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# FLUORESCENCE AND FLOW DICHROISM OF F-ACTIN–ε-ADP: THE ORIENTATION OF THE ADEMINE PLANE RELATIVE TO THE LONG AXIS OF F-ACTIN

Masao MIKI and Koshin MIHASHI
*Department of Physics, Faculty of Science, Nagoya University, Nagoya 464, Japan*

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The excitation polarization spectrum of ε-ADP bound to F-actin shows that two absorption dipoles at 260 nm and 340 nm are oriented in different directions relative to the emission dipole. On the other hand, the linear dichroism of F-actin–ε-ADP gives that the dichroic ratio of the bound ε-ADP is approximately constant (about −0.5) in the wavelength region from 250 to 350 nm. Furthermore, the fluorescence polarization of ε-ADP bound to F-actin which is oriented in the field of flow shows that the emission dipole is nearly perpendicular to the long axis of F-actin. From these observations we conclude that the adenine plane of the bound nucleotide is almost perpendicular to the long axis of F-actin.

## 1. Introduction

G-actin from skeletal muscle has one specific site for binding of nucleotides per molecule [1]. Among nucleotides so far investigated, ATP has the highest affinity to this site. In the course of polymerization of G-actin to F-actin, the bound ATP is hydrolysed and the resultant ADP is tightly incorporated in the structure of F-actin. Higashi et al. found that F-actin oriented by flow shows a negative linear dichroism at 260 nm indicating a geometrically regular binding of ADP to F-actin [2]. The negative dichroism gave information about the direction of the electric transition moment of the absorption of ADP at 260 nm. However, the direction of the plane of adenine was not uniquely determined. Recently we have demonstrated that ε-ATP (1:$N^6$-ethenoadenosine-5′-triphosphate) replaces ATP in the binding site of G-actin and the polymerization of G-actin having ε-ATP occurs in a way quite similar to G-actin–ATP [3]. This afforded an opportunity for a further study on the binding manner of nucleotide to F-actin; an advantage of ε-ADP lies in the fact that it has absorption bands around 340 nm as well as at 260 nm, and emits light centered at 410 nm. From the measurement of fluorescence polarization of ε-ADP, one can calculate the angle between two transition moments at 260 nm and 340 nm. On the other hand from the linear dichroism at 260 nm and at 340 nm of ε-ADP bound to F-actin oriented by flow, one can determine the direction of two absorption dipoles relative to the long axis of F-actin.

Also, from the fluorescence polarization of F-actin–ε-ADP which is oriented by flow, we can find the direction of the emission dipole relative to the long axis of F-actin. Then from these parameters, we can determine the angle between the adenine plane and the long axis of F-actin. The result of our study along this line is presented in this paper.

## 2. Experimental

Actin of rabbit skeletal muscle was prepared from acetone dried powder of minced muscle. Details of the purification of actin has been described elsewhere [4]. ε-ATP was synthesized from ATP (Sigma) according to the method of Secrist et al. [5]. The spectrum of ε-ATP obtained was in good agreement with that reported by these authors. F-actin–ε-ADP was

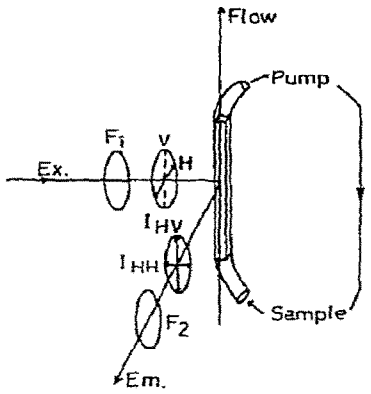


Fig. 1. Schematic presentation of the flow-cell apparatus and its optical system. $F_1$ is UV-D25 and $F_2$ is UV-39 filter. Excitation light is polarized horizontally.

obtained in the same way as described previously [3]. The apparatus for ultraviolet flow dichroism measurements was the same one as used previously [2]. Corrections to subtract the scattering anisotropy were made by the method used in the previous paper [2]. Static fluorescence intensity and polarization were measured with Hitachi MPF 2A. The direction of the emission dipole was measured with a home-made fluorometric flow cell attached to a Hitachi MPF 2A; the optical system of this apparatus is shown in fig. 1. F-actin–ϵ-ADP was flowed in a 0.2 cm × 0.2 cm quartz cell at a constant flow rate by using a microtube pump (Tokyo Rikakiki Co Ltd.). The flow rate was 7 ~ 25 ml/min which gave a constant value of polarization of F-actin–ϵ-ADP.

Both fluorescence and anisotropy decays were determined using nanosecond pulsefluorimetry (ORTEC 9200). All measurements were performed at room temperature, unless stated otherwise.

## 3. Results and discussion

### 3.1. The excitation polarization spectrum of ϵ-ATP

The excitation spectrum of ϵ-ATP is composed of a broad band above 300 nm and a few peaks at shorter wavelength [5] (see also fig. 2). According to the principle of fluorescence polarization, when excited by linearly polarized light, the degree of polarization of emitted light is related to the angle $\beta$ between the absorption and emission transition dipole moments. If the fluorophore is fixed with random orientation, the angle $\beta$ is related to $P_0$ as

$$\cos^2\beta = (1 + 3P_0)/(3 - P_0), \quad (1)$$

where $P_0$ is the degree of polarization under the limit where the brownian motion of the fluorophore is absent. Therefore, to obtain $P_0$, it is desirable to measure the fluorescence in a highly viscous solvent at a very low temperature. The degree of polarization of emission $P$ of ϵ-ATP after excitation at various wavelengths was already measured in propylene glycol at −50°C [5], where it was found that the polarization $P$ was positive above 300 nm and became negative below 275 nm. This suggested that transition moments responsive to absorption at long and short wavelengths form a large angle between them. The corresponding angle of ϵ-ADP bound to F-actin is determined as follows.

Actin-bound ϵ-ADP shows an excitation spectrum similar to that of free ϵ-ATP except for an apparent red shift of the broad band above 300 nm (fig. 2) [3]. The fluorescence polarization of this broad band is positive similar to free ϵ-ATP in propylene glycol at −50°C. However, the degree of polarization of actin-bound ϵ-ADP is apparently smaller than that of free ϵ-ATP; that is, after excitation at 340 nm, $P$ is 0.31 for actin-bound ϵ-ADP in comparison with 0.42 of free ϵ-ATP in propylene glycol at −50°C [5]. One may consider that the low value of $P$ of actin bound ϵ-ADP might result from the brownian motion of ϵ-ADP in the binding site of F-actin; but this is not the case, because the anisotropy decay of actin-bound ϵ-ADP measured with nanosecond pulsefluorimetry revealed no motional freedom in the binding site. Furthermore, the rotational correlation time calculated from the anisotropy decay curve is larger than 5 μs; these are already reported [6] and it is confirmed under the present experimental condition [11]. This implies that the static polarization $P$ of actin-bound ϵ-ADP is approximately equal to $P_0$ which in turn corresponds to $r_0$, the fluorescence anisotropy at zero time. As a matter of fact, the value $P = 0.31$ which is obtained in the present study (fig. 2) is practically the same as $P_0$ calculated from the following equation using $r_0 = 0.234$ which is determined from the anisotropy decay measurements [6,11].

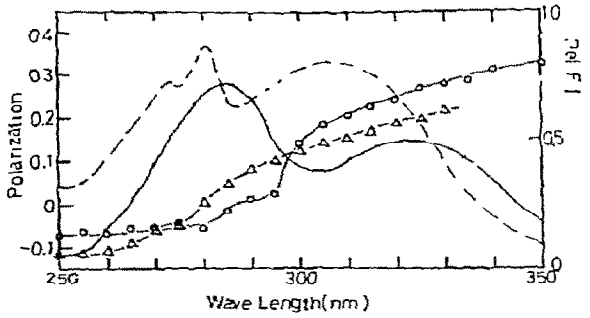


Fig. 2. The excitation spectra and the excitation polarization spectra of (1) free ϵ-ATP, and (2) F-actin–ϵ-ADP observed at 440 nm.

(1) ϵ-ATP 30 μM, dissolved in aqueous solution of 95% glycerol containing phosphate buffer 20 mM at pH 7.0;

(2) F-actin–ϵ-ADP 1.0 mg/ml in aqueous solution containing KCl 0.1 M, $MgCl_2$ 2 mM and phosphate buffer 20 mM at pH 7.0.

Excitation spectra; (– – –) ϵ-ATP, (——) F-actin–ϵ-ADP. Excitation polarization spectra; (△) ϵ-ATP, (○) F-actin–ϵ-ADP. Measurements were made at 20°C. To exclude the scattered light from the emission light, filters UVD 25 and UV 39 were used and fluorescence cell of 3 mm was used.

$$P_0 = 3r_0/(2 + r_0). \qquad (2)$$

We also found that the fluorescence decay of ϵ-ATP in 95% glycerol (at 5°C) gave $r_0$ which is indistinguishable from that of actin-bound ϵ-ADP. Therefore it seems likely that the large value of $P = 0.42$ in propylene glycol (–50°C) obtained by Secrist et al. [5] is an indication of temperature dependent electronic configuration of ϵ-ATP at the energy level of the near-ultraviolet region [6,7]. From above considerations, we conclude that $P_0$ at 340 nm of F-actin-bound ϵ-ADP is 0.314. We then have $\beta_1 = 32°$ using eq. (1) for the angle between the emission and the absorption dipole moments at 340 nm.

Below 300 nm, the excitation polarization of F-actin-bound ϵ-ADP decreases drastically, and after a little plateau at 290 nm it reaches a level of $P = -0.08$. This multiple change in the polarization may be explained by a complex electronic structure of ϵ-ATP which is already suggested by Secrist et al. [5]. In addition to this, the fluorescence of F-actin–ϵ-ADP solution after the excitation at 250–300 nm includes the contribution from tryptophan and tyrosine residues of F-actin. This contribution is not only due to a strong fluorescence of tryptophan whose tail at the emission wavelength (440 nm) is still not negligible in comparison with the fluorescence of ϵ-ADP but also comes from an excitation energy transfer from the aromatic group of F-actin to the bound ϵ-ADP. We do not know the proportion of these contributions. Furthermore at these wavelengths, optical density is very large, so that it is difficult to determine the exact value of the fluorescence polarization of bound ϵ-ADP alone at the wavelength between 300 nm and 250 nm.

Apart from this difficulty, if we look at the polarization at 260 nm where the absorption of protein aromatic groups is weak in comparison with that of the bound ϵ-ADP, we find that the degree of polarization of actin-bound ϵ-ADP is close to that of free ϵ-ATP in 95% glycerol solution (fig. 2). The polarization of ϵ-ATP in propyrenglycol at –50°C is also of the same order of magnitude [5]. Then we approximate $P_0 = -0.10$ for the actin-bound ϵ-ADP at 260 nm. On this basis, we obtain $\beta_2 = 62°$ which determines the angle between the emission and the absorption (at 260 nm) dipole moments using eq. (1). Finally we obtain the angle $\omega$ between absorption dipole moments at 340 nm and 260 nm of actin-bound ϵ-ADP using equation $\omega = \beta_2 \pm \beta_1$. In this calculation, we assume that the emission and the absorption dipoles are in a single plane of adenine group. In the above equation, the positive or negative sign corresponds to the case where two absorption dipoles are placed on the opposite or the same side of the emission dipole in the adenine plane respectively. The calculated value is 86° or 30° according to the sign. We have no principle to choose any one of these unequivocally.

### 3.2. Flow dichroism of F-actin–ϵ-ADP

The absorption spectrum of F-actin–ϵ-ADP is shown in fig. 3. The long tail of the spectrum above 300 nm is due to bound ϵ-ADP (fig. 2). The linear dichroism of F-actin–ϵ-ADP oriented by flow was measured in the range of 250–360 nm wavelength. As shown in fig. 3, the dichroism was negative from 310 nm to 340 nm where only ϵ-ADP contributes. The dichroic ratio $\Delta\epsilon/\epsilon$ averaged in this range was $-0.52 \pm 0.07$ (table 1). The dichroism was more negative at 295 nm and then reversed to a positive value

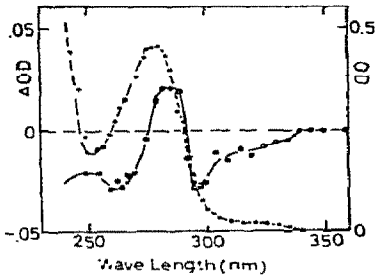


Fig. 3. The absorption spectrum (•) and the flow dichroism spectrum (o) of F-actin–ϵ-ADP. F-actin–ϵ-ADP 3.5 mg/ml. The solution contained 0.1 M KCl, 2 mM $MgCl_2$ and 20 mM phosphate buffer (pH 7.0). The spectra were measured at room temperature.

at 290 nm with a peak at about 280 nm. It became negative again at 277 nm and had a negative trough at 260 nm. This dichroism spectrum of F-actin–ϵ-ADP below 300 nm is very similar to that of F-actin–ADP previously obtained [2]. That is, F-actin–ADP gave a small negative trough at 295 nm, a positive peak at 280 nm and a large negative trough at 260 nm. The first and the second were attributed to tryptophan and/or tyrosine residues in F-actin and the third was attributed to bound ADP. The value of dichroism of F-actin–ϵ-ADP between 300 nm and 270 nm where amino acid residues have main contribution was smaller at 280 nm and more negative at 295 nm than that of F-actin–ADP. However, the profile of the dichroic spectra of these two F-actin are very similar and the height of the positive peak at 280 nm, when measured from the negative trough at 295 nm, is nearly equal. If the contribution of bound ϵ-ADP to the dichroism is subtracted from the total dichroism, the spectra of two F-actins are expected to become identical.

Let us assume that the dichroism below 300 nm is expressed as the sum

$$\Delta\epsilon = \Delta\epsilon_1 + \Delta\epsilon_2,$$

where $\Delta\epsilon_1$ comes from bound ϵ-ADP and $\Delta\epsilon_2$ comes from tryptophan and/or tyrosin residues. If the dichroism at 260 nm is assumed to be wholly due to the bound ϵ-ADP, its dichroic ratio $\Delta\epsilon/\epsilon$ is $-0.55 \pm 0.09$. If the same value of the dichroic ratio of ϵ-ADP is taken in the range from 295 nm to 260 nm, then the dichroic ratio of tryptophan and/or tyrosine residues at 295 nm and 280 nm is found to be $-0.14 \pm 0.02$ and $+0.08 \pm 0.01$, respectively. Thus, as shown in table 1, the dichroic ratios of F-actin–ϵ-ADP at 260 nm, at 280 nm and at 295 nm are almost equal to those of F-actin–ADP. This means that between two F-actins, the structure of the protein moiety is not significantly different which was already suggested in a previous study [3].

The most important finding is that the dichroic ratios of ϵ-ADP in 260–290 nm and in 300–340 nm both have large negative values, around −0.5; in spite of the different direction of the corresponding transition moments in the adenine plane, there is no remarkable difference in the dichroic ratio. In the following section, this will be interpreted in terms of the direction of the adenine plane of ϵ-ADP relative to the long axis of F-actin.

### *3.3. Direction of transition moments and the adenine plane of the bound ϵ-ADP with respect to the long axis of F-actin*

When F-actin filaments are completely oriented

Table 1
Flow dichroism ($\Delta\epsilon/\epsilon$) of F-actin–ϵ-ADP and F-actin–ADP

| λ (nm) | F-actin–ϵ-ADP | | F-actin–ADP | |
|---|---|---|---|---|
| | ϵ-ADP | protein | ADP | protein |
| ~ 260 | −0.55 ± 0.09 | – | −0.52 ± 0.08 | – |
| ~ 280 | – | +0.08 ± 0.01 | – | +0.08 ± 0.01 |
| 295 | – | −0.14 ± 0.02 | – | −0.14 ± 0.02 |
| ~ 340 [a] | −0.52 ± 0.07 | – | – | – |

[a] Averaged between 305 ~ 335 nm (at each 5 nm).

in parallel, the dichroic ratio is given by

$$\Delta\epsilon/\epsilon = 3(3\cos^2\alpha - 1)/2, \quad (3)$$

where $\alpha$ describes the angle between the transition moment of absorption and the long axis of F-actin. In this equation, the helical symmetry of F-actin was taken into consideration. In the case of the apparatus used here, F-actin is aligned in the gap between two concentric cylinders and the extinction angle of F-actin solution is very small (about 3°) [9]. This indicates that F-actin filaments are well oriented in the plane parallel to the cylinder surface. However, it should be noted that within this plane, the filaments are not completely oriented parallel to the flow. In such a case, the dichroic ratio is given by

$$\Delta\epsilon/\epsilon = 3(2\sin^2\theta - 1)(3\cos^2\alpha - 1)/2, \quad (4)$$

where $(\pi/2-\theta)$ is the angle between the long axis of F-actin and the direction of flow in the plane parallel to the cylinder surface. (The formula for $\Delta\epsilon/\bar{\epsilon}$ which can be applied to the case of larger values of the extinction angle can be found in ref. [8].) The average of $\sin^2\theta$ is called the orientation factor and can be related to the degree of flow birefringence. In the case of independent rigid filaments, the orientation factor was calculated as a function of the ratio of the rotary diffusion constant and the shear rate [10]. If we approximate F-actin as a rigid rod whose diameter is 80 Å and the length is 1.0 $\mu$m, the rotary diffusion

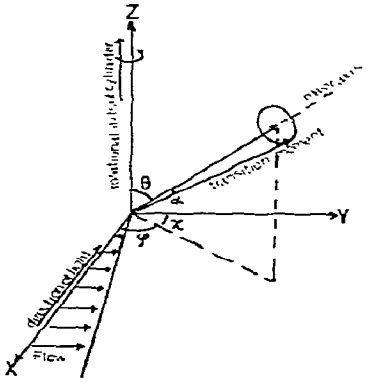


Fig. 4. The schematic presentation of the relation of angles $\alpha, \theta, \phi$ and $\chi$ used in the text. The long axis of F-actin is oriented in the direction of the major axis shown in this figure.

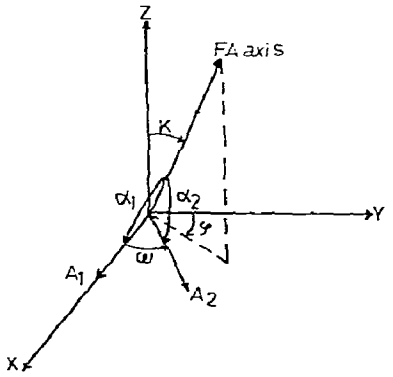


Fig. 5. The schematic presentation of the relation between the adenine plane and the long axis of F-actin. The adenine ring is placed in the X–Y plane. $A_1$, $A_2$ are the absorption dipoles around 340 nm and 260 nm. $\alpha_1$, $\alpha_2$ are the angles between each absorption dipole and the long axis of F-actin. $\omega$ is the angle between two absorption dipoles. In the text, $(\beta_1 \pm \beta_2)$ is $\omega$.

constant becomes 20 $s^{-1}$. This is very much smaller than the shear rate of the order of $10^3$ $s^{-1}$, where the dichroism was measured. Under such a condition, the orientation factor becomes insensitive to the shear rate. Using the result of a calculation made by Kasai and Oosawa [9], the value of the orientation factor or $\sin^2\theta$ is estimated to be 0.70. Inserting this value and the observed value of $\Delta\epsilon/c$ into eq. (4), we obtain $\alpha_1 = 78° \pm 5°$ for the absorption around 340 nm and $\alpha_2 = 80° \pm 6°$ around 260 nm.

From the geometrical relation of $\alpha_1, \alpha_2$ with $\omega$, we obtain finally the angle $K$ which is determined by the direction normal to the adenine plane of the bound ε-ADP and by the long axis of F-actin. The relation of these angles $\alpha_1, \alpha_2, \omega$ is illustrated in fig. 5 where the adenine ring is placed in the X–Y plane and the long axis of F-actin has the azimuthal angle $(K, \phi)$. We obtain the relations

$$\cos\alpha_1 = \sin K \sin\phi,$$

$$\cos\alpha_2 = \sin K \sin\phi \cos\omega + \sin K \cos\phi \sin\omega.$$

From these equations, we obtain

$$\sin K = \left(\frac{\sin\omega}{\sqrt{\cos^2\alpha_1 + \cos^2\alpha_2 - 2\cos\alpha_1 \cos\alpha_2 \cos\omega}}\right)^{-1}. \quad (5)$$

As was stated, $\omega$ is not chosen uniquely. It should be noted that we have to consider the angle $\omega' = \pi - \omega$

as well as $\omega$, because of the geometrical relation of the angles between absorption dipoles relative to the long axis of F-actin. Then all possible values of $K$ corresponding to four cases of $\omega$ and $\omega'$ are calculated using eq. (5). The results obtained are

(1) $\omega = 86°$, $K = 15° \pm 2°$,

(2) $\omega' = 86°$, $K = 16° \pm 2°$,

(3) $\omega = 30°$, $K = 12° \pm 6°$,

(4) $\omega' = 30°$, $K = 90° \pm 1°$.

Apparently the first three cases give approximately the same value of $K$, while the last differs. Without any other parameter, it is difficult to decide between them. However, this could be done, if we know the direction of emission dipole relative to the long axis of F-actin. Let us consider the fluorescence polarization of ε-ADP bound to F-actin which is oriented by flow. We excite the oriented F-actin–ε-ADP with light polarized perpendicularly to the long axis of F-actin (fig. 1). If the emission dipole lies perpendicular to the long axis of F-actin, $I_{HV}$ will be lower than the fluorescence intensity perpendicular to the F-actin $I_{HH}$. This corresponds to the cases 1, 2, 3. On the other hand, in case 4, we can see from geometrical consideration that the emission dipole lies at an angle less than $\cos^{-1}\sqrt{1/3}$ with the long axis of F-actin and it will give $I_{HV} > I_{HH}$. It is also apparent that if the orientation of F-actin is randomised after stopping the flow, $I_{HV}$ and $I_{HH}$ will relax to the same value. The experimental results are shown in fig. 6. It is evident that $I_{HV} < I_{HH}$ when F-actin is oriented and they become essentially identical after stopping the flow. Then we can exclude the case 4. It is difficult to choose among cases 1, 2, 3 because of the limitation of the experimental precision. Therefore we conclude that the nucleotide is bound to F-actin with its adenine plane lying almost perpendicularly to the long axis of F-actin. The details of the analysis of the emission dipole will be given elsewhere by us and Drs. Yanagida and Oosawa.

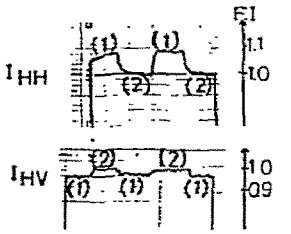


Fig. 6. The fluorescence intensity ($I_{HH}$, $I_{HV}$) of F-actin–ε-ADP. Curve (1), F-actin–ε-ADP is oriented by the flow. Curve (2), the orientation of F-actin–ε-ADP is randomised after stopping the flow. The concentration of F-actin–ε-ADP is 0.2 mg/ml; 0.1 M KCl, 20 mM phosphate buffer (pH 7.0), 2 mM $MgCl_2$. The flow rate is 20 ml/min. Excitation is at 320 nm and emission is at 440 nm.

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