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# FLUORESCENCE ANISOTROPY OF LABELLED F-ACTIN

# INFLUENCE OF $Ca^{2+}$ ON THE FLEXIBILITY OF F-ACTIN

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We measured the fluorescence static anisotropy and the time-resolved fluorescence anisotropy decay of F-actin labelled with *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine at 20°C in solutions containing 100 mM KCl and free $Ca^{2+}$ at various concentrations. The average fluorescence anisotropy and the fluorescence rotational correlation time of actin decreased in the presence of micromolar concentrations of free $Ca^{2+}$. The change of the rotational correlation time of labelled actin could not be explained by a variation of the actin critical concentration. We concluded therefore that F-actin undergoes a conformational change induced by $Ca^{2+}$ binding. The binding constant was $6 \times 10^6$ $M^{-1}$.

## 1. Introduction

The actin monomer (G-actin) found at low salt concentration polymerizes into F-actin upon addition of salt. It has been shown that actin has a high-affinity site for divalent cations which regulates the affinity of ATP fixation [1]. The divalent cations which are free in solution rapidly exchange with $^{45}Ca^{2+}$ bound to G-actin, and slowly when bound to F-actin [1–5].

It has been recently reported that EGTA induces the polymerization of G-actin at low ionic strength in the presence of ATP [6–8]. On the other hand, the ATPase activity of F-actin in the presence of 90 mM KCl under an ultrasonic field varies as a function of the free $Ca^{2+}$ concentration in the micromolar range [6].

It has been shown that the rapidly reacting sulfhydryl groups of F-actin can be labelled with the fluorescent derivative *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine [9]. The fluorescence anisotropy decay of the labelled F-actin could be fitted with a sum of exponential terms, the prevailing term of which was characterized by a correlation time of several hundred nanoseconds [9–13]. We found in the present work that this correlation time, as well as the average fluorescence anisotropy, decreased when the free $Ca^{2+}$ concentration increased in the micromolar range.

## 2. Materials and methods

### 2.1. Reagents

All solutions were prepared with doubly distilled water. *N*-Iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine was purchased from Aldrich-Europe, ATP from Sigma, and other chemicals were of analytical grade from Merck.

### 2.2. Preparation of actin

Acetone-dried powder of rabbit skeletal muscle was prepared by the method of Straub [14] with

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the following modification: the regulatory proteins discovered by Ebashi and Ebashi [15] were carefully removed from myosin-extracted muscle mince by incubation in doubly distilled water overnight at 4°C before acetone treatment. G-Actin was extracted from acetone-dried powder at 4°C with 20 ml of 1 mM Tris-HCl (pH 8.0) per g dried muscle powder, and was purified by the procedure of Mommaerts [16] except that G-actin was polymerized with 30 mM KCl in order to avoid contamination by tropomyosin. SDS-polyacrylamide gel electrophoresis showed that there was no other protein present.

Actin concentration was determined by the biuret reaction using an absorbance value at 540 nm of 0.070 for 1 mg/ml of protein.

### 2.3. Preparation of actin labelled with N-iodoacetyl-N'-(5-sulfo-1-naphthyl)ethylenediamine

F-Actin was labelled with *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine in 60 mM KCl, 1 mM $MgCl_2$, 20 mM phosphate buffer (pH 7.0) at 4°C for 48 h [11,13]. The labelled F-actin was collected by centrifugation at 80000 *g* for 2.5 h and dissolved in buffer G (0.2 mM ATP, 0.5 mM mercaptoethanol, 1 mM sodium azide, 2 mM Tris-HCl (pH 8.0)), plus 0.2 mM $CaCl_2$, and dialysed against the same buffer solution. Labelled G-actin was purified by passing through Sephacryl S-200 chromatography column in order to remove the denatured actin and other contaminating proteins [13,17]. We measured the molar ratio of dye to actin by absorption, using the molar coefficient of 6100 $M^{-1}$ $cm^{-1}$ at 336 nm given by Hudson and Weber [27]. This molar ratio varied from 0.4 to 0.2 according to the preparations.

For the physical measurements, stock solutions of 1 mg/ml of F-actin were prepared as follows: the purified labelled G-actin was polymerized in solvents comprised of 20 mM Tris-HCl (pH 7.6), 1 mM sodium azide, 1 mM ATP, 0.5 mM mercaptoethanol and various concentrations of KCl. These solutions were diluted with appropriate solvents and measurements were made after several hours in order to allow the solutions to reach their equilibrium.

### 2.4. $Ca^{2+}$ buffer

Free $Ca^{2+}$ concentrations were adjusted with p*Ca* buffers containing 0.5 mM $CaCl_2$ and various amounts of EGTA. The p*Ca* values were computed according to the method of Schwarzenbach et al. [18] with a modification described by Ogawa [19].

### 2.5. Viscosity measurements

The viscosity of F-actin was measured at 20°C by means of an Ostwald viscosimeter having an outflow time of 68 s for water.

### 2.6. Absorption and steady-state fluorescence measurements

Absorption spectra and absorbances were measured with a Beckman Acta 111 spectrophotometer. Steady-state fluorescence measurements were performed with a Jobin Yvon spectrofluorimeter JY 3C connected to a Tektronix Desk computer 4051. For static anisotropy measurements, each polarized fluorescence component ($I_{vv}$, $I_{vh}$, $I_{hh}$, $I_{hv}$) was automatically measured 20 or 32 times and averaged by the desk computer. One measurement of the average anisotropy took 3 min. The excitation wavelength was 370 nm ($\Delta\lambda = 6$ nm) and the emission wavelength 480 nm ($\Delta\lambda = 10$ nm).

### 2.7. Pulse fluorimetry

The fluorescence decay experiments were carried out by the single-photoelectron counting method. The conditions of the measurements were the same as previously described [11,13]. For each experiment, we measured the following three experimental curves: the response function $g(t)$ obtained with a reference compound, the parallel $i_{\parallel}(t)$ and perpendicular $i_{\perp}(t)$ components of the polarized fluorescence [13]. The fluorescence decay $S(t)$ and the anisotrophy decay $R(t)$ were assumed to be sums of exponential functions, $\Sigma_i\ a_i \exp(-t/\tau_i)$, and $\Sigma_i\ \alpha_i \exp(-t/\theta_i)$, respectively. These curves were satisfactorily fitted by sums of two exponentials, the parameters of which were

obtained by the least-squares method [20]. The analysis was controlled by examining the appropriate residuals $\chi^2$ and deviation functions as previously described [13]. In the case of $S(t)$, the two fluorescence lifetimes $\tau_1$ and $\tau_2$ were close to each other and could not be accurately determined. More significant was the average lifetime defined as follows:

$$\langle\tau\rangle=(a_1\tau_1+a_2\tau_2)/(a_1+a_2)$$

where $a_1$ and $a_2$ are the preexponential terms. The values of the anisotropy decay parameters obtained by computation were appreciated by examining the shape of the deviation function and the residual $\chi^2$ which were defined as follows:

$$DV_k=(r_k^{ex}-r_k^c)/\sigma_k$$

$$\chi^2=\frac{1}{n}\sum_i^n (DV_k)^2$$

where $r_k^{ex}$ and $r_k^c$ were the experimental and computed values of $r(t)$ at channel $k$, $\sigma_k$ the variance of $r_k^{ex}$ and $n$ the channel number. For a good fit, $DV_k$ oscillates randomly around zero with an average amplitude of 1, and $\chi^2$ is close to unity.

## 3. Results

The static fluorescence anisotropy of F-actin (0.2 mg/ml) dissolved in buffer F (0.1 M KCl, 20 mM Tris-HCl (pH 7.6), 1 mM sodium azide, 1 mM ATP, 0.5 mM mercaptoethanol) plus 25 $\mu$M $CaCl_2$ was measured at 20°C. When we added a small volume of concentrated EGTA (100 mM) to a final concentration of 0.1 mM to this solution, the static anisotropy increased with time and reached the equilibrium value within 60 min. This process was reversible. When a small volume of concentrated $CaCl_2$ (0.5 M) was added to this solution in order to obtain a final concentration of 525 $\mu$M, the static anisotropy decreased again to its initial value (fig. 1).

We then measured the static anisotropy of F-actin solutions obtained at equilibrium, as a function of free $Ca^{2+}$ concentration. The anisotropy decreased when the p$Ca$ decreased from 6 to 7.2. The half amplitude of the variation was obtained

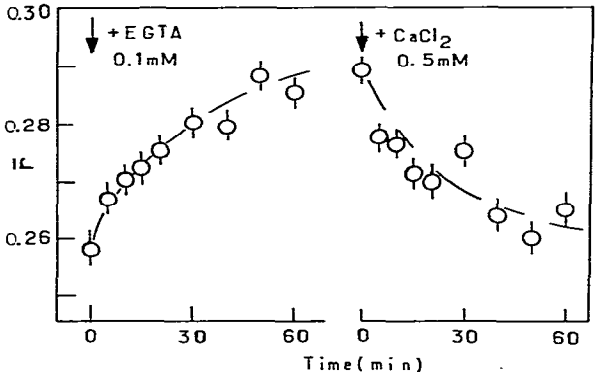


Fig. 1. Kinetics of the change of average anisotropy of F-actin after adding EGTA and $CaCl_2$ at 20°C. F-Actin concentration was 0.2 mg/ml in buffer F. Initial $Ca^{2+}$ concentration was 25 $\mu$M.

at p$Ca$ 6.8, corresponding to a $Ca^{2+}$ concentration of $1.6\times10^{-7}$ M (fig. 2).

The fluorescence and fluorescence anisotropy decays of F-actin solutions in buffer F were also measured at 20°C at various free $Ca^{2+}$ concentrations. The average fluorescence lifetime and the parameters of the anisotropy decays are given in table 1. The inverse of the longer correlation time $\theta_2$ varied in the same concentration range of free $Ca^{2+}$ as did the static anisotropy; the midpoint of the variation was also the same.

From these results we concluded that actin has a high-affinity site for $Ca^{2+}$. The binding of $Ca^{2+}$

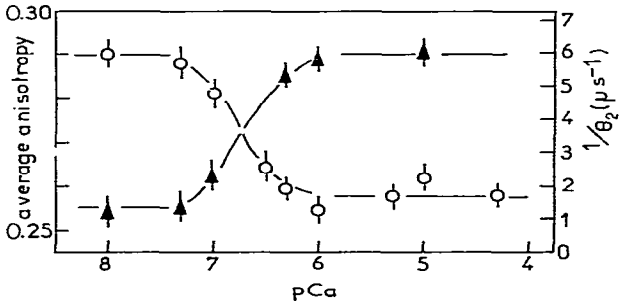


Fig. 2. Average anisotropy of F-actin (○) and the inverse of the correlation time ($1/\theta_2$) of F-actin (▲) as a function of free $Ca^{2+}$. F-Actin concentration was 0.2 mg/ml in buffer F at 20°C.

Table 1

Parameters of the anisotropy decays $R(t)$ of F-actin at various free $Ca^{2+}$ concentrations
0.2 mg/ml F-actin in buffer F, 525 $\mu$M $CaCl_2$ and various amounts of EGTA.

| p*Ca* | $\alpha_1$ | $\theta_1$ (ns) | $\alpha_2$ | $\theta_2$ (ns) | $\langle\tau\rangle$ (ns) | $\chi^2$ |
|---|---|---|---|---|---|---|
| 5 | 0.055 | 13.4 | 0.205 | 163 | 19.7 | 1.1 |
| 6 | 0.060 | 8.0 | 0.200 | 175 | 19.5 | 1.7 |
| 6.3 | 0.063 | 9.0 | 0.195 | 195 | 19.3 | 1.5 |
| 7 | 0.053 | 12.0 | 0.205 | 440 | 18.8 | 1.2 |
| 7.3 | 0.040 | 11.0 | 0.232 | 705 | 19.4 | 1.01 |
| 8 | 0.040 | 6.3 | 0.244 | 885 | 19.6 | 0.90 |

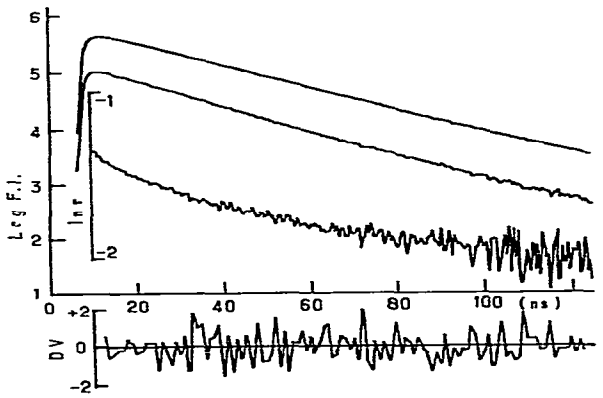


Fig. 3. Transient fluorescence of labelled F-actin (0.2 mg/ml) in buffer F plus 0.1 mM $CaCl_2$ at 20°C. From top to bottom: $s^{ex}(t)$, $d^{ex}(t)$, $r^{ex}(t)$ and the deviation function relative to $r^{ex}(t)$ with the following parameters of $R(t)$: $\alpha_1=0.057$, $\theta_1=14.0$ ns; $\alpha_2=0.207$, $\theta_2=186$ ns. F.I., fluorescence intensity.

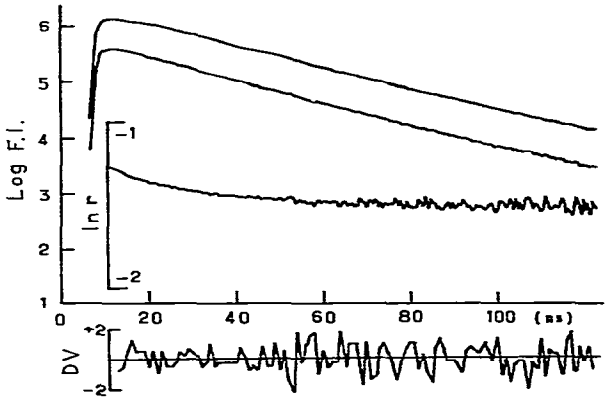


Fig. 4. Transient fluorescence of labelled F-actin (0.2 mg/ml) in buffer F plus 0.1 mM $CaCl_2$ and 0.5 mM EGTA at 20°C. The curves have the same meaning as in fig. 3. The parameters of $R(t)$ used in the deviation function are $\alpha_1=0.055$, $\theta_1=12.0$ ns; $\alpha_2=0.236$, $\theta_2=697$ ns.

Table 2

Parameters of the anisotropy decays of $R(t)$ of F-actin before and after subtraction of G-actin contribution at various protein concentration in buffer F and 0.1 mM $CaCl_2$ at 20°C

| Protein concentration (mg/ml) | Without subtraction | | | | | $C_c=0.015$ mg/ml | | $C_c=0.03$ mg/ml | |
|---|---|---|---|---|---|---|---|---|---|
| | $\alpha_1$ | $\theta_1$ | $\alpha_2$ | $\theta_2$ (ns) | $\chi_2$ | $\theta_2$ | $\chi_2$ | $\theta_2$ | $\chi_2$ |
| 0.2 | 0.057 | 14.0 | 0.207 | 186±12 | 0.98 | 192±13 | 0.99 | 221±18 | 1.00 |
| 0.25 | 0.064 | 14.3 | 0.211 | 273±45 | 1.25 | 284±46 | 1.24 | 309±97 | 1.24 |
| 0.3 | 0.054 | 13.0 | 0.221 | 294±25 | 1.00 | 306±27 | 1.00 | 338±36 | 1.00 |
| 0.4 | 0.062 | 13.4 | 0.220 | 353±22 | 1.03 | 382±27 | 1.01 | 386±25 | 1.03 |

Table 3

Parameters of the anisotropy of $R(t)$ of F-actin at various protein concentrations in buffer F plus 0.1 mM $CaCl_2$ and 0.5 mM EGTA at 20°C

| Actin concentration (mg/ml) | $\alpha_1$ | $\theta_1$ (ns) | $\alpha_2$ | $\theta_2$ (ns) | $\chi_2$ |
|---|---|---|---|---|---|
| 0.1 | 0.045 | 11.1 | 0.241 | 673± 46 | 1.17 |
| 0.2 | 0.055 | 12.0 | 0.236 | 697± 60 | 1.09 |
| 0.3 | 0.041 | 9.6 | 0.241 | 1400±320 | 1.03 |
| 0.4 | 0.050 | 12.0 | 0.236 | 1008±130 | 1.02 |

to this site in the presence of 100 mM KCl increased the mobility of the fluorescent label attached to the molecule.

The effect of $Ca^{2+}$ on the fluorescence anisotropy decay of labelled actin dissolved in buffer F was also determined at various actin concentrations. Measurements were performed at $Ca^{2+}$ concentrations above (fig. 3) and below (fig. 4) the range in which the fluorescence anisotropy varied. The results are given in tables 2 and 3.

According to the work of Oosawa and Kassai [23], the F-actin solutions contain also G-actin. In these solutions the concentration of G-actin is equal to the critical concentration.

In order to take into account, therefore, the contribution of G-actin to the emission of the F-actin solutions, we applied a correction procedure based on the critical concentration value and on the fluorescence decay properties of a G-actin solution. The critical concentration of labelled actin was determined at p*Ca* 5.6 and p*Ca* 7.6 by measuring the specific viscosity as a function of the protein concentration (table 4).

At low ionic strength (10 mM KCl); the chelating of $Ca^{2+}$ with EGTA induced an increase in the specific viscosity as previously reported by Dancker and Löw [6] and Avissar et al. [7]. In addition, we found a decrease in the critical concentration.

At high ionic strength (60 or 100 mM KCl), the difference between the specific viscosities measured at both p*Ca* values was small (table 4). It can be seen in table 4 that the critical concentration was less than 0.015 mg/ml at p*Ca* 5.6.

By absorbance measurements, Cooke [24] found a value of 0.03 mg/ml for the critical concentration of actin dissolved in 0.1 M KCl, 0.1 mM $CaCl_2$, pH 8, at 20°C. The difference between this value and ours may be due to the difference in pH.

The transient fluorescence components $i_{\parallel}(t)$ and $i_{\perp}(t)$ of a labelled G-actin solution in buffer G were measured under the same optical conditions as those under which F-actin solutions had been measured. The average lifetime of G-actin was $18.8 \pm 0.2$ ns and the anisotropy was characterized by the following parameters: $\alpha_1 = 0.05 \pm 0.01$, $\theta_1 = 3.6 \pm 2$ ns, $\alpha_2 = 0.210 \pm 0.01$, $\theta_2 = 28 \pm 1$ ns.

Table 4

Critical concentrations and reduced viscosities of labelled actin

| [KCl] (mM) | p*Ca* 5.6 | | p*Ca* 7.6 | |
|---|---|---|---|---|
| | $C_c$ (mg/ml) | $\eta_r$ (ml/g) | $C_c$ (mg/ml) | $\eta_r$ (ml/g) |
| 10 | 0.03±0.005 | 1100 | ≤0.01±0.005 | 1200 |
| 60 | 0.01±0.005 | 1370 | ≤0.005±0.005 | 1370 |
| 100 | 0.01±0.005 | 1370 | ≤0.005±0.005 | 1370 |

These results are in agreement with our previous work [13]. Here $\theta_2$ corresponds to the rotation of the G-actin molecule as a whole.

The G-actin transient curves $i_{\parallel}(t)$ and $i_{\perp}(t)$ were multiplied by appropriate factors and subtracted from the corresponding transients of F-actin solutions. The areas under the subtracted curves (G-actin) and the F-actin curves were in the ratio of $\beta_{\parallel} C_c/C_t$ for $i_{\parallel}(t)$ and $\beta_{\perp} C_c/C_t$ (for $i_{\perp}(t)$), where $C_c$ and $C_t$ are the critical concentration and the total protein concentration, respectively, and $\beta_{\parallel}$ and $\beta_{\perp}$ were calculated as follows: from the definition of fluorescence intensity $S$ and anisotropy $r$, one can introduce eqs. 1 and 2:

$$\bar{I}_{\parallel} = \bar{S}(1+2\bar{r})/3 \quad (1)$$

$$\bar{I}_{\perp} = \bar{S}(1-\bar{r})/3 \quad (2)$$

where $\bar{I}_{\parallel}$, $\bar{I}_{\perp}$, $\bar{S}$ and $\bar{r}$ are the time averages of the polarized parallel and perpendicular components, the fluorescence intensity and anisotropy, respectively. $\bar{S}$ is proportional to the quantum yield and average lifetime. Then $\beta_{\parallel}$ and $\beta_{\perp}$ are given by eqs. 3 and 4:

$$\beta_{\parallel} = \bar{S}_G(1+2\bar{r}_G)/\bar{S}_F(1+2\bar{r}_F) \quad (3)$$

$$\beta_{\perp} = \bar{S}_G(1-\bar{r}_G)/\bar{S}_F(1-\bar{r}_F) \quad (4)$$

where the ratio $\bar{S}_G/\bar{S}_F$ was taken equal to the ratio of the average lifetime of the G-actin and F-actin solutions; $\bar{r}_G$ and $\bar{r}_F$ are the average anisotropy values of G-actin and F-actin determined from the experiments.

The correction procedure we have just described, which takes into account the G-actin contribution, was applied to the fluorescence decay measurements of F-actin solutions having a low p*Ca*. Two different values of the critical concentration were assumed in these calculations, namely the limiting value measured by us (0.015 mg/ml) and the value reported by Cooke (0.03 mg/ml [24]). The correlation times $\theta_2$ obtained after the correction are given in table 2. It can be seen that even for $C_c = 0.03$ mg/ml, the increase in $\theta_2$ was less than 20%. The other parameters of the anisotropy decay (not given here) were changed by less than 10%. We can conclude that the change of $\theta_2$ in response to the change of free $Ca^{2+}$ concentration is mainly due to a conformation change of F-actin.

## 4. Discussion

As in our previous works [11,13], we found here that the anisotropy decay of F-actin solutions in which the protein was covalently labelled with *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine could be characterized by two correlation times: the shorter correlation time (of about 10 ns) and a longer one $\theta_2$ greater than 100 ns. The main finding of the present work was that $\theta_2$ varied with the free $Ca^{2+}$ concentration in solutions of F-actin containing 100 mM KCl. $\theta_2$ was about 3-times greater at p*Ca* 7.2 than at p*Ca* 6. The midpoint of the $\theta_2$ variation was found at a free $Ca^{2+}$ concentration of $1.6 \times 10^{-7}$ M. In order to interpret this finding we discuss first what kind of rotational motion can be associated with $\theta_2$. We found in a previous work [13] and we confirm here that our labelled F-actin samples have the same viscosity as the nonlabelled purified F-actin. In addition, the labelling ratio was reasonably high so that we can assume that the labelled monomers have the same polymerizability as the nonlabelled one and that they are randomly distributed in F-actin filaments. According to the theory of Oosawa and Kassai [23], the actin solutions contain G-actin, short linear oligomers and long helical F-actin polymers under our solvent conditions. The amount of G-actin present in F-actin solutions is given by the critical concentration [2,3]. We found that micromolar concentration of free $Ca^{2+}$ increases the critical concentration of actin. However, in a solvent containing 100 mM KCl, the critical concentration was small even in the presence of $Ca^{2+}$. In order to take into account the contribution of G-actin to the fluorescence emission of F-actin solutions, we used a subtraction procedure in which an upper limit of the critical concentration was introduced. We determined this critical concentration (0.015 mg/ml) by viscosity measurements. The $\theta_2$ value of F-actin was not significantly modified by this correction. This was also true when we introduced in our correction the larger critical concentration measured by Cooke (0.03 mg/ml [24]) and by Rich and Estes (0.05 mg/ml [25]). In the latter case, $\theta_2$ showed an increase of only 25%.

It has been recently shown that native G-actin exists in a polymerizable and in a nonpolymeriz-

able conformation [25,26]. According to these results, we must deduce that in the G-actin solvent used for our fluorescence decay measurements, G-actin was in the nonpolymerizable conformation. In a previous work, we did not find any significant difference between the parameters of polarized fluorescence decay of labelled G-actin solutions containing various KCl concentrations [13]. The latter results justify the subtraction procedure used here to correct the transient F-actin emission.

The theory of Oosawa and Kassai [23] predicts that the concentration of actin oligomers is much smaller than the G-actin concentration. Nevertheless, we simulated anisotropy curves with correlation times $\theta_2$ equal, respectively, to 2-, 3- and 4-times the value of the G-actin correlation time. Subtraction of these curves from the experimental anisotropy decay curves of F-actin solutions leads to smaller modifications than subtraction of the G-actin anisotropy curve. We conclude that oligomers, even if present at the same concentration as G-actin, will contribute very little to the $\theta_2$ value of F-actin solutions.

The F-actin molecules are very long helical polymers of actin monomers of various degree of polimerization [28]. The correlation time corresponding to the overall rotation of these molecules is given by $2\langle i\rangle\theta_m$ where $\langle i\rangle$ is the average degree of polymerization and $\theta_m$ the correlation time of the overall rotation of the monomer [11]. According to the measurements of Kawamura and Maruyama [28], $\langle i\rangle$ is about 400 which leads to a correlation time of 20 $\mu$s for F-actin solution at 20°C. This value is much higher than the measured $\theta_2$ value. Therefore, we conclude that $\theta_2$ characterizes mainly some internal motions of labelled F-actin. However, it remains to be elucidated why $\theta_2$ increases with actin concentration. As in a previous work [13], we attribute this variation to the interactions between F-actin filaments. Let us remark that Kawamura and Maruyama [28] found that the average degree of polymerization of F-actin decreases when actin concentration increases. Therefore, the variation of the correlation time characterizing the overall rotation of the F-actin molecule will be contrary to the variation of $\theta_2$ with the protein concentration. Since $\theta_2$ characterizes an internal motion of F-actin, its change with p*Ca* must be due to a molecular conformation change. Finally, we note that $\alpha_2$ also increased with p*Ca*. This variation came from an increase in $r_0$ and can be explained by a decrease in the amplitude of the fluorescent label motions characterized by subnanosecond correlation times. One can also attribute this change of $r_0$ to the same conformational change of the F-actin molecule.

Dancker and Löw [6] have shown that the ATPase activity of sonicated actin polymerized in 90 mM KCl increased with the concentration of free $Ca^{2+}$. The half activity of the ATPase was obtained at a free $Ca^{2+}$ concentration of $2\times 10^{-7}$ M, which is very close to the midpoint value of the variation of $\theta_2$. This suggests that the $\theta_2$ and ATPase activity measurements reveal the same conformation change controlled by $Ca^{2+}$ binding to a high-affinity site of F-actin. The affinity constant of this site is of the order of $5\text{–}6\times 10^6$ $M^{-1}$. It should be noted that this value is higher than the highest affinity constant of $Ca^{2+}$ binding to G-actin, which is of the order of $10^5$ $M^{-1}$ [21,22]. In order to explain their results, Dancker and Löw [6] proposed that F-actin polymerized by KCl becomes more flexible by addition of free $Ca^{2+}$ at micromolar concentrations. We may interpret our results as follows: $Ca^{2+}$ binding to F-actin increases the internal Brownian motion in the molecule. This effect as well as the ATPase effect under an ultrasonic field may result from a decrease in the strength of the interaction which link the protomers in the F-actin filaments.

It is well known that the divalent cation bound to G-actin is rapidly exchanged with other divalent cations present in the solution [1,2,4,5], while the exchange rate of cations bound to F-actin is very slow, the half-lifetime of the exchange being of the order of several hours. However, the rate of the average anisotropy change of F-actin was fast when EGTA was added to the solution containing $Ca^{2+}$. It reached an equilibrium value within 1 h. However, this does not mean that all bound divalent cations were released when the anisotropy had reached its plateau value. This question will be studied in future.

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