Kinetic heterogeneity of F-actin polymers

Further evidence that the elongation reaction may occur through condensation of the actin filaments with small aggregates

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We have shown that F-actin, polymerized in 50 mM-KCl at 20 °C and pH 8.0, can be resolved by centrifugation into two polymer populations, which differ morphologically as well as kinetically. The first population represents about 10 % of the overall polymer and is composed of small amorphous aggregates. It rapidly exchanges the bound nucleotide with free ATP in the medium, either directly or through the monomers. The second population is composed of long actin filaments. These are labelled by free ATP in the medium only through condensation with labelled small amorphous aggregates.

INTRODUCTION

The original theory on fibrous and globular aggregation of charged macromolecules predicts the formation, above a critical concentration, of small amounts of globular aggregates before long fibrous aggregation (Oosawa, 1957).

According to this theory, the kinetic treatment of the polymerization of actin is generally based on the following assumptions: (a) actin filaments represent almost the totality of the polymeric species (Ooi, 1960; Kasai *et al.*, 1960); (b) polymer elongation occurs by addition of monomers only (Arisaka *et al.*, 1975; Wegner & Engel, 1975; Frieden & Goddette, 1983); (c) the elongation rate constant is independent of the polymer length for oligomers above the dimer (Wegner & Engel, 1975), the trimer (Arisaka *et al.*, 1975) and the hexamer (Frieden & Goddette, 1983).

It is a common experience, however, that actin filament preparations, when observed by electron microscopy, reveal the presence of significant amounts of very short filaments as well as of amorphous aggregates (Hanson & Lowy, 1963; Depue & Rice, 1965; Nachmias *et al.*, 1970; Mabuchi, 1976; Bryan & Kane, 1978; Cooke, 1982; Strzelecka-Golaszewska *et al.*, 1985).

We show here that these aggregates can be resolved, by differential centrifugation from long filaments, giving rise to two polymer populations, which are morphologically as well as kinetically different. The first actin population, composed of amorphous aggregates, is capable of rapidly taking up free ATP from the medium. The second actin population, composed of long actin filaments, is labelled only indirectly through condensation with labelled amorphous aggregates. This finding gives further support to our proposal (Grazi *et al.*, 1983, 1984; Grazi, 1984) that filament elongation takes place, not only by reaction with monomers, but also by condensation of the filaments with short amorphous aggregates.

MATERIALS AND METHODS

G-actin from rabbit muscle was prepared as described by Spudich & Watt (1971) and further gel-filtered through Sephadex G-150 (McLean-Flechler & Pollard, 1980). Actin was kept at the concentration of 5 mg/ml in 0.2 mM-ATP/0.2 mM-CaCl₂/0.5 mM-2-mercaptoethanol/2 mM-NaN₃/2 mM-Tris/HCl buffer, pH 8.2. Actin concentration was measured from the absorbance at 290 nm, the absorbance of 1 mg of pure actin/ml (light-path 1 cm) being taken to be 0.62 (Gordon *et al.*, 1976). Alternatively, the Coomassie Blue method was used (Bradford, 1976). Molar concentrations of G-actin were calculated on the basis of an *M_r* of 42000 (Collins & Elzinga, 1975). [2,8-³H]ATP ammonium salt (specific radioactivity 50 Ci/nmol) was purchased from Amersham International, Amersham, Bucks., U.K.

Centrifugations were performed at 366000 *g* in the TLA-100 rotor of the Beckman TL-100 centrifuge for the times indicated in each individual experiment, plus the acceleration and the deceleration times. The TL-100 centrifuge accelerates and decelerates to and from full speed in 1 min.

To measure the exchange of labelled ATP with ADP bound to polymeric actin, F-actin solutions (0.2 ml) were sedimented by centrifugation and the protein pellets were dissolved in 0.2 ml of 5 mM-Tris/HCl buffer, pH 8.0. Protein content was then determined by the Coomassie Blue method (Bradford, 1976), and radioactivity was measured in 10 ml of a Packard emulsifier scintillation cocktail in a Packard Tri-Carb liquid-scintillation counter.

For electron-microscopic observations, 1 drop of the polymerized actin solution was diluted with 9 drops of the polymerization buffer. 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 same procedure was employed for the analysis of the supernatant solution

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obtained after centrifugation for 3 min at 366 000 *g* of F-actin polymerized in 10 mM-KCl. The supernatant solution obtained after centrifugation for 3 min of F-actin polymerized in 50 mM-KCl was, however, applied to the grid without dilution. The actin solutions were transferred by Pasteur pipette to minimize mechanical damage to the filaments. Electron microscopy was performed with a Hitachi H-800 electron microscope.

RESULTS

Resolution of different polymeric actin populations by differential centrifugation

The polymeric population of actin, in 50 mM-KCl, is clearly heterogeneous. Long actin filaments are found, together with a large number of small aggregates 8–12 nm wide and 10–40 nm long (Fig. 1*a*). These aggre-

Fig. 1. Electron micrographs of F-actin polymerized in KCl and negatively stained with uranyl acetate

(*a*) F-actin polymerized in 50 mM-KCl; (*b*) supernatant solution obtained by centrifugation for 3 min at 366 000 *g* of F-actin polymerized in 50 mM-KCl; (*c*) F-actin polymerized in 10 mM-KCl; (*d*) supernatant solution obtained by centrifugation for 3 min at 366 000 *g* of F-actin polymerized in 10 mM-KCl. The bar represents 500 nm.

gates are easily separated from the long filaments by centrifugation for 3 min at 366 000 *g*. The supernatant solution after centrifugation contains only small aggregates (Fig. 1*b*) [Please notice that the sample, in this case, was not diluted 10-fold, as in the case of the sample of Fig. 1*a*), but was applied directly to the grid.] After 10 min of centrifugation at 366 000 *g* the aggregates are also sedimented (results not shown). The comparison of the protein content of the pellets obtained after 3 min and after 10 min of centrifugation gives estimates of the relative amount of the small aggregate fraction compared with the total polymers. The protein content of the 3 min protein pellet (obtained from 1 ml of a 500 $\mu\text{g/ml}$ actin solution) is found to be $366 \pm 30 \mu\text{g}$ (mean $\pm$ S.E.M. for 48 samples) and that of the 10 min protein pellet $407 \pm 36 \mu\text{g}$ (mean $\pm$ S.E.M. for 43 samples). The amount of the small aggregates (difference between the 10 min and the 3 min

protein pellets) is thus about the 10% of the total polymeric forms.

The possibility of resolving the polymeric populations of a 24 μM -actin solution, polymerized for 16 h in 10 mM-KCl, was also investigated. It was found that, after 3 min of centrifugation at 366 000 *g*, the protein pellet (obtained from 1 ml of a 1 mg/ml actin solution) contained $234 \pm 43 \mu\text{g}$ of protein (mean $\pm$ S.E.M. for 26 experiments) and the protein pellet obtained after 100 min of centrifugation contained $511 \pm 16 \mu\text{g}$ of protein (mean $\pm$ S.E.M. for 12 experiments). Electron micrographs of the actin solution (Fig. 1*c*) showed that the polymeric species were composed of filaments as well as of a very large amount of amorphous aggregates. The amorphous aggregates were of quite varied sizes, from very large aggregates to aggregates of small dimensions (8–12 nm wide and 10–40 nm long). The supernatant solution after 3 min of

Fig. 2. Kinetic analysis of the nucleotide exchange on F-actin polymerized in 50 mM-KCl

For this 12 μM -ATP-G-actin, in 0.1 mM-ATP/0.1 mM- CaCl_2 /0.5 mM-2-mercaptoethanol/0.4 mM- NaN_3 /50 mM-KCl/5 mM-Tris/HCl buffer, pH 8.0, was polymerized for 16 h at 20 $^\circ\text{C}$ and pH 8.0. At the start of the experiment, 0.005 ml of 40 μM -[2,8- ^3H]ATP (specific radioactivity 500 000 d.p.m./nmol) was added per ml of solution. At various time intervals 0.2 ml samples (in quadruplicate) were taken and centrifuged either for 3 min or for 3 min plus an additional 7 min, at 366 000 *g*. The pellets were dissolved in 0.2 ml of 5 mM-Tris/HCl buffer, pH 8.0. Specific radioactivity was determined as described in the Materials and methods section. Δ , 3 min pellets; $\bullet$, 10 min pellets. Inset: $\ln(1 - x_t/x_\infty)$ is plotted against time; straight lines were fitted by the least-squares method.

centrifugation (Fig. 1d) was composed of small aggregates and, rarely, of short filaments. It thus appears that both the large aggregates and the long filaments are sedimented after 3 min of centrifugation.

Exchange of free nucleotide with the nucleotide bound to F-actin polymerized in 50 mM-KCl

The exchange of free ATP with ADP bound to F-actin, polymerized for 16 h in 50 mM-KCl at pH 8.0 and 20 °C, was studied. The increase, as a function of time, of the specific radioactivity of the nucleotide bound to F-actin was determined on the polymer pellets, obtained by centrifugation either for 3 min at 366 000 *g* (long filaments) or for 10 min at the same speed (long filaments plus short filaments plus small aggregates) (Fig. 2).

The rate of exchange of labelled nucleotide was then calculated from the equation:

$$-\ln(1 - x_t/x_\infty) = k_{\text{obs.}} \cdot t$$

by plotting $\ln(1 - x_t/x_\infty)$ against time (Fig. 2 inset). In this equation x_t is the specific radioactivity, at time t , of the nucleotide bound to the pellet, and x_∞ is the specific radioactivity of the nucleotide bound to the pellet at the isotopic equilibrium and is given by:

$$x_\infty = \text{initial specific radioactivity of free nucleotide} \times \frac{[\text{free nucleotide}]}{([\text{free nucleotide}] + [\text{actin-bound nucleotide}])}$$

As shown in Fig. 2, the specific radioactivity of the 3 min pellets (long filaments fraction) begins to increase after a lag of about 40 min, with $k_{\text{obs.}} = 8.3 \times 10^{-4} \text{ min}^{-1}$. The gross exchange rate of the nucleotide can be calculated from the equation (Frost & Pearson, 1961):

$$\text{Rate} = k_{\text{obs.}} \cdot ab/(a+b)$$

(where a is the concentration of free nucleotide and b is the concentration of actin-bound nucleotide) and was found to be $8.92 \times 10^{-9} \text{ M}^{-1} \cdot \text{min}^{-1}$.

The exchange of the nucleotide in the 10 min pellets (long filaments plus short filaments plus small aggregates) follows a different pattern. It is characterized by a rapid phase, which involves about 10 % of the whole polymer population (a fraction that corresponds to the relative amount of the small aggregates) and continues with a slow phase ($k_{\text{obs.}} = 7.67 \times 10^{-4} \text{ min}^{-1}$), with a nucleotide gross exchange rate of $8.2 \times 10^{-9} \text{ M}^{-1} \cdot \text{min}^{-1}$.

Exchange of free nucleotide with the nucleotide bound to F-actin polymerized in 10 mM-KCl

The exchange of free ATP with ADP bound to F-actin, polymerized for 16 h in 10 mM-KCl at 20 °C and pH 8.0, was also studied. The increase of the specific radioactivity, as a function of time, was determined on the polymeric fractions obtained by centrifugation either for 3 min or for 8 min at 366 000 *g* (Fig. 3).

Fig. 3. Kinetic analysis of the nucleotide exchange on F-actin polymerized in 10 mM-KCl

For this 24 μM -ATP-G-actin, in 0.1 mM-ATP/0.1 mM- CaCl_2 /0.5 mM-2-mercaptoethanol/0.4 mM- NaN_3 /10 mM-KCl/5 mM-Tris/HCl buffer, pH 8.0, was polymerized for 16 h at 20 °C and pH 8.0. At the start of the experiment, 0.005 ml of 40 μM -[2,8- ^3H]ATP (specific radioactivity 500 000 d.p.m./nmol) was added per ml of actin solution. At various time intervals 0.2 ml samples (in duplicate) were taken and centrifuged either for 3 min ($\blacktriangle$ and $\bullet$, two experiments) or for 8 min ($\triangle$) at 366 000 *g*. The pellets were dissolved in 0.2 ml of 5 mM-Tris/HCl buffer, pH 8.0. Specific radioactivity was determined as described in the Materials and methods section. Inset: $\ln(1 - x_t/x_\infty)$ is plotted against time.

The plot of $\ln(1 - x_t/x_\infty)$ against time (Fig. 3) shows that both the 3 min and the 8 min fractions display very similar, biphasic exchange patterns, characterized by the rate constants $k_{\text{obs.}} = 3.1 \times 10^{-2} \text{ min}^{-1}$ and $k_{\text{obs.}} = 4.1 \times 10^{-3} \text{ min}^{-1}$ respectively, which correspond to nucleotide gross exchange rates of $0.6 \times 10^{-6} \text{ M}^{-1} \cdot \text{min}^{-1}$ and $0.79 \times 10^{-7} \text{ M}^{-1} \cdot \text{min}^{-1}$ respectively.

DISCUSSION

F-actin, polymerized in 50 mM-KCl, was resolved, by differential centrifugation, into two polymer populations, which display different morphology as well as different kinetic properties.

The following points should be considered. (a) We have shown that F-actin polymers, usually considered to be kinetically homogeneous, display different kinetic properties, which can be attributed to morphologically different polymers. This is evidence of gross structural differences of the two polymer populations, differences that may influence other kinetic properties as well, such as the elongation reaction. (b) Our separation method, which must, in any case, be monitored by electron microscopy, is, however, not of general use. We have failed, in fact, to resolve the kinetically different polymer populations present in F-actin polymerized in 10 mM-KCl. (c) The incorporation of the label into the long F-actin filaments, obtained in 50 mM-KCl, takes place with a delay of at least 40 min, whereas no delay is observed in the labelling of the amorphous aggregates. This behaviour is explained only by assuming that the label is not incorporated directly into the long filaments, but is incorporated indirectly via the small amorphous aggregates. This implies that the elongation reaction may occur also by condensation of the filaments with small aggregates and not only by reaction with monomers. This proposal has been made previously (Grazi *et al.*, 1983, 1984; Grazi, 1984) and is strengthened by the present finding. (d) With respect to points (a), (b) and (c) above, it is unclear whether labelling of the amorphous aggregates occurs directly or through the actin monomers. It is clear, however, that labelling of the long

filaments does not occur directly from the nucleotide of the medium.

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