Effects of temperature on actin polymerized by Ca^{2+}

Direct evidence of fragmentation

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When the temperature is lowered from 20 to 4 °C, the specific viscosity of actin polymerized in the presence of either 4 mM- CaCl_2 or 2 mM- MgCl_2 , but not of actin polymerized in the presence of 90 mM-KCl, is decreased by 50% in the absence of free ATP. Addition of ATP restores the viscosity of the actin polymerized by Mg^{2+} , but not that of actin polymerized by Ca^{2+} , to the original value. The effect of temperature on actin polymerized in the presence of Ca^{2+} is due to (a) polymer-into-monomer conversion, (b) latero-lateral aggregation of filaments, and (c) fragmentation of the filaments. Fragmentation, as demonstrated by fractional centrifugation and electron microscopy, was the most important of these.

INTRODUCTION

The aim of this work, which extends previous findings of Grant (1965) and of Grazi *et al.* (1984), is to understand the mechanism of the effect of temperature on Ca-F-actin. The system has been studied by viscosity, light-scattering, fractional centrifugation and electron microscopy. The study has provided a direct demonstration that lowering the temperature induces fragmentation of Ca-F-actin, thus confirming the prediction by Wegner (1982).

MATERIALS AND METHODS

G-actin from rabbit muscle was prepared as described by Spudich & Watt (1971) and stored as a concentrated solution (5 mg/ml) at 0 °C in 0.2 mM-ATP/0.2 mM- CaCl_2 / 1 mM-2-mercaptoethanol / 1 mM- NaN_3 / 2 mM-Tris/HCl buffer, pH 8.2.

Actin concentration was measured from A_{290} , the absorbance of 1 mg of pure actin/ml (light path 1 cm) being taken to be 0.62 (Gordon *et al.*, 1976), or alternatively by the method of Bradford (1976). The molar concentration of G-actin was calculated on the basis of an M_r of 42000 (Collins & Elzinga, 1975).

Viscosity was measured with a thermostatically controlled Ostwald viscometer (water flow time 60 s at 20 °C). Light-scattering was determined at 546 nm with a 90° observation angle by using a thermostatically controlled Perkin-Elmer MPF3 spectrofluorimeter equipped with a Linseis recorder. The light-scattering intensity of the cells containing pure buffer was subtracted from the total scattering intensity. Centrifugation was performed in a thermostatically controlled Beckman TL100 centrifuge with a TLA100 rotor. P_i was determined by the method of Tashima & Yoshimura (1975) after precipitation of the protein with 0.15 M-trichloroacetic acid. To remove free nucleotide, ATP-G-actin solutions were treated with Dowex 1 (X8; 100–200 mesh) resin (150 mg/ml of solution) equilibrated with a solution containing 0.2 mM- CaCl_2 and 2 mM-Tris/HCl buffer, pH 8.2.

For electron-microscopic observations, one drop of a 12 μM -Ca-F-actin solution was diluted with 9 drops of a solution containing 10 mM-Tris/HCl buffer, pH 8.2, and 4 mM- CaCl_2 ; one drop of the diluted solution was immediately applied on a carbon-coated 400-mesh grid and after 60 s stained with 5 drops of 1% (w/v) uranyl acetate, pH 4.25.

The supernatant solutions obtained after 2 and 6 min of centrifugation at 95000 rev./min of a 12 μM -Ca-F-actin solution were applied without dilution to the grids and stained as described above. The actin solutions were transferred by a Pasteur pipette to avoid mechanical damage to the filaments. Electron microscopy was performed on a Hitachi H-800 electron microscope.

RESULTS

Effect of temperature on Ca-, Mg- and K-F-actin

In the absence of free ATP, the viscosity of actin solutions, polymerized with either 2 mM- MgCl_2 or 4 mM- CaCl_2 , decreases when the temperature is lowered from 20 to 4 °C. The viscosity of the solution remains unchanged when the actin had been polymerized in 90 mM-KCl. The initial viscosity of Mg-F-actin is rapidly (10 min) restored when 0.2 mM-ATP is added to the samples at 4 °C. This phenomenon is accompanied by the release of P_i from ATP. Addition of ATP has no effect, however, on the viscosities of either Ca-F-actin or K-F-actin (Table 1).

This experiment indicates that the properties of F-actin depend on the conditions of the polymerization (Strzelecka-Golaszewska *et al.*, 1978; Ferri & Grazi, 1981) and show that Ca-F-actin is a convenient system for the study of effects of temperature, even in the presence of free ATP.

The mechanism of the effect of temperature on Ca-F-actin

The effect of temperature on the viscosity of Ca-F-actin was studied as a function of actin concentration, and it became clear that it could not be explained by the simple

Abbreviations used: Ca-F-actin, Mg-F-actin and K-F-actin, actin polymerized in the presence of 4 mM- CaCl_2 , 2 mM- MgCl_2 and 90 mM-KCl respectively.

Table 1. Effect of temperature on the viscosity of F-actin

ATP-G-actin (12 μM), dissolved in a solution containing 20 μM -ATP, 20 μM - CaCl_2 and 2 mM-Tris/HCl buffer, pH 8.2, was treated with Dowex-1 resin to remove free ATP and was polymerized at 20 °C in the presence of 90 mM-KCl (K-F-actin) or 2 mM- MgCl_2 (Mg-F-actin) or 4 mM- CaCl_2 (Ca-F-actin). The temperature was then lowered to 4 °C. After 30 min, 0.2 mM-ATP was added. Samples were taken for determination of viscosity at 20 and 4 °C before, and 10 min after, the addition of ATP. P_i released is the difference between the amount found immediately and 10 min after the addition of ATP.

	Specific viscosity ($\eta_{\text{sp.}}$) at:			P_i released after addition of 0.2 mM-ATP (μM)
	20 °C	4 °C	4 °C, after addition of 0.2 mM-ATP	
K-F-actin	0.36	0.33	0.34	0
Mg-F-actin	0.35	0.18	0.36	2.4
Ca-F-actin	0.35	0.16	0.16	—

Table 2. Effect of temperature on viscosity as a function of actin concentration

Actin was polymerized at 20 °C in a solution containing 10 mM-Tris/HCl buffer, pH 8.2, 40 μM -ATP and 4 mM- CaCl_2 . The temperature was then lowered to 4 °C and samples were taken for determination of viscosity at different times, the final value being reached after 30 min of incubation at 4 °C.

[Actin] (μM)	Specific viscosity ($\eta_{\text{sp.}}$) at:	
	20 °C	4 °C
24	0.82	0.56
12	0.35	0.16
6	0.19	0.09
3.6	0.13	0.06

polymer-into-monomer conversion (i.e. by the increase of the critical concentration of actin) (Table 2). If this was the case, then after allowing for the decrease of viscosity of 24 μM -actin the expected specific viscosity of 6 and 3.6 μM -actin at 4 °C would have been zero.

This conclusion was confirmed when viscosity changes were compared with changes in the amount of protein sedimented after 10 min of centrifugation at 95000 rev./min. As Fig. 1 shows, the specific viscosity of a 12 μM -Ca-F-actin solution fell from 0.35 to 0.16 when the temperature was lowered from 18 to 4 °C. The amount of actin found in the supernatant solution after centrifugation at 95000 rev./min for 10 min was 1.4 μM at 18 °C and 2.3 μM at 4 °C. (Under these conditions of centrifugation, ATP-G-actin was fully recovered in the supernatant solution.) Light-scattering increased from 10.5 arbitrary units (a.u.) at 18 °C to 11.5 a.u. at 4 °C. 'Intrinsic light-scattering' relative to polymer population (calculated as the ratio between light-scattering and the difference between total actin and actin found in the supernatant solution after 10 min of centrifugation at

Fig. 1. Effect of temperature on viscosity, sedimentation and light-scattering of Ca-F-actin

ATP-G-actin (12 μM) was polymerized at 18 °C in a solution containing 10 mM-Tris/HCl buffer, pH 8.2, 40 μM -ATP and 4 mM- CaCl_2 . The temperature was then lowered stepwise to 14, 10.5, 5 and 4 °C. Measurements were made 30 min after attainment of each temperature. Protein was determined in the supernatant solution obtained after 10 min of centrifugation at 95000 rev./min.

Fig. 2. Fractional centrifugation of Ca-F-actin at 20 and 4 °C

ATP-G-actin (12 μM) was polymerized at 20 °C in a solution containing 10 mM-Tris/HCl buffer, pH 8.2, 40 μM -ATP and 4 mM- CaCl_2 . After 120 min a sample was taken and cooled at 4 °C for 60 min. Both samples were then submitted to centrifugation at 95000 rev./min for the periods indicated in the Figure. Protein in the supernatant solution was then assayed by the Bradford (1976) method. ●, Samples at 20 °C; ○, samples at 4 °C.

Fig. 3. Electron micrographs of Ca-F-actin negatively stained with uranyl acetate

a, Ca-F-actin at 20 °C; b, Ca-F-actin at 4 °C; c, Ca-F-actin at 4 °C: supernatant solution after 2 min of centrifugation at 95000 rev./min; d, Ca-F-actin at 4 °C: supernatant solution after 6 min of centrifugation at 95000 rev./min. The bar (shown in d) represents 276 nm in b and 368 nm in a, c and d.

95000 rev./min) increased even more. It was 0.99 a.u./nmol of actin in the polymers at 18 °C and 1.18 a.u./nmol of actin in the polymers at 4 °C. This shows that light-scattering, even though less sensitive than viscosity to changes in polymer population, is nevertheless not fully insensitive.

Fractional centrifugation of Ca-F-actin solutions kept at 20 °C and at 4 °C confirmed that the polymer population differs considerably at the two temperatures. After 2 and 4 min of centrifugation at 95000 rev./min,

60.8 and 81.6% of the actin respectively was found in the sediment at 20 °C, and only 28 and 53.3% of actin respectively was found in the sediment at 4 °C (Fig. 2).

Characterization, by electron microscopy, of Ca-F-actin at 20 and at 4 °C

Electron-microscopic observation shows that Ca-F-actin at 20 °C is characterized by long filaments as well as by ropes formed by latero-lateral aggregation of single filaments (Fig. 3a). At 4 °C the rope-like structures

prevail over single filaments (Fig. 3b). The increase of the latero-lateral aggregation between filaments certainly contributes to decrease the viscosity of the solution. This phenomenon was noticed also in the course of the formation of the actin-protamine heteropolymers (Grazi *et al.*, 1982).

Ca-F-actin at 4 °C was also centrifuged for 2 min and 6 min at 95000 rev./min, and the supernatant solutions, which contained 71.6% and 41% of the total protein respectively, were observed in the electron microscope. After 2 min of centrifugation, fragments of the rope-like structures and amorphous aggregates of irregular shape and dimension represented a substantial proportion of the polymer population (Fig. 3c). After 6 min of centrifugation, amorphous aggregates represented by far the greatest proportion of the polymer population in the supernatant solution (Fig. 3d).

DISCUSSION

In the absence of free ATP, when the temperature is lowered from 20 to 4 °C, both Mg-F-actin and Ca-F-actin depolymerize reversibly. Addition of ATP, at 4 °C, to the mixture promotes repolymerization of Mg-F-actin but not of Ca-F-actin. This is due to the exceedingly slow rate of exchange of the actin-bound nucleotide at high Ca^{2+} concentration (Kuehl & Gergely, 1969). The viscosity of K-F-actin is insensitive to these changes of temperature.

The effect of temperature on Ca-F-actin is only partially explained by the conversion of the polymers into monomers. Two other factors contribute to the phenomenon: latero-lateral aggregation of the filaments and, most important, fragmentation. This event was documented by fractional centrifugation as well as by electron microscopy. Furthermore, comparison of the behaviour of Mg-F-actin and of Ca-F-actin in the presence of free ATP showed that the rate of exchange of the actin-bound nucleotide is an important regulatory factor of the fragmentation process, at least at low temperature.

Recently, spontaneous fragmentation of actin filaments was suggested by Wegner (1982) and by Wegner & Savko (1982) on purely kinetic grounds. Fragmentation was predicted to occur when actin is made to polymerize in the presence of either Ca^{2+} or Mg^{2+} , but not of K^{+} . Cooper *et al.* (1983) presented similar conclusions.

The analysis made by Wegner (1982) and Wegner & Engel (1975), which treats fragmentation as an irreversible process, was criticized by Frieden & Goddette (1983). The latter authors argue not only that fragmentation must be a reversible process because of re-annealing, but also that, under some conditions, re-annealing may be rapid enough so that fragmentation is not apparent. They observed that the rate of re-annealing depends on the protein concentration, such that the effect of fragmentation is expected to be larger at low actin concentration.

We think, however, that the most serious criticism is the methodological one. To provide a faithful description of the polymerization process, the increase of light scattering, used by Wegner (1982) in his study, should be proportional to the amount of the monomers incorporated into the polymers, and insensitive to changes in the average length of the polymer population. On this premise, it is hard to understand how the method can give information on fragmentation. In fact fragmentation, which occurs when the temperature is lowered from 20 to 4 °C, increases (and not vice versa) the 'intrinsic light-scattering' of a 12 μM -Ca-F-actin solution from 0.99 to 1.18 a.u./nmol of actin.

In spite of all these criticisms, Wegner's (1982) prediction was confirmed. Ca-F-actin spontaneously fragments at low temperature. The same probably occurs also with Mg-F-actin, which also depolymerizes at low temperature.

We wish to conclude this paper by stressing that the polymerization of actin is a phenomenon too complex to be explained by observation of a single parameter. A realistic description requires the integrated use of measurements of all the available parameters.

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