NANOSECOND PULSEFLUOROMETRY IN POLARIZED LIGHT OF G-ACTIN-ε-ATP AND F-ACTIN-ε-ADP

Koshin MIHASHI and Philippe WAHL
Centre de Biophysique Moleculaire, F. 45 045 Orleans Cedex France

Received 3 February 1975
Revised version received 24 February 1975

1. Introduction

It has been recently found by Miki et al. [1] and Thames et al. [2] that ATP, the natural ligand of G-actin, can be replaced by $1,N^6$-ethenoadenosine triphosphate, ε-ATP. In the present work, the anisotropy decay of the fluorescence of ε-ATP bound to G-actin as well as of ε-ADP bound to F-actin are investigated. From these studies, correlation times characterising the Brownian rotation of G-actin and flexibility of F-actin are obtained.

2. Experimental

2.1. *G-actin-ε-ATP*

Actin from rabbit skeletal muscles was prepared in the manner already described [3]. ε-ATP was a gift from M. Miki, and the incorporation of ε-ATP into actin was done as follows:

A pellet of F-actin having bound ADP (about 0.5 ml) was depolymerized in 6 ml of cold distilled water containing ε-ATP (207 μM) at neutral pH. The solution had been dialysed against 100 ml of cold distilled water containing ε-ATP (55 μM), tris–acetate (5 mM; pH 7.0) for 65 hr in an ice-bath. The solution had finally been centrifuged at $4 \cdot 10^4$ rev/min for 90 min in order to remove aggregates.

Protein concentration was determined by a modified Lowry method proposed by Hartree [4]. The total actin concentration was 5.5 mg/ml. An aliquot of solution having been taken, the active fraction of G-actin was polymerized by adding KCl (final concentration 100 mM) and collected by centrifugation. 3.8 mg/ml of G-actin was found to be active.

The amount of ε-ATP was determined by measuring optical absorption at 315 nm where the absorbance of ε-ATP does not depend on the binding to G-actin [1].

The total amount of ε-ATP in the G-actin-ε-ATP solution was 120 μM and the amount of ε-ATP in the dialysate was 64 μM. Then we obtained 56 μM of ε-ATP bound to G-actin. Judging from the binding constant reported by Thames et al. [2], the above value is apparently smaller than expected. Presumably, this is due to the presence of ε-ADP in the solution.

2.2. *F-actin-ε-ADP*

A pellet of F-actin-ADP (about 0.5 ml) had been depolymerized in 5 ml of cold distilled water containing ε-ATP (207 μM) and dialysed for 110 min in an ice-bath, against 50 ml of a solution containing ε-ATP (100 μM) and bicarbonate (0.5 mM). Free nucleotide having been removed by the method of Asakura [5], actin was polymerized by the addition of KCl (60 mM) and collected by centrifugation.

F-actin was then dissolved in 4 ml of cold buffer solution containing KCl (100 mM), $MgCl_2$ (1 mM) and Tris–acetate (20 mM; pH 7.0). The solution had been dialysed against a large vol of the same buffer solution for two days in the cold room.

F-actin-ε-ADP thus obtained was diluted with the same buffer solution to an appropriate concentration (0.98 mg/ml) and had been centrifuged for 30 min at 10^4 rev/min in order to remove large aggregates just prior to the fluorescence measurements. The molar ratio of ε-ADP to F-actin was 0.82.

2.3. *Determination of fluorescence decay and anisotropy*

A brief description only will be given here, since details of the method can be found elsewhere [6,7].

 North-Holland Publishing Company – Amsterdam

The exciting light was provided by a spark bursting in Nitrogene gas. The exciting beam was vertically polarized and filtered through an interference filter with a maximum transmission at 335 nm, while an interference filter centered at 410 nm was placed on the measured beam.

The experimental decay curves $i_{\parallel}(t)$ and $i_{\perp}(t)$ were measured separately and the two following curves were computed:

$$s(t) = i_{\parallel}(t) + 2\, i_{\perp}(t)$$

$$d(t) = i_{\parallel}(t) - i_{\perp}(t)$$

In the case of G-actin-ϵ-ATP, we were interested in the fluorescence of the bound ϵ-ATP. It was necessary to eliminate the contribution from ϵ-ATP free in solution. The fluorescence decay of a dialysate previously equilibrated with the G-actin-ϵ-ATP solution, was then measured. This dialysate emission had a negligible polarization. Its decay was multiplied by an appropriate factor in order to take into account the ratio of the dialysate fluorescence intensity to that of the G-actin-ϵ-ATP solution. The apparatus response function g(t) was obtained with a reference compound: the 2-5 diphenyloxazole (decay time 1.38 nsec) by a method already described [8].

When s(t) and d(t) correspond to single exponential functions, they are related to g(t) by the following convolution products:

$$s(t) = A_S\, g(t) * \exp\,[-t/\tau_S]$$

$$d(t) = A_D\, g(t) * \exp\,[-t/\tau_D]$$

with

$$\frac{1}{\tau_D} = \frac{1}{\tau_S} + \frac{1}{\theta} \qquad (1)$$

τ_S is the lifetime of the excited state and θ the Brownian correlation time. The static anisotropy $\bar{r}$ and the anisotropy at time zero r_o are given by the following relations:

$$\bar{r} = \frac{\int_o^\infty d(t)dt}{\int_o^\infty s(t)dt} \qquad (2)$$

$$r_o = A_D/A_S \qquad (3)$$

τ_S, τ_D, A_S and A_D were obtained by a computer program based on the modulation function method [9]. In the case of F-actin-ϵ-ADP, this method was not applicable, because a peak of scattering light perturbed the initial part of the decay curve. The anisotropy decay was calculated in the time range not perturbed by the scattering light, according to the relation:

$$r(t) = d(t)/s(t) \qquad (4)$$

The function Ln r(t) was fitted with a straight line by a least square procedure. θ was given by the inverse of the slope, and r_o by the ordinate of the extrapolated least square line at the abscissa of the scattering peak maximum.

3. Results and discussion

3.1. *Fluorescence lifetime of free ϵ-ATP*

The fluorescence of ϵ-ATP free in aqueous solution of neutral pH showed a single exponential decay, the lifetime of which was 25.4 ± 0.3 nsec at 25°C and 27.5 ± 0.3 nsec at 5°C.

3.2. *Fluorescence decays of ϵ-ATP bound to G-actin*

The measurements have been performed at 5°C during 10 hr. About 90% of G-actin has been found to retain the polymerizability after the fluorescence measurements.

The parameters resulting from computer analysis of the experimental data are given in table 1.

Since s(t) and d(t) are satisfactorily reproduced by single exponential decays, the rotational Brownian motion is characterized by a single correlation time given by equation (1). This would correspond to a

Table 1
Fluorescence parameters of ϵ-ATP bound to G-actin (5°C)

τ_S (nsec)	τ_D (nsec)	θ (nsec)	$\bar{r}$	r_o
34.9 ± 0.3	18.4 ± 0.3	39 ± 1	0.123	0.234

spherical molecule, the volume of which is given by the Einstein–Perrin relation, $V_e = 98\,000$ Å^3.

A spherical molecule for G-actin seems a good approximation, since electron micrographs of F-actin show molecular subunits having a very small elongation [10]. On the other hand, the dry volume V_o, computed from the mol. wt (43 000) [11,12] and the partial specific volume (v = 0.732 ml/g) [13], is equal to 53 000 Å^3. The difference between V_e and V_o should be attributed to an hydration which amounts about to 60%. Similar high hydration values have been found for several other globular proteins studied by this method [7].

Thames et al. [2] recently found a value of τ_S of 35 nsec at 10°C, which is equal to our value at 5°C. By the static polarization method, they have determined a correlation time value at 20°C which corresponds to a spherical volume 20% smaller than the volume determined in the present work. However in their determination these authors do not take into account the change of τ_S with temperature or the possibility of desorption of bound ϵ-ATP. These factors should cause a diminution of the apparent correlation time.

Following comments are worth making. Since there is a preferential binding of nucleotide to active G-actin [5,11], the denatured actin present in the G-actin-ϵ-ATP solution, (which accounts for 32% of the total actin in our present sample) did not influence the fluorescence of ϵ-ATP bound to active G-actin. Denatured actin seems to form aggregates [14] and even a small amount of aggregates would lead to and overestimation of the molecule axial ratio determined by viscosity measurements.

An asymmetrical picture of G-actin given in previous studies [13,14] may be explained in that way.

3.3. *Fluorescence decay of ϵ-ADP bound to F-actin*

Decay times of s(t) curves were found to be equal to 32 nsec and 35 nsec at 21 and 5°C respectively. Convolution calculated with these time constants fit the experimental curves except in their initial parts where a sharp peak due to the scattering light is

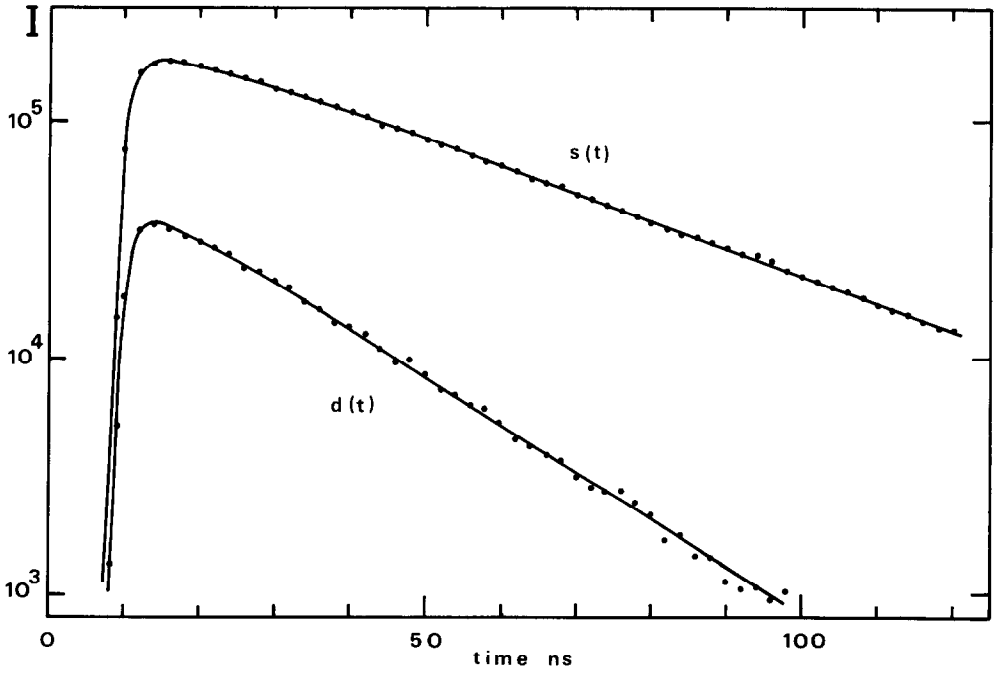

Fig.1. Decay curves, s(t) and d(t) for G-actin-ϵ-ATP at 5°C. The circles are experimental values and the solid lines are calculated convolution products.

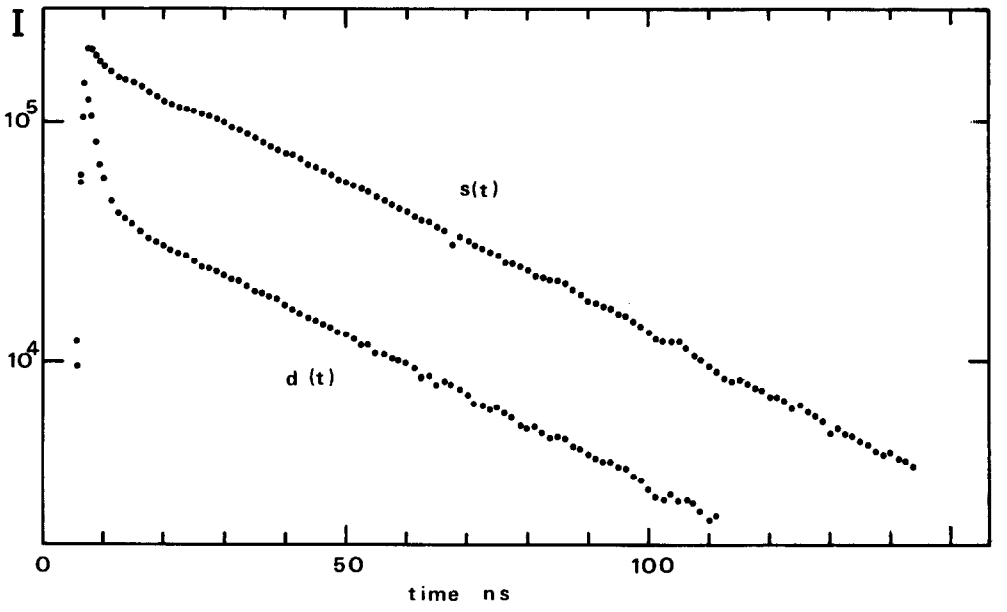

Fig.2. Decay curves, s(t) and d(t) for F-actin-ϵ-ADP at 21°C. The circles are experimental values.

present. A still more prominent peak appears in the d(t) curve because the scattering is strongly polarized. In the part which is not disturbed by the scattering light, the d(t) curve is almost parallel to the s(t) curve. This indicates that correlation times are in a range of value greater than several hundred nanosec.

We attempted to obtain more precision about correlation times by calculating the anisotropy decay curve as described in the experimental section. From such study we conclude that θ must be greater than 10 μsec, while the value of r_0 is equal to the one determined with G-actin-ϵ-ATP.

It seems likely that this r_0 value is the fundamental anisotropy characteristic of the electronic transition centered at 315 nm. This transition has a very small oscillator strength, which can explain why its fundamental anisotropy is much smaller than the maximum value 0.4 [16].

Using the static polarization method, Thames et al. found for F-actin-ϵ-ADP a smaller r_0 value than the one they found for G-actin-ϵ-ATP [2]. These authors conclude that energy transfers occur between ϵ-ADP bound to F-actin. However, our measurements have been performed with a sample which contained about 0.8 mol of ϵ-ATP per mol of actin and still we did not see any evidence of energy transfers.

We rather think that the small polarization measured by Thames et al. [2] comes from emission of free chromophores or chromophores bound to depolymerized material. We found indeed that such a material was present after polymerisation of actin, and that it could be eliminated by centrifugation. Depolymerized material was in the supernatant and was then discarded.

In conclusion, one may stress that 1-N^6 ethenoadenosine chromophore is rigidly bound to G-actin and F-actin. No motion of the chromophore in its site can be detected with a correlation time smaller than several microseconds. Furthermore the protein molecule forms a rigid structure. The behavior contrasts with the one observed in the complexes DNA-dyes where a correlation time in the nanosecond scale has been detected [15].

Acknowledgements

We thank Professor Sadron and Professor Oosawa for their interest in our work. We greatly appreciate the technical assistance of J. C. Auchet.

The present work has been supported in part by the "Délégation Générale à la Recherche Scientifique

et Technique" A.C.C "Interactions moléculaires en Biologie".

One of us (K.M) thanks the Délégation Générale à la Recherche Scientifique et technique and the Ministry of Education in Japan for the award of a fellowship.

References

[1] Miki, M., Ohnuma, H. and Mihashi, K. (1974) FEBS Lett. 46, 17–19.
[2] Thames, K. E., Cheung, H. C. and Harvey, S. C. (1974) Biochem Biophys. Res. Commun. 60, 1252–1261.
[3] Mihashi, K. (1972) Biochim. Biophys. Acta 267, 409–421.
[4] Hartree, E. F., (1972) Anal. Biochem. 48, 422–427.
[5] Asakura, S. (1961) Arch. Biochem. Biophys. 92, 140–149
[6] Wahl, Ph. (1969) Biochem. Biophys. Acta 175, 55–64.
[7] Yguerabide, J. (1972) Nanosecond fluorescence spectroscopy of macromolecules in: Methods in Enzymology (Hirs, C. H W. and Timasheff, S. N., eds) 26, part C. pp 498–578.
[8] Wahl, Ph., Auchet, J. C. and Donzel, B. (1974) Rev. Scient. Instrum. 45, 28–32.
[9] Valeur, B. and Moirez, J. (1973) J. Chim. Phys. 70, 500–506.
[10] Hanson, J. and Lowry, J. (1963) J. Mol. Biol. 6, 46.
[11] Tsuboi, K. (1968) Biochim. Biophys. Acta 160, 420–434.
[12] Elzinga, M., Collins, J. H., Kuehl, W. M. and Adelstein, R. S. (1973) Proc. Natl. Acad. Sci. U.S. 70, 2687–2691.
[13] Mihashi, K. (1964) Arch. Biochem. Biophys. 107, 441–448.
[14] Lewis, M., Maruyama, K., Carroll, W., Kominz, D. and Laki, K. (1963) Biochemistry 2, 34–39.
[15] Wahl, Ph., Paoletti, J. and Le Pecq, J. B. Proceed, Natl. Acad. Sci. (1970) 65, 417–421.
[16] Albrecht, A. C. (1961) J. Mol. Spectroscopy 6, 84.