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# SUBSTOICHIOMETRIC CONCENTRATIONS OF ATP-G-ACTIN ARE REQUIRED TO ANNEAL ACTIN POLYMERIZED BY CALCIUM IONS

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Summary : At 3°C and pH 7.0, the addition of 40 nM ATP-G-actin to F-actin (12 $\mu$M as the monomer), polymerized in the presence of 4 mM $CaCl_2$, determines a substantial and rapid increase of the viscosity of the solution, which is accompanied by the incorporation of the ATP-G-actin added into the polymer.

The hypothesis that the presence of ATP-actin at the filament end(s) promotes the annealing reaction is substantiated by the finding that, after the addition of ATP-G-actin, the average filament length is increased. This finding is relevant, not only because it provides evidences in favour of the existence of annealing but also because it shows that the concentration of ATP-G-actin influences the filaments length distribution through a mechanism different from the elongation reaction.


Because of the presence of many ancillary proteins, it is difficult to compare the features of microfilaments *in vivo* with those of actin filaments *in vitro* . Nevertheless, it is hoped that some of the properties of the microfilaments can be traced back to the intrinsic properties of F-actin. Among these, spontaneous fragmentation and annealing are definitely important in the determination of the rheological properties of the actin network.

We have now found conditions in which the actin filament length distribution as well as the steady state of the fragmentation-annealing process are influenced by the concentration of ATP-G-actin. This behaviour is particularly evident at low temperature, with actin polymerized by 4 mM $CaCl_2$.

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It is likely, however, that a similar behaviour is displayed in other conditions as well.

## MATERIALS AND METHODS

G-actin from rabbit muscle was prepared as described by Spudich and Watt (1) and further gel-filtered through Sephadex G-150 (2). Actin was kept at the concentration of 5 mg/ml in 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM 2-mercaptoethanol, 2 mM $NaN_3$, 2 mM tris-HCl buffer, pH 8.2 ("dialysis buffer"). 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 (3). Alternatively, the Coomassie Blue method was used (4). Molar concentrations of G-actin were calculated on the basis of an $M_r$ of 42,000 (5). N-Ethylmaleimide-actin (6) was prepared allowing to react $^3H$-labelled 0.5 mM N-ethylmaleimide with 0.1 mM ATP-G-actin dissolved in 2 mM tris-HCl buffer, containing 0.2 mM ATP, 0.2 mM $CaCl_2$ and 0.2 mM ascorbate. The reaction was stopped by the addition of 2 mM mercaptoethanol and the labelled protein was thoroughly dialysed against the "dialysis buffer". Under these conditions, approximately one cysteine residue per molecule of actin was modified by ethylmaleimide. N-(1-pyrenyl)iodoacetamide labelled actin was prepared essentially as described by Kouyama and Mihashi (7). Centrifugations were performed in the TLA-100 rotor of the Beckman TL-100 centrifuge for the times indicated in each individual experiment. To measure the incorporation of labelled actin into polymeric actin, F-actin solutions (0.2 ml) were sedimented by centrifugation and the protein pellets were dissolved in the "dialysis buffer". Protein content was then determined by the Coomassie Blue method and radioactivity was measured in 10 ml of a Packard emulsifier scintillation cocktail in a Packard Tri-Carb liquid-scintillation counter. Viscosity was measured with Ostwald viscosimeters (water flow time 60 s at 20°c), maintained in thermostated water baths. Viscosity measurements of the same incubation mixture, at different times, were always performed on newly taken samples. Transfer of the samples was performed by means of large tip, digital adjusted micropipettors to avoid mechanical breaking of the filaments. Rapid temperature adjustements, either in the process of cooling or warming actin solutions, were followed by means of a thermocouple.
The relative length as well as the relative number concentration of actin filaments were measured by following, at 20°C, the decay of the fluorescence of samples of pyrenyl-F-actin (12 μM as the monomer) which had been diluted 100 times with the same buffer employed for the polymerization (8).
Fluorescence measurements were performed with a Perkin Elmer MPF3 spectrofluorimeter equipped with a Linseis recorder. A cut off filter for the removal of the scattered light (wavelength below 390 nm) was inserted in front of the photomulti-

plier. Excitation was at 365 nm, emission was detected at 407 nm.

RESULTS

It is known that actin, polymerized in the presence of 4 mM $CaCl_2$, undergoes spontaneous fragmentation with the concomitant decrease of viscosity,when the solution is cooled to 3°C (9, 10). We show now that the addition to this cold F-actin solution (12 μM as the monomer) of 0.12 μM ATP-G-actin increases specific viscosity to the value found at 20°C (Fig. 1). The phenomenon is usually complete in about 5 min, lasts for 10 to 20 min, then viscosity returns to the base value. Addition of an equal volume of the "dialysis buffer" to F-actin at 3°C does not change the viscosity of the solution. The increase of viscosity is accompanied by the incorporation of added ATP-G-actin into the polymer (Table I).

The effect of the addition of ATP-G-actin is much more pronounced at pH 7.0 than at pH 8.2. At pH 7.0, in fact, the effect of cooling is reversed even by the addition of 30 nM ATP-G-actin to a 12 μM (as the monomer) F-actin solution (Table II).

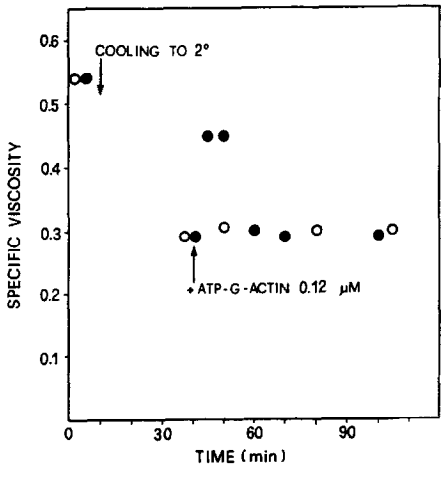


Fig. 1. Effect of the addition of ATP-G-actin on the viscosity of F-actin at 3°C - The incubation mixtures (20 ml) contained 12 μM ATP-G-actin, 20 μM ATP, 4 mM $CaCl_2$ and 10 mM tris-HCl buffer. pH was 7.5. After 3 hours of polymerization at 20°C, the mixtures were cooled to 3°C. After 30 min either 0.02 ml of 120 μM ATP-G-actin ( ● ) or an equal volume of the dialysis buffer ( ○ ) were added. Viscosity was then measured.

TABLE I

The repolymerization of F-actin by ATP-G-actin, at 3°C, is accompanied by the incorporation of the monomer into the polymer

| Addition | Temperature | Specific Viscosity | ATP-G-actin bound ($\mu$M) |
|---|---|---|---|
| None | 20°C | 0.38 | ----- |
| None | 3°C | 0.24 | ----- |
| $^3$H N-ethylmaleimide ATP-G-actin | | | |
| 0.09 $\mu$M | 3°C | 0.30 | 0.055 |
| 0.24 $\mu$M | 3°C | 0.39 | 0.11 |

12 $\mu$M ATP-G-actin was polymerized for 3 hours at 20°C in the presence of 20 $\mu$M ATP, 4 mM $CaCl_2$ and 10 mM tris-HCl. pH was 7.5. The solutions were then cooled to 3°C. After 30 min of incubation $^3$H N-ethylmaleimide-ATP-G-actin (specific radioactivity 8780 dpm/nmol), at the concentrations indicated, was added. 10 min after the addition specific viscosity was measured. To measure the amount of $^3$H N-ethylmaleimide-G-actin taken up by the polymer, 0.2 ml samples were taken, centrifuged for 10 min at 366,000 g, pellets were dissolved in 0.2 ml of the "dialysis buffer" and radioactivity was measured as described in the methods section.

TABLE II

Effect of the addition of ATP-G-actin on the specific viscosity of F-actin at 3°C

| Addition | | Temperature | Specific Viscosity | |
|---|---|---|---|---|
| | | | pH 8.2 | pH 7.0 |
| None | | 20°C | 0.39 | 0.38 |
| None | | 3°C | 0.21 | 0.24 |
| ATP-G-actin | 1.2 $\mu$M | 3°C | 0.28 | --- |
| | 0.6 $\mu$M | 3°C | 0.32 | --- |
| | 0.24 $\mu$M | 3°C | 0.20 | --- |
| | 0.12 $\mu$M | 3°C | 0.2 | 0.36 |
| | 60 nM | 3°C | --- | 0.37 |
| | 36 nM | 3°C | --- | 0.40 |
| | 24 nM | 3°C | --- | 0.34 |
| | 12 nM | 3°C | --- | 0.29 |

12 $\mu$M ATP-G-actin was polymerized for 3 hours at 20°C in the presence of 20 $\mu$M ATP, 4 mM $CaCl_2$ and either 10 mM tris-HCl buffer (pH 8.2) or 10 mM triethanolamine-HCl buffer (pH 7.0). The solutions were then cooled to 3°C. After 30 min of incubation ATP-G-actin, at the concentrations indicated, was added and specific viscosity was measured 5 min after the addition.

The transient increase of viscosity, that follows the addition of ATP-G-actin to cold F-actin solutions, is mostly due to annealing of short filaments. This is shown by the result of the measurement of the relative actin filament length before and after the addition of ATP-G-actin. In the experiment described in fig. 2, a 12 μM actin solution, polymerized at 20°C in 4 mM $CaCl_2$ ($\eta_{sp}$=0.38), displays five filament classes accounting for 89% of total polymer mass) of relative lengths : 10, 19, 23, 54 and 107. A second aliquot of the same sample, cooled for 30 min at 3°C ( $\eta_{sp}$=0.23), displays five classes of filaments (accounting for 88% of total polymer mass) of relative lengths : 6.5, 13.5, 23, 37.5 and 55. An aliquot of the cooled sample, 10 min after the addition of 51 nM ATP-G-actin, displays a $\eta_{sp}$=0.34 and three classes of filaments (accounting

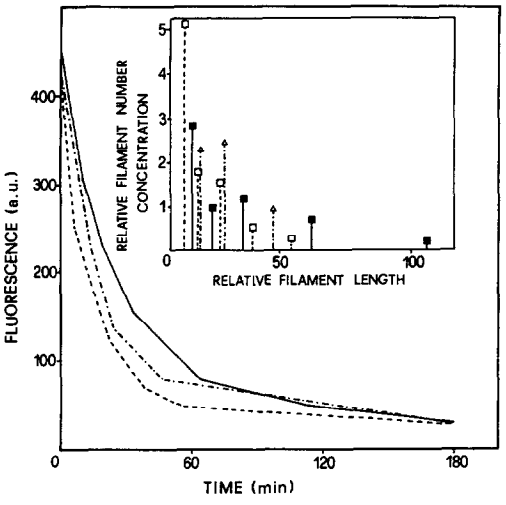


Fig. 2. Effect of the addition of ATP-G-actin on the filament length distribution of F-actin in the cold - The incubation mixtures contained 12 μM ATP-pyrenyl-G-actin, 20 μM ATP, 4 mM $CaCl_2$, and 10 mM tris-HCl buffer. pH was 7.5. After 3 hours of polymerization at 20°C, an aliquot of the polymerization mixture was taken and cooled to 3°C for 30 min. To an aliquot of the cooled sample 51 nM ATP-pyrenyl-G-actin was added and the sample was incubated for additional 10 min at 3°C. F-actin samples (0.03 ml) were taken either from the solution at 20°C (■———), or from that at 3°C (□-----), or from that at 3°C 10 min after the addition of ATP-G-actin (Δ-·-·-·-), diluted into 2.97 ml of the polymerization buffer and the decay of the flucrescence was followed at 20°C. Inset : Relative length and number concentration of the filaments.

for 82% of total polymer mass) of relative lengths : 14, 25 and 47. The main cause of the increase of the viscosity of the cooled sample, after the addition of substoicheiometric concentrations of ATP-G-actin, is thus the increase of the average length of the shorter classes of filaments.

## DISCUSSION

According to Pollard and Cooper (11), the existence of actin filament annealing is problematical. Positive evidence was derived from electron microscopy (12, 13), while the quantitative measurement of filament number concentration, during the recovery of ADP-F-actin from sonication, apparently, was inconsistent with annealing (14). The latter conclusion, however, is open to some methodological reservations (15).

Our present finding, that the addition of substoichiometric concentrations of ATP-G-actin increases the viscosity of F-actin at 3°C, is particularly relevant in this respect. At pH 7.0, even the addition of one molecule of ATP-G-actin for every 300 protomers in the filaments reverses the effect of cooling on the specific viscosity of the solution. The phenomenon is concomitant with the increase of the average length of the filaments, which is more pronounced for the shorter filament classes. If we take into consideration the very small amount of ATP-G-actin added, the change of the average length of the filaments can hardly be explained by models different from annealing.

As a conclusion, we have shown that the concentration of ATP-G-actin influences the filament length distribution by a mechanism which is different from the elongation reaction. Furthermore, by regulating the steady state of the fragmentation - annealing process, the concentration of ATP-G-actin influences the rheological properties of the actin network and, therefore, of the cytosol.

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