Characterization of the ATP–G-actin aggregates formed at low potassium chloride concentration

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The ATP–G-actin aggregates formed by incubation of ATP–G-actin in 7.5 mM-KCl were characterized by electron-microscopical observation, by high-pressure liquid chromatography and by the study of the 1,*N*⁶-etheno-ATP–ATP exchange reaction between the free and the actin-bound nucleotide. In 30 mM-KCl the initial rate of the reduced-viscosity increase is found to be directly related to the amount of the aggregates formed in the course of the preincubation in 7.5 mM-KCl.

In the last few years the studies on the polymerization of actin have been focused particularly on the first steps of the process. Rich & Estes (1976) and Rouyarenc & Travers (1981) have called attention to conformational modifications of the actin monomer that may favour the nucleation process. Maruyama (1981) has stressed the importance of the presence of submicromolar concentrations of Mg²⁺ for the nucleation. Barden *et al.* (1982) have detected directly, by liquid chromatography, the formation of nuclei. Ferri & Grazi (1982) have found conditions that favour the accumulation of nuclei, and Grazi *et al.* (1983) have provided evidence for the consecutive formation of two different nuclear species: one that is not directly susceptible to elongation, and the activated nuclei, which exist in very low concentration. The activated nuclei are directly susceptible to elongation, and they have a short life and decay rapidly into the ground state unless the elongation reaction occurs.

We provide here further characterization of the first nuclear species. It is represented by ATP–G-actin oligomers of irregular shape, which exchange 1,*N*⁶-etheno-ATP of the medium for bound ATP at a rate slightly greater than that with G-actin.

Materials and methods

G-actin from rabbit muscle (myokinase-free) was prepared as described by Spudich & Watt (1971). Before each experiment actin preparations were dialysed overnight against medium containing 100 μ M-CaCl₂, 100 μ M-ATP, 0.5 mM-2-mercaptoethanol, 0.5 mM-NaN₃ and 2 mM-Tris/HCl buffer, pH 7.8. The preparation was then centri-

fuged at 28 000 rev./min (66 000 *g*) for 3 h at 2°C in the Spinco S30 rotor.

Actin concentration was measured from the *A*₂₉₀, the absorbance of 1 mg of pure actin/ml (light-path 1 cm) being taken as 0.62 (Gordon *et al.*, 1976). Molar concentration of G-actin was calculated on the basis of an *M_r* of 42 000 (Collins & Elzinga, 1975). 1,*N*⁶-Etheno-ATP was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Inorganic orthophosphate was determined by the method of Tashima & Yoshimura (1975) after precipitation of the protein with 0.1 M-trichloroacetic acid. Viscosity was measured with an Ostwald viscosimeter (water flow time 60 s at 20°C).

For electron microscopy, 2 μ l drops were adsorbed for 60 s on carbon-coated 400-mesh grids rendered hydrophilic by glow discharge in air at decreased pressure. The grids were then washed for 30 s on several drops of 7.5 mM-KCl and stained for 30 s with several drops of 1% (w/v) uranyl acetate, pH 4.25. Electron microscopy was performed in a Siemens Elmiskop IA electron microscope.

The high-pressure liquid chromatography was performed with a Jasco Trirotar apparatus equipped with an Uvidec-100 u.v. spectrophotometer and a Houston Omniscrite recorder. An I-250 high-pressure liquid chromatography column (Waters Associates, Milford, MA, U.S.A.) was employed. The mobile phase consisted of 20 mM-sodium acetate, the pH was 7.75 and the temperature was 22°C. The flow rate was 1 ml/min; the back pressure was 1.5 MPa.

Fluorescence measurements were performed with a Perkin–Elmer MPF3 spectrofluorimeter equipped with a Linseis recorder. A cut-off filter for removal of the scattered light (wavelengths

Fig. 1. *Electron micrograph of negatively stained actin aggregates formed in 7.5mM-KCl*
 ATP-G-actin ($27\ \mu\text{M}$) dissolved in a solution containing 0.2mM-ATP, 0.2mM- CaCl_2 , 0.5mM-2-mercaptoethanol and 2mM-Tris/HCl buffer was supplemented with 7.5mM-KCl. The pH was 7.8 and the temperature was 22°C . At time intervals (a, 0h; b, 2h; c, 4h; d, 6h) after the addition of KCl, samples were taken, diluted 20-fold with a 2mM-Tris/HCl buffer solution containing 7.5mM-KCl, pH 7.8, and grids were prepared as described in the Materials and methods section. The bar represents 150nm (a) and 100nm (b, c and d).

below 390 nm) was inserted in front of the photomultiplier. The exciting wavelength was 360 nm and the emission wavelength was 410 nm.

Results

ATP-G-actin in the presence of low concentrations of KCl (7.5 mM) polymerizes very slowly and, at least for the first few hours, the viscosity of the solution does not increase. The viscosity of the sample, however, begins to rise, at a rate depending on the preincubation time in 7.5 mM-KCl, as soon as the concentration of KCl is raised to 20 mM. This was explained by an association reaction with the formation of small aggregates (Ferri & Grazi, 1982; Grazi *et al.*, 1983).

We present here electron-microscopical evidence that this is indeed the case (Fig. 1). Immediately after the addition of 7.5 mM-KCl to ATP-G-actin (Fig. 1a), a very dense field of actin monomers in a random distribution is revealed. After 2 h of incubation at 22°C (Fig. 1b), a large number of aggregates of different shape and size is evident. Some of the aggregates are very small and look like monomers associated along the longitudinal axis to form dimers (indicated by the arrow). In some cases the monomers group together (the association being always along the longitudinal axis) to form short segments 40–50 nm

long and 10–11 nm wide. In most cases, however, the aggregates are irregular bodies (diameter 10–20 nm). These bodies are by far the most predominant species after 4 h of incubation (Fig. 1c). When the incubation is prolonged, increasingly the aggregates we have just described line up to form nascent filaments (Fig. 1d).

The amount of the actin aggregates was estimated by high-pressure liquid chromatography. In the absence of KCl, ATP-G-actin was eluted as a single sharp peak with a retention time of 7.5 min (Fig. 2a). The actin samples injected on the column immediately after the addition of 7.5 mM-KCl displayed a shoulder, accounting for about 14% of the total protein, which was eluted immediately before G-actin. In actin samples incubated for 75 min and 150 min in 7.5 mM-KCl, the total amount of the oligomeric species was found to represent respectively 30% and 42% of the total protein (Figs. 2b, 2c and 2d). In agreement with the electron-microscopical observation, the chromatographic analysis showed that the oligomer population was heterogeneous: three shoulders are clearly shown in Fig. 2(d). No attempts were made, however, to estimate the size of aggregates. The retention time of F-actin was 6.5 min (not shown); thus in our chromatographic system the larger oligomeric species were found in the same position as F-actin. In all the samples submitted to chromatography,

Fig. 2. Elution profile of actin samples incubated in 7.5 mM-KCl

ATP-G-actin (15 μ M) dissolved in a solution containing 25 μ M-ATP, 25 μ M- CaCl_2 , 0.5 mM-2-mercaptoethanol and 2 mM-Tris/HCl buffer was supplemented with 7.5 mM-KCl. The pH was 8.0 and the temperature was 22°C. At time intervals (b, 0 min; c, 75 min; d, 150 min) after the addition of KCl, 0.02 ml samples were taken and submitted to high-pressure liquid chromatography as described in the Materials and methods section. (a) Control sample of ATP-G-actin without KCl.

however, specific viscosity was the same as that of G-actin and no hydrolysis of the ATP bound to actin was detectable. This latter observation shows that the aggregates formed, which, after 150 min of incubation in 7.5 mM-KCl, represent 42% of total protein, are composed exclusively of ATP-actin.

The exchange of 1, *N*⁶-etheno-ATP for ATP on the actin aggregates formed in 7.5 mM-KCl was also studied by exploiting the increase of fluorescence that occurs when 1, *N*⁶-etheno-ATP binds to actin (Waechter & Engel, 1975). To 15 μ M-ATP-G-actin dissolved in a solution containing 20 μ M-ATP, 20 μ M-CaCl₂, 0.5 mM-2-mercaptoethanol and 2 mM-Tris/HCl buffer, pH 8.0, was added KCl (final concn. 7.5 mM). After different times of incubation the solutions were supplemented with 0.1 mM-1, *N*⁶-etheno-ATP and the increase of the fluorescence was monitored at 410 nm. The increase of fluorescence occurred as a single transient with a *t*₁ of 150 s independently of the time of preincubation (up to 4 h) in the presence of KCl. Under the same conditions, but in the absence of KCl, *t*₁ was 210 s for ATP-G-actin. The small difference in the rate of the exchange seems therefore to be due to the addition of KCl as such and not to the formation of the aggregates.

The relationship between the formation of the aggregates and the susceptibility to polymerization of the actin solutions preincubated in 7.5 mM-KCl was also studied. Portions of the actin samples of the experiment described in Fig. 2 were supplemented with KCl to a final concentration of 30 mM and the increase of viscosity was measured. The initial specific-viscosity increase per min was 0.013, 0.016 and 0.019 for the samples preincubated in 7.5 mM-KCl for 0, 75 and 150 min respectively. The increase in the rate of polymerization thus accompanies the increase of the amount of the actin aggregates. This is good evidence for the aggregates being competent intermediates of the polymerization reaction.

Discussion

In previous studies (Ferri & Grazi, 1982; Grazi *et al.*, 1983) we provided kinetic evidence for the accumulation of actin nuclei from ATP-G-actin incubated in 7.5 mM-KCl. It was also proposed that these nuclei were not directly susceptible to elonga-

tion but had to be first converted into activated nuclei, present in very low concentration at the steady state.

We were unable at that time to provide a quantitative estimation of the nuclear species accumulating in 7.5 mM-KCl. This has been done in the present study by means of high-pressure liquid chromatography. It has been shown that up to 42% of the total actin may accumulate as the oligomeric species and that in the meantime hydrolysis of the ATP bound to actin does not occur. These two data together indicate that the oligomers spontaneously formed in 7.5 mM-KCl are composed exclusively of ATP-G-actin, in contrast with the activated nuclei, which appear to be mixed oligomers of ATP-G-actin and ADP-G-actin (Grazi *et al.*, 1983).

The electron-microscopical observation has further confirmed the formation of oligomers that appear as irregular bodies (diameter 10–20 nm) capable of lining up to form nascent filaments.

It has also been shown that the concentration of the aggregates formed in 7.5 mM-KCl is directly related to the capability for polymerization once KCl concentration is increased to 30 mM.

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