzed with the indicated decay models. Uncertainties in lifetimes were calculated by χ^2 surface analysis.¹⁰ Sample temperature was maintained at 0.6 °C. ^b In this fit, the value of the short-lived lifetime component was allowed to vary but was linked throughout the data sets, as described in Results and Discussion.

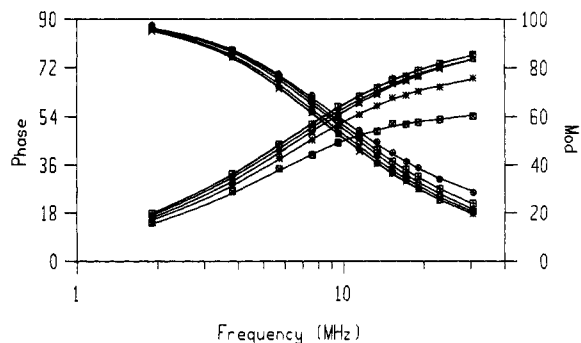


Figure 2. Phase-modulation lifetime measurements for ϵ -ATP at different glycerol concentrations. Data were acquired at 0.6 °C at glycerol concentrations ranging from 0 to 90% (v/v). Solid lines are fits according to the double-exponential fluorescence decay model from Table 1 (see text for details). Glycerol concentrations (v/v) were as follows: (+, \times) no glycerol; (\square , \circ) 20%; (\triangle , \times) 40%; (\star , \diamond) 60%; (\ast , \boxplus) 80%; (\boxtimes , \oplus) 90%.

wavelength excitation band (see below) and decreases toward shorter wavelengths (indicating that contribution from the strong absorption bands at shorter wavelengths is not negligible³).

Effects of Glycerol on the Fluorescence Decay of ϵ -ATP. Figure 2 shows multifrequency phase-modulation plots for ϵ -ATP at different concentrations of glycerol ranging from 0 to 90% (v/v). Table 1 summarizes the results obtained in the analysis of these data. A single-exponential lifetime was inadequate to describe the fluorescence decay, as indicated by the high χ^2 values obtained in the fits (Table 1). In fact, the single-exponential fit became significantly worse at high glycerol concentrations, indicating the need for a different decay model. Adding a second exponential lifetime to the fit resulted in marked decreases in χ^2 . The double-exponential analysis yielded a major lifetime component at 23–27 ns and an additional minor component at 2–6 ns. The relative fractional intensity of the minor component increased (from ca. 1 to ca. 15%) at high glycerol concentrations, suggesting the existence of a viscosity-dependent equilibrium between the long-lived and short-lived lifetime species. In order to decrease the uncertainties associated with the short lifetime, we have performed the double-exponential analysis by linking the short lifetime throughout the data sets (Table 1). This resulted in χ^2 and fractional intensities very similar to those obtained in the unlinked analysis (not shown). At increasing concentrations of glycerol, slight quenching of the major long-lived lifetime occurred (Table 1). In addition, it should be noted that the fraction of the short-lived component was small and essentially unchanged up to 60% (v/v) glycerol. The increase in fractional intensity of the short lifetime occurred in parallel with the large and abrupt increase in viscosity above 60% glycerol (see Table 2). These results suggest that the short lifetime component is likely associated with a particular configuration of the molecule in which the fluorescence

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(6) Beechem, J. M.; Gratton, E.; Ameloot, M.; Knutson, J. R.; Brand, L. In *Topics in Fluorescence Spectroscopy*, Vol. 2, Principles; Lakowicz, J. R., Ed.; Plenum Press: New York, 1991; pp 241–305.

Table 2. Fluorescence Anisotropy Decay of ϵ -ATP at Different Viscosities^a

[glycerol], % (v/v)	η (cp) ^b	single-exponential fit		double-exponential fit			
		Φ (ns)	χ^2	Φ_1 (ns)	f_1	Φ_2 (ns)	χ^2
0	1.79	0.3	2.7	0.1	0.95	7.6	2.4
20	3.44	0.5	1.2	0.4	0.94	0.2	1.2
40	8.25	0.8	2.5	0.7	0.99	65	2.4
60	29.9	5.2	1.4	4.5	0.95	72	1.1
80	255	34	1.6	35	0.91	0.4	1.6
90	1310	181	1.2	176	0.93	0.1	1.2

^a Differential polarized phase data were acquired as described under Materials and Methods, and data were analyzed in terms of one or two exponential rotational correlation times (see text). The lifetimes at each glycerol concentration (from the double-exponential analysis in Table 1) were incorporated in the analysis, Φ , rotational correlation times obtained in the fits; f_1 , fractional intensity corresponding to Φ_1 in the double-exponential fit. ^b Viscosity data from ref 11.

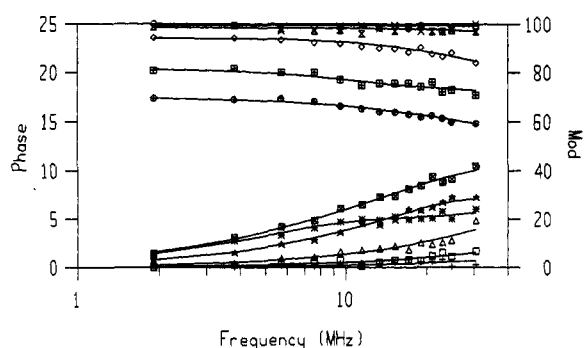


Figure 3. Differential polarized phase measurements for ϵ -ATP at different glycerol concentrations. Data were acquired at 0.6 °C at the same glycerol concentrations as in Figure 1. Solid lines are fits of the data with the rotational correlation times shown in Table 2. Glycerol concentrations (v,v) were as follows: (+, ×) no glycerol; (□, ○) 20%; (Δ, X) 40%; (☆, ◇) 60%; (*, □) 80%; (□, ⊕) 90%.

emission is quenched. This configuration is not significantly populated under conditions in which internal motions of the ϵ -ATP molecule are fast, given the fact that in the absence of glycerol or below 60% glycerol, the fractional intensity of the quenched lifetime is negligible. From the increase in fractional intensity of the short lifetime at high viscosities, it appears that restriction of internal motions of ϵ -ATP results in stabilization of the quenched configuration.

In the absence of glycerol, the fluorescence decay of ϵ -ATP was described by a lifetime of 27.0 ± 0.4 ns, with a fractional intensity of 98.9% (Table 1). This value is in good agreement with previous measurements at a single modulation frequency.³

Rotational Diffusion of the Adenine in ϵ -ATP. Direct measurements of the rotation of the adenine moiety in ϵ -ATP were performed by measuring the decay of the fluorescence anisotropy. Measurements were carried out with the differential polarized phase method⁷ at increasing glycerol concentrations. Figure 3 shows differential phase and modulation data for samples containing 0–90% (v/v) glycerol. It is clear from the data that increasing concentrations of glycerol promoted a significant increase in differential phase angles (Figure 3), indicating slower rotations of the fluorophore. The marked decrease in modulation at high glycerol concentrations (Figure 3) further indicated a decrease in rotational mobility at high viscosities. Thus, independently of any model to fit the fluorescence anisotropy of ϵ -ATP, the anisotropy decay data show a strong viscosity dependence.

Table 2 summarizes the results of the analysis of anisotropy decay data. For this analysis, the lifetime values at each concentration of glycerol (from the double-exponential fit shown

in Table 1) were used, and data were analyzed in terms of one or two exponential rotational correlation times (as described in Materials and Methods). For the single-exponential rotational correlation time fit, the limiting anisotropy, r_0 , was linked throughout the data sets, and very good fits to the data were obtained with an r_0 value of 0.106 recovered in this analysis (Table 2). We have independently measured the limiting anisotropy of ϵ -ATP at -70 °C in the presence of 80% glycerol and found an r_0 value of 0.118. The slightly lower r_0 value recovered in the analysis of anisotropy decay data can probably be explained by a slight deviation (of as little as 1 nm) from 290 nm in the calibration of the excitation wavelength provided by the tunable dye laser source. Table 2 shows that the rotational correlation time (Φ , from the single-exponential correlation time analysis) of the adenine moiety of ϵ -ATP increased with increasing viscosity of the medium. Furthermore, the marked increase in viscosity above 60% (v/v) glycerol was paralleled by a large increase in rotational correlation time.

We have also analyzed the fluorescence anisotropy decay data in terms of two exponential rotational correlation time (Table 2). No improvements in the fits were achieved with such a double-exponential model relative to the single-exponential analysis, as indicated by the similar χ^2 values obtained with the two models (Table 2). Furthermore, it is noteworthy that in the fits with two correlation times, the fraction of one of the correlation times (Φ_2) ranged from only 1 to 9%, and the values recovered for the major correlation time (Φ_1) were extremely similar to those obtained in the single-exponential correlation time analysis. These results indicate that the data do not permit resolution of two closely spaced rotational correlation times from the measurements with ϵ -ATP. As discussed below, this is probably due to the relatively small difference in molecular volumes for the adenine moiety and the whole ATP molecule, which results in nonresolvable rotational correlation times for rotation of the adenine and for overall molecular rotation.

It is instructive to consider the origin of the rotational motions (i.e., rotations of the adenine moiety alone versus overall rotation of the ϵ -ATP molecule) revealed by the anisotropy decay measurements. For the rotational diffusion of a spherical molecule,

$$\Phi = \eta V / RT \quad (2)$$

where Φ is the rotational correlation time of the molecule, η is the viscosity of the medium, V is the hydrated volume of the equivalent sphere, and R and T have their usual meanings. Thus, a plot of Φ versus the ratio η/T should yield a straight line. This is shown in Figure 4 using the single-exponential rotational correlation time (from Table 2) for the adenine in ϵ -ATP. A volume of $310 \text{ cm}^3/\text{mol}$ (or ca. $515 \text{ \AA}^3/\text{molecule}$) was obtained from the slope of Figure 4. Assuming a hydration of 0.59–0.84 g $\text{H}_2\text{O}/\text{gram}$ of ϵ -ATP (values usually found for nucleoside moieties in nucleic acid⁸) and a density of 1.3 – $1.5 \text{ g}/\text{cm}^3$, the calculated molar volume for ϵ -ATP (MW = 619) ranges from 656 to $876 \text{ cm}^3/\text{mol}$. For the ethenoadenine moiety (MW = 163), the calculated molar volume ranges from 173 to $231 \text{ cm}^3/\text{mol}$. Thus, the volume obtained from Figure 4 is less than one-half of the expected volume for the whole ϵ -ATP molecule and is closer to the volume expected for rotation of the adenine base alone. This indicates that, although it is not possible to resolve the rotations of the adenine and overall rotation of ϵ -ATP, the rotational motions of the adenine moiety give the major contribution to our measurements.

The linear dependence of the rotational correlation time on viscosity was further confirmed by measurements of the steady-state anisotropy of the samples at increasing glycerol concentra-

(7) Weber, G. J. *Chem. Phys.* 1977, 66, 4081–4091.

(8) Cantor, C. R.; Schimmel, P. R. *Biophysical Chemistry, Part II: Techniques for the study of biological structure and function*; W. H. Freeman and Co.: San Francisco, CA, 1980; p 552.

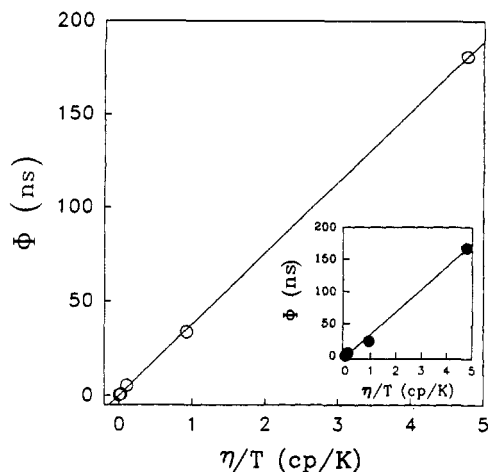


Figure 4. Viscosity dependence of the rotational diffusion of the adenine in ϵ -ATP. Rotational correlation times (from Table 2) are plotted as a function of the ratio η/T (see text). Sample temperature was maintained at 0.6 °C. The line through the points was obtained by linear regression. Inset: rotational correlation times, calculated from steady-state anisotropy measurements at 0.6 °C at different glycerol concentrations (see text), are plotted as a function of the ratio η/T .

tions. From the anisotropy values thus obtained, the average correlation time may be calculated according to the Perrin equation as:

$$\Phi = \tau / ((r_0/r) - 1) \quad (3)$$

where τ is the fluorescence lifetime and r_0 is the limiting anisotropy of ϵ -ATP at the excitation wavelength of 290 nm. The inset in Figure 4 shows a plot of the rotational correlation times calculated with the Perrin equation as a function of η/T . The calculated correlation times were in excellent agreement with those obtained directly from the anisotropy decay measurements in Figure 4 and confirm the linear dependence on viscosity.

Anisotropic Rotations in ϵ -ATP. Fluorescence anisotropy measurements monitor the decay of the angular orientation of the emission dipole relative to the absorption during the excited state of the fluorophore. On the other hand, NMR measurements monitor the reorientational effects on the ^{13}C - ^1H dipolar interaction tensor and the ^{13}C chemical shift anisotropy tensor.¹ Thus, the directions of the axis probed by fluorescence and NMR measurements relative to the axis of rotation of the adenine moiety could be different. In particular, if the angle between the axis of rotation and the electronic transition dipole was small, the persistence of fast rotations at high viscosity (such as those detected by NMR measurements¹) might go undetected in our fluorescence measurements. In such a case, the glycosidic rotation would not have much effect on the fluorescence anisotropy. To address this question, we have performed fluorescence anisotropy measurements across the excitation band of ϵ -ATP at increasing solvent viscosity.

Figure 5 shows the excitation anisotropy spectra of ϵ -ATP at increasing viscosities. For the motionless molecule (-70 °C in the presence of 80% glycerol), the anisotropy reaches a value of 0.312 at the longest wavelength and decreases to a negative value of -0.090 toward shorter excitation wavelengths. This is in agreement with previous reports³ and indicates significant contribution from the strong absorption band at 275 nm. In addition, the negative anisotropy at 275 nm indicates that the transition moment for this band must lie at a large angle (approximately 65°) to the emission moment. It is clear from Figure 5 that regardless of the angle between absorption and emission (i.e., the excitation wavelength employed), the measured anisotropy was markedly dependent on viscosity. In fact, in the absence of glycerol, rotation of the adenine moiety was very fast,

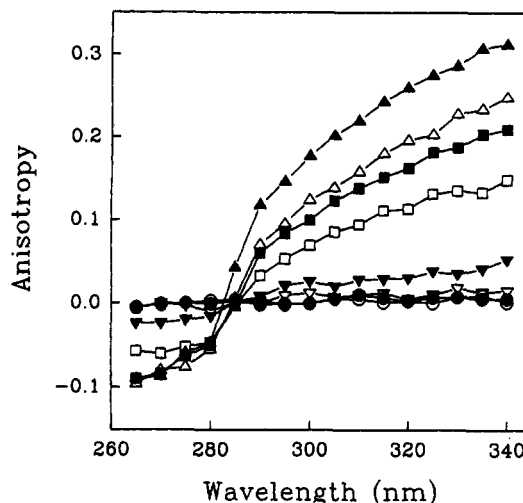


Figure 5. Anisotropy spectra of ϵ -ATP at increasing solvent viscosity. Excitation anisotropy spectra of ϵ -ATP were measured at 5 °C in the presence of the following concentrations (v/v) of glycerol: (○) no glycerol; (●) 20%; (▽) 40%; (▽) 60%; (□) 80%; (■) 90%; (△) 95%. The curve indicated by ▲ indicates data at -70 °C in the presence of 80% (v/v) glycerol.

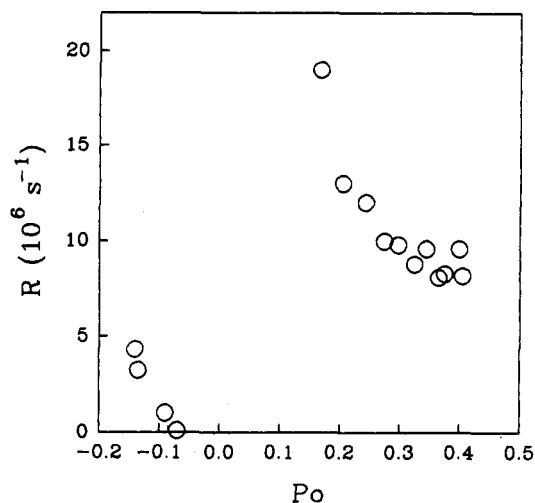


Figure 6. Apparent rate of rotation of ϵ -ATP as a function of P_0 . The apparent rate of rotation was calculated as described in Results and Discussion at 5-nm intervals across the excitation spectrum of ϵ -ATP. Data were obtained at 5 °C in the presence of 80% (v/v) glycerol and are plotted as a function of the limiting polarization (P_0) at each excitation wavelength, as described by Shinitsky et al. (1971).⁹

leading to very low steady-state fluorescence anisotropy. Addition of glycerol increased the anisotropy across the entire excitation band. Given the wide range of angular displacement between absorption and emission that could be achieved under the conditions of Figure 5, we conclude that the possibility of alignment between the electronic dipole moment and the axis of rotation about the glycosidic bond is not likely to explain the differences between our results and those obtained from NMR measurements.¹

As originally pointed out by Leonard et al.,³ the anisotropy spectrum of ϵ -ATP (Figure 5) indicates the existence of anisotropic rotations. In the present work, we have quantified the asymmetry in rotational diffusion of the molecule by calculating the apparent rate of rotation as a function of excitation wavelength (i.e.,

(9) Shinitsky, M.; Dianoux, A.-C.; Gitler, C.; Weber, G. *Biochemistry* 1971, 10, 2106-2113.

(10) Beechem, J. M.; Gratton, E. Time-resolved laser spectroscopy in biochemistry. *Proc. SPIE-Int. Soc. Opt. Eng.* 1988, 909, 70-81.

(11) Minor, C. S.; Dalton, N. N. *Glycerol*; Reinhold Publ. Corp.: New York, 1953; pp 278-283.

selecting different orientations of the electronic dipole moment). The apparent rate of rotation, R , can be calculated from the expression:⁹

$$R = ((r_0/r) - 1)/6\tau \quad (4)$$

Figure 6 shows the apparent rates of rotation in 80% (v/v) glycerol as a function of the limiting polarization at each excitation wavelength, as proposed by Weber and co-workers.⁹ The changes in rotational diffusion with excitation wavelength indicate that the depolarizing motions are strongly anisotropic. The value of R at P_0 equal to 0.143 represents the rate of out-of-plane motions, while the limiting value at P_0 equal to 0.5 represents the average rate of in-plane and out-of-plane rotations.⁹ From Figure 6, it is apparent that out-of-plane motions in ϵ -ATP ($R \approx 20 \times 10^6 \text{ s}^{-1}$, corresponding to a rotational correlation time $\sim 8 \text{ ns}$) are considerably faster than in-plane rotations (the average rate of the two motions being *ca.* $(8-10) \times 10^6 \text{ s}^{-1}$, corresponding to rotational correlation time of 17–21 ns).

While the above discussion takes into account solely the existence of in-plane and out-of-plane rotations of the adenine moiety in ϵ -ATP, it is important to notice that we cannot exclude the possibility that the slower rotational rate revealed in Figure 6 may also contain a contribution from rotation of the adenine moiety coupled to the rotation of the whole ϵ -ATP molecule (as discussed above). The relevant finding to be stressed here is that all motions are dependent on solvent viscosity (Figure 5).

IV. Conclusions

From our data on both steady-state and time-resolved anisotropy of ϵ -ATP in viscous glycerol solutions, we have found no evidence for viscosity-independent rotational diffusion of the adenine moiety. Selective excitation of the fluorescence at different relative orientations between the absorption and emission transition moments enabled detection of anisotropic rotations in ϵ -ATP, with out-of-plane rotation being considerably faster than in-plane rotation. These measurements showed that the fluorescence anisotropy is sensitive to viscosity over a wide range of angular displacements between excitation and emission dipole moments. This rules out the possibility of fast, undetected rotations of the adenine that could arise from alignment of the electronic dipole with the axis of rotation. We conclude that the rotational diffusion of the adenine moiety in ϵ -ATP is linearly attenuated by solvent viscosity. With respect to a comparison with the results previously reported by Rao and Ray,¹ it should be noted that the ϵ -ATP molecule we have studied has a chemical structure which is not identical to that of ATP. Thus, there is still a possibility that this small structural difference could affect the hydrodynamics of the molecule.

Acknowledgment. We would like to thank Dr. B. D. Nageswara Rao for pertinent comments on a preliminary version of the manuscript and Mr. Jason Sutin for help with measurements of the limiting anisotropy of ϵ -ATP at $-70 \text{ }^\circ\text{C}$.