NANOSECOND PULSE FLUOROMETRY IN POLARIZED LIGHT OF DANSYL-L-CYSTEINE LINKED TO A UNIQUE SH GROUP OF F-ACTIN; THE INFLUENCE OF REGULATORY PROTEINS AND MYOSIN MOIETY

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1. Introduction

In a previous pulse fluorometry study, we found that the anisotropy decay of ϵADP bound to F-actin was characterized by a correlation time of the order of 10 μs This correlation time was attributed to some macromolecular motion of F-actin [1].

With the same technique we performed a study of the dansyl group covalently linked to the unique reactive cysteine of F-actin [2,9–11]. In addition we examined how the anisotropy decay is influenced by components of the regulatory system (tropomyosin, troponin and Ca^{2+} ions) as well as by the binding of heavy meromyosin and the subunit S_1. This study is presented in this paper.

2. Experimental

2.1. *Materials*

Actin of rabbit skeletal muscle was prepared as previously described [3]. Purified F-actin was equilibrated with buffer containing KCl 75 mM $MgCl_2$ 2 mM, Tris-acetate 20 mM (pH 7.0). This composition of solvent was used throughout all our measurements.

Di-dansyl-L-cystine, 5-dimethyl-amino-naphtalene-I-sulfonyl-L-cystine (Sigma) was dissolved in a few drops of 1 mM bicarbonate and then quickly mixed with F-actin. F-actin concentration in the mixture was 3 to 4 mg/ml and the molar ratio of dye to actin was limited to 0.3–0.8 to minimize secondary reactions. The conjugation reaction was continued in cold room for 2–3 days with a gentle stirring. Unreacted dye was separated from F-actin by repeating ultracentrifugation. Finally the F-actin conjugate was dialysed against a large volume of buffer solution in cold room for at least 30 h. The molar ratio of the conjugated dye to F-actin was determined according to Cheung et al. [2]. Absorption and emission spectra of the conjugated dye were identical to the result of Cheung et al. [2]. Tropomyosin troponin and heavy meromyosin were prepared according to the methods previously described [4–6]. Myosin subunit S_1 was prepared by chymotryptic digestion of myosin following the method of Onodera and Yagi [7]. After fractionation on DEAE cellulose column, S_1 was purified by $(NH_4)_2SO_4$ fractionation at 67% saturation.

2.2. *Determination of fluorescence decay and anisotropy*

The exciting light was provided by a spark bursting in N_2 gas. The exciting beam was filtered through an interference filter with a maximum transmission at 336 nm, $\Delta\lambda$ = 4 nm and the emission and detected through a low band pass filter having its half transmission at 495 nm. The exciting light was not polarized. The scattering light was entirely eliminated by this set of filters. The experimental curves $i_{\parallel}(t)$ and $i_{\perp}(t)$ were alternatively measured and the two following curves were computed:

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

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Measurements were made at 22°C, unless otherwise noted. Other details of measurement were previously given [8,12].

We found that the conjugated dansyl group was gradually released into solution during the storage time at 0°C, presumably because of reduction of the SS link. In some cases an abnormal peak produced by the emission of the freed dansyl group, appeared in the initial part of the s(t) curve. Because of this deformation of s(t) a computation of the correlation time by a deconvolution method was not feasible. We then calculated the anisotropy decay in the time range of 35 ns to 110 ns where the perturbation by freed dye was negligible, according to the following relation:

$$r(t) = d(t) / s(t)$$

The function ln r(t) was fitted with a straight line by a least square procedure. The correlation time was obtained by the inverse of the line slope. The initial anisotropy factor $r_n(\circ)$ was taken equal to the straight line ordinate extrapolated at the time of the s(t) maximum. $r_n(\circ)$ is one half of the anisotropy factor corresponding to a polarized excitation.

3. Results and discussion

3.1. *Fluorescence anisotropy decay of F-actin dansyl conjugate*

Typical experimental plots of s(t), d(t) and r(t) are shown in fig.1. The average life time calculated from s(t) was 15.8 ns and the correlation time θ obtained from r(t) was 411 ± 22 ns. Since the molar ratio of the conjugated dye to actin was 0.08, low enough to eliminate the energy transfers between dansyl groups, the above value of θ corresponded to the Brownian motion of F-actin conjugate.

Cheung et al. [2] measured the static fluorescence polarization as a function of temperature, of a dansyl-F-actin conjugate. This conjugate was prepared in the same way as ours. They could not detect any measurable correlation time with this method. This is certainly due to the fact that the resolving power of the static method is lower than the resolving power of the anisotropy decay method. θ was found to vary with F-actin as shown in table 1. In another preparation in which F-actin concentration was ten times greater

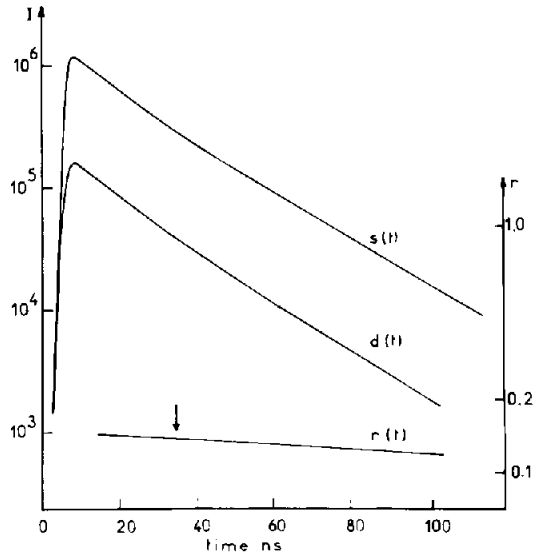

Fig.1. Pulse fluorometry in polarized light of dansyl-cystine linked to F-actin. The ordinate scales are logarithmic. The scale for s(t) and d(t) is on the left, while for the r(t) curve it is on the right of the figure.

(2 mg/ml), the correlation time was 550 ns, that is to say in the range of the other measurements.

It is important to note that the correlation time values obtained here are significantly smaller than the

Table 1

Parameters obtained from fluorescence anisotropy decay measurements of F-actin-dansyl cysteine complex (FA*) under the influences of the regulatory proteins, tropomyosin (TM), troponin (TN) and heavy meromyosin (HMM)

Proteins	θ (ns)	$r_n(\circ)$
FA* (2)	411 ± 22	0.145
FA* (2) + HMM	551 ± 88	0.148
FA* (3)	650 ± 64	0.130
FA* (3)/TM/TN (+ Ca)	409 ± 43	0.129
FA* (3)/TM/TN (– Ca)	760 ± 142	0.129

FA* (2) and FA* (3) are obtained from different preparation of F-actin. Concentration of actin is 0.2 mg/ml. The molar ratio of DN cysteine/F-actin: 0.08 for FA* (2) and 0.09 for FA* (3). The solvent composition is common to all these preparation (KCl 75 mM, $MgCl_2$ 2 mM, Tris-acetate 20 mM; pH 7.0) except (+ Ca): $CaCl_2$ 100 μM and (– Ca) EGTA 5.2 mM.

values obtained previously with ϵ ADP bound to F-actin. This can be explained by the fact that the two fluorescent chromophores are attached in different ways, to two different protein sites. One may then assume that they participate to different kinds of protein motion. Our results suggest that the cystein residue linked to the D.N.S. moiety is included in a mobile part of the protein molecule. Let us remark that this residue, cystein 373, is next to the C-terminal chain of the actin monomer [11]. There is then the possibility that only a few terminal residues praticipate to the brownian motion involved in the fluorescence measurements. The relatively high correlation time value obtained could be explained by interactions of the kinetics unit with the surrounding structure. At the present time it is not possible to estimate the size of the kinetics unit involved in this motion.

By measuring the anisotropy decay of *N*-(3-pyrene) maleimide bound to the same SH site of F-actin, Kawasaki [16] has obtained a correlation time of 560 ns which is in the range of values determined in the present work. Two different probes bound to the same cystein residue give essentially the same correlation time.

3.2. *F-actin D.N.S. cystein in presence of the regulatory system*

We measured the anisotropy decay of F-actin–D.N.S. conjugate in presence of the regulatory proteins tropomyosin and troponin in the proportion prevailing in the physiological conditions. Results are also presented in table 1.

If Ca^{2+} is present at a concentration of 100 μM, the correlation time is smaller than the correlation time measured for the same preparation of dansyl F-actin in absence of regulatory protein. On the contrary when troponin is devoid of Ca^{2+} the diminution of correlation time is suppressed (table 1). The same behaviour was repeatedly found in several preparations.

3.3. *The rigor complex of F-actin with HMM and S_1*

We also examined the fluorescence anisotropy of the F-actin-dansyl conjugate under the influence of heavy meromyosin. In all our measurements the binding of heavy meromyosin increases the correlation time (table 1). This corresponds to an immobilisation of the kinetics unit. This effect could be due either to a direct interaction of the myosin head with the C-terminal region of the actin peptide chain or to a conformational change of actin induced by interactions with myosin. This change of mobility might be in relation to the reactivity change of this SH group under the influence of myosin.

4. Conclusion

The order of magnitude of the correlation time, which characterizes the dansyl cysteine residue linked to F-actin is ten times greater than the correlation time of the G-actin monomer [1]. Still it is much smaller than the correlation times of the F-actin polymer as a whole. The dansyl chromophore reveals that the C terminal end of the actin peptide chain, is mobile.

As Ebashi and his co-workers have shown (13), Ca^{2+} triggers muscular contraction by acting on F-actin through the mediation of the regulatory proteins troponin and tropomyosin. By using spin label technique, Tonomura et al. [14] found that Ca^{2+} induces a conformational change on the troponin, tropomyosin actin complex. The quasi elastic scattering of laser light measurement of Fujime and Ishiwata [15] showed that troponin-tropomyosin F-actin has a rotational correlation time in the millisecond range which characterizes the flexibility of this complex; Ca^{2+} induces an increase of this flexibility. The present pulse fluorometry study shows an increase of mobility of the fluorescent probe induced by Ca^{2+}. It seems difficult to correlate the results of the two kinds of measurements as long as we do not know the exact nature of the fluorescent kinetics unit.

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