The polymerization of actin

A study of the nucleation reaction

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(Received 18 January 1983/Accepted 16 May 1983)

We compared the properties of the nuclei that accumulate in 7.5 mM-KCl in ATP-G-actin solutions and of the oligomers that are formed by sonication of either G-actin or F-actin. We found that the ability of the above species to prime the polymerization of actin decays with different rates. The nuclei are stable in 7.5 mM-KCl (they decay with a rate constant of $1.5 \times 10^{-3} \text{ s}^{-1}$ at pH 7.8 at 22°C in the absence of KCl). The oligomers formed by sonication of either G-actin or F-actin, once the sonication is stopped, revert to simpler structures or evolve into F-actin, depending on the KCl concentration in which they are kept. In 10.5 mM-KCl at pH 7.8 at 22°C their priming ability decays with a rate constant of $6 \times 10^{-3} \text{ s}^{-1}$. We propose that the nuclei that form spontaneously in 7.5 mM-KCl are not directly susceptible to elongation. They must first be converted into activated nuclei, which exist in very low concentration at the steady state. The activated nuclei are directly susceptible to elongation, they have a short life and they decay rapidly into the ground state unless the elongation reaction occurs. Sonication displaces the steady-state concentration in favour of the activated state.

The polymerization of actin has been thoroughly and intensively studied by a number of authors; however, because of the lack of specific and direct signals, the study of the nucleation step has been somewhat neglected. In the present paper we provide evidence that the nucleation step may proceed through the consecutive formation of two different intermediates. The fundamental observations that provide the experimental basis of our contribution have been known for a long time. These are the delay of the viscosity response to the polymerizing agent, and the 'explosive polymerization' (Kasai *et al.*, 1962). In their studies Kasai *et al.* (1962) showed that the initial velocity of viscosity increase of the transformation of G-actin into F-actin rises proportionally to the third to fourth power of the G-actin concentration. They also demonstrated that the addition of F-actin accelerates the polymerization of G-actin. They called this phenomenon 'explosive polymerization'. In further experiments it was shown that the priming effect could be enhanced by sonication of F-actin, the ends of the F-actin fragments acting as primers for the polymerization of G-actin.

We have further developed these observations and have found that, by a suitable choice of the concentration of the polymerizing agent (KCl), it is

possible to identify the first intermediate species of the polymerization, which accumulates in 7.5 mM-KCl and is unable to trigger the 'explosive polymerization'. We have also detected a second intermediate, which accumulates under sonication and is able to trigger the 'explosive polymerization'.

We propose that the nuclei that form spontaneously in 7.5 mM-KCl are not directly susceptible to elongation. They must first be converted into activated nuclei, which exist in very low concentration at the steady state. The activated nuclei are directly susceptible to elongation, they have a short life and they decay rapidly into the ground state unless the elongation occurs. Sonication displaces the steady-state concentration in favour of the activated state.

Materials and methods

Rabbit muscle ATP-G-actin was prepared by the method of Spudich & Watt (1971). The actin preparations were homogeneous on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis performed by the technique of Maizel (1971). Electrophoresis was done for 4 h at 2°C in 7.5%-cross-linked polyacrylamide gel polymerized in 10 mm × 0.5 mm tubes. The current was 2 mA/tube. A 60 µg

portion of protein was used for each run. The buffer was 0.2 M-glycine/25 mM-Tris/HCl buffer, pH 8.8. Protein was detected by staining with Amido Schwarz (purchased from Carlo Erba, Milan, Italy). Before each experiment actin preparations were dialysed overnight against 60 μ M-CaCl₂/60 μ M-ATP/0.5 mM-2-mercaptoethanol/0.5 mM-NaN₃/2 mM-Tris/HCl buffer, pH 7.8 (this mixture is referred to as 'actin buffer' in the text below). The preparation was then centrifuged at 28 000 rev./min for 3 h at 2°C in the 30 Spinco rotor.

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). Molar concentration of G-actin was calculated on the basis of an M_r value of 42 000 (Collins & Elzinga, 1975).

Viscosity was measured with an Ostwald viscometer (water flow time 60 s at 20°C) maintained at 22°C. Sonication of either G-actin or F-actin was performed with an MSE ultrasonic disintegrator (operating frequency 23 kHz) equipped with a 9.5 mm-diameter titanium probe (4 μ m oscillation amplitude) maintained at 22°C by a water bath.

In some experiments the polymerization of actin was stimulated by circulating, with the aid of a peristaltic pump, the actin solution in silicone-rubber tubing (1.1 mm diameter) at the rate of 5.5 cm/s. To do this the tube was first filled with the actin solution by aspiration, the pump was stopped, the two ends of tubing were connected and the pump was started again. Mechanical stimulation was employed only for practical convenience, since it was shown previously (Ferri & Grazi, 1982) that it increases the rate of the polymerization.

Results

Formation of actin nuclei in ATP-G-actin solutions supplemented with 7.5 mM-KCl

We show here that, at pH 7.8 at 22°C in the presence of 7.5 mM-KCl, 14 μ M-ATP-G-actin undergoes an association reaction with the formation of nuclei at a rate that is faster than the rate of the subsequent elongation reaction.

When actin is kept in actin buffer containing 7.5 mM-KCl for up to 6 h, no increase of viscosity or light-scattering, or of hydrolysis of actin-bound ATP, was detectable. The viscosity of the sample, however, begins to increase as soon as the concentration of KCl is raised to 20 mM (Fig. 1). Furthermore, the increase of the viscosity takes place with a rate depending on the preincubation time in 7.5 mM-KCl. This demonstrates that during the preincubation in 7.5 mM-KCl a species is formed that is more susceptible to elongation than is the actin monomer itself.

Fig. 1. Effect of the time of preincubation of ATP-G-actin in 7.5 mM-KCl on the rate of the viscosity increase in 20 mM-KCl

Two 8 ml samples of a 15.4 μ M-ATP-G-actin solution, dissolved in the actin buffer supplemented with 7.5 mM-KCl, were preincubated at pH 7.8 at 22°C for 50 min (●) and 100 min (O) respectively. After the preincubation to the two samples, 0.8 ml of 145 mM-KCl was added to adjust actin to 14 μ M and KCl to 20 mM. For each time indicated in the Figure a fresh sample of the actin solution was taken and viscosity was measured. A control sample was also prepared containing 20 mM-KCl and 14 μ M-actin not preincubated in 7.5 mM-KCl (■). The horizontal line indicates the final value of the specific viscosity of a 14 μ M-actin solution in 20 mM-KCl.

Fig. 2 shows that the effect of the preincubation in 7.5 mM-KCl increases with the increase of the initial concentration of G-actin. In these experiments 21 μ M- and 42 μ M-ATP-G-actin solutions were first incubated for various times in 7.5 mM-KCl. The samples were then diluted to 19 μ M-ATP-G-actin, the KCl concentration was raised to 20 mM and the samples were immediately stimulated by flow. The increase of the specific viscosity was larger in the samples that were originally more concentrated, as would be predicted for an association phenomenon between actin monomers to form actin nuclei. No viscosity increase was found in preincubated control samples that were submitted to mechanical stimulation in the presence of 7.5 mM-KCl. (In these experiments the polymerization of G-actin in 20 mM-KCl was stimulated by flow only for practical convenience, i.e. to decrease the length of the observation.)

Decay of the actin nuclei spontaneously formed in 7.5 mM-KCl

The dissociation of the actin nuclei spontaneously formed in 7.5 mM-KCl was also studied. A

Fig. 2. Effect of the concentration of actin preincubated in 7.5 mM-KCl on the polymerization in 20 mM-KCl

The incubation mixtures contained either 21 μ M-ATP-G-actin (O) or 42 μ M-ATP-G-actin (●) dissolved in the actin buffer supplemented with 7.5 mM-KCl. The pH was 7.8, the temperature 22°C. At the times indicated in the Figure samples were taken, actin was diluted to 19 μ M with identical buffer and in the meantime the concentration of KCl was raised to 20 mM. The samples were then immediately stimulated to polymerize by flow, and specific viscosity was measured immediately thereafter. Control samples containing either 21 μ M-actin (Δ) or 42 μ M-actin ($\blacktriangle$) were also prepared, which were preincubated in the actin buffer supplemented with 7.5 mM-KCl and then submitted to flow stimulation.

37 μ M-ATP-G-actin solution was first incubated for 4 h in 7.5 mM-KCl to allow the formation of the nuclei. The solution was then filtered through a Sephadex G-50 column to remove KCl. At different times after the filtration 20 mM-KCl was added to the solution, and polymerization was stimulated by flow for 150 s. As shown in Fig. 3, the effect of the stimulation by flow on the polymerization decreases with the increase of the time interval during which the actin nuclei are kept in the absence of KCl. This decrease, which takes place with a rate constant of $1.5 \times 10^{-3} \text{ s}^{-1}$, is clearly a measure of the decay of the nuclei in the absence of KCl. In parallel experiments, where the filtration was performed against different KCl concentrations, it was found that the half-time of dissociation of the nuclei was 10 min in 0.1 mM-KCl, whereas in 1.5 mM-KCl it was longer than 2 h.

Decay of the priming of polymerization after sonication of F-actin or G-actin

It is well known that the fragments obtained by sonication of F-actin are very efficient primers of the polymerization of G-actin. The phenomenon is known as 'explosive polymerization' (Kasai *et al.*, 1962). We have extended these studies and have reproduced this same phenomenon also by

sonication of G-actin. Furthermore, we have investigated the decay of the priming effect as a function of time after sonication, and have found that it decreases rapidly independently of the KCl concentration in which the actin fragments are kept.

In these experiments (Figs. 4 and 5) the sonication of actin was performed for 30 s in 44 mM-KCl, starting from either 83 μ M-G-actin or from F-actin (83 μ M as the monomer). After the sonication the samples were immediately diluted to 14 μ M-actin and 10.5 mM-KCl. The ability to induce the 'explosive polymerization' was tested by adding 0.05 ml of sonicated diluted solution to 0.83 ml of 14 μ M-ATP-G-actin just supplemented with 20 mM-KCl (Fig. 4). As shown in Fig. 5(a), the capability of the sonicated sample to induce the 'explosive polymerization' decreases with the increase of the time elapsed after the dilution of KCl to 10.5 mM. The decay occurs with a rate constant of $6 \times 10^{-3} \text{ s}^{-1}$.

The ability to stimulate the polymerization is lost also when the sonicated sample is kept at KCl

Fig. 3. Rate of polymerization in 20 mM-KCl of the actin preincubated in 7.5 mM-KCl as a function of the time elapsed in the absence of KCl

ATP-G-actin (37 μ M), dissolved in the actin buffer supplemented with 7.5 mM-KCl, was incubated for 4 h at 22°C. The solutions (1.5 ml) were then filtered through Sephadex G-50 (coarse grade) columns (1 cm $\times$ 60 cm) equilibrated with actin buffer without KCl. Elution was performed with the same buffer. The protein peak was eluted after 3 min. The two fractions most rich in protein were pooled: actin was 15 μ M. At the times (t) indicated in the Figure, to 0.6 ml portions of the filtrate 0.05 ml of 260 mM-KCl was added (final concentrations 14 μ M-actin and 20 mM-KCl), the samples were stimulated by flow and the increase of the specific viscosity per time unit (v) was determined. v_0 indicates the increase of the specific viscosity per time unit of a similarly stimulated sample of 37 μ M-actin incubated for 4 h in 7.5 mM-KCl and adjusted to 14 μ M-actin in 20 mM-KCl without previous filtration. The straight line is the semi-logarithmic plot of the reciprocals of the data points (●) with the right-hand ordinate.

Fig. 4. Time course of the 'explosive polymerization' induced either by sonicated F-actin or by sonicated G-actin

F-actin ($83\ \mu\text{M}$ as the monomer), dissolved in the actin buffer supplemented with $44\ \text{mM}$ -KCl, was sonicated for 30 s at 22°C and immediately diluted to $20\ \mu\text{M}$ -actin and $10.5\ \text{mM}$ -KCl. At the earliest time (10 s) after dilution $0.05\ \text{ml}$ ($1\ \text{nmol}$ of actin) of the above solution and $0.01\ \text{ml}$ of $1.73\ \text{M}$ -KCl were added to $0.83\ \text{ml}$ of $20\ \mu\text{M}$ -ATP-G-actin (final concentrations $20\ \mu\text{M}$ -actin and $20\ \text{mM}$ -KCl). Specific viscosity was then measured after the incubation times indicated in the Figure. In parallel experiments actin fragments were produced by sonication in $44\ \text{mM}$ -KCl of $83\ \mu\text{M}$ -G-actin instead of F-actin. Conditions were the same as described above. ●, Polymerization induced by the fragments obtained by sonication of F-actin; ○, polymerization induced by the fragments obtained by sonication of G-actin.

concentrations greater than $10.5\ \text{mM}$ (Fig. 5b); in this case the fragments evolve into F-actin, as shown by the rapid increase of the viscosity of the sample.

The triggering ability and the rate of the decay of the triggering ability were identical, whether starting from G-actin or from F-actin (Figs. 4 and 5a).

Comparison of the priming effect on actin polymerization of the actin nuclei formed spontaneously in $7.5\ \text{mM}$ -KCl with that of the actin fragments obtained by sonication

In the preceding subsections of this paper we have shown that both the nuclei formed spontaneously from G-actin incubated in $7.5\ \text{mM}$ -KCl and the fragments formed by sonication of either G-actin or F-actin are capable of priming the elongation reaction. In the experiment illustrated in Table 1 the priming effects of the above species are compared, and it is found that the effect is larger

with sonicated actin. (As stated above, priming effects of the same magnitude were obtained by sonication of either F-actin or G-actin.)

We have also found that the priming in the two cases is due to two different species, as is shown by the fact that these display very different half-lives. The nuclei formed spontaneously in $7.5\ \text{mM}$ -KCl keep their priming activity for hours, even in $1.5\ \text{mM}$ -KCl. The priming activity of the fragments obtained by sonication of actin, on the contrary, decays rapidly at the same KCl concentration.

Time course of the induction of the 'explosive-polymerization'-triggering activity and the hydrolysis of ATP in sonicated G-actin

The release of inorganic phosphate from actin, as well as the onset of the 'explosive-polymerization'-triggering activity, were studied as a function of the time of sonication. In these experiments (Fig. 6), which were performed in the presence of $14\ \mu\text{M}$ -ATP-G-actin, longer sonication times were needed to obtain maximal triggering activity, as compared with the experiments performed with $42\ \mu\text{M}$ -ATP-G-actin, where maximal triggering ability was obtained after 15 s of sonication (half-maximal ability after 2 s). Furthermore, a clear lag phase was detected. The dephosphorylation begins after a lag time of about 15 s, and clearly occurs in a rapid and a slow phase. In the rapid phase about 40% of the ATP bound to actin is dephosphorylated; at this time the 'explosive-polymerization'-triggering activity has reached its maximum. The extent of the rapid phase was estimated by the linear regression of the slow phase to the vertical axis crossing the abscissa at the end of the lag time. The slow phase is caused by a continuous formation of actin oligomers with concomitant ATP hydrolysis.

The same pattern in the release of orthophosphate is repeated also when the experiment is performed with $28\ \mu\text{M}$ -actin (results not shown). Thus, independently of the initial actin concentration, the dephosphorylation takes place in a rapid phase and a slow phase, the rapid phase is over when the triggering activity reaches its maximum and its amplitude accounts for 40% of the ATP originally bound to actin. This seems to indicate that during sonication the reaction is strongly displaced in favour of the formation of short oligomers of ADP-G-actin plus ATP-G-actin.

Discussion

We have previously shown (Ferri & Grazi, 1982) that, in the polymerization of actin, conditions can be found in which the nucleation is faster than the elongation, and therefore nuclei accumulate in the actin solution. The presence of the nuclei is deduced from the finding that the rate of polymerization of

Fig. 5. Characterization of the actin fragments formed by sonication: (a) formation and decay of the actin fragments, and (b) conversion of the fragments into F-actin

(a) Either F-actin or G-actin was sonicated and diluted as described in Fig. 4 legend. At the times indicated in the Figure 0.05 ml (1 nmol of actin) of the sonicated and diluted solution and 0.01 ml of 1.73 M-KCl were added to 0.83 ml of 20 μ M-ATP-G-actin (final concentrations 20 μ M-actin and 20 mM-KCl). Specific viscosity was measured 2 min after mixing. ●, Polymerization induced by the fragments obtained by sonication of F-actin; ○, polymerization induced by the fragments obtained by sonication of G-actin. (b) F-actin was sonicated under the conditions described in Fig. 4 legend and immediately diluted to 14 μ M-actin with the actin buffer containing KCl at the appropriate concentration to give the final KCl concentrations reported in the Figure. After dilution the increase of the specific viscosity was followed as described in the Materials and methods section. Curve A, 20 mM-KCl; curve B, 18 mM-KCl; curve C, 15 mM-KCl; curve D, 13 mM-KCl; curve E, 10.5 mM-KCl.

Table 1. Priming effects on the polymerization of actin
For Expts. 1–3 the experimental conditions were as described in Fig. 1 legend. For Expts. 4 and 5 sonicated actin at the concentration indicated was added to 14 μ M-ATP-G-actin dissolved in the actin buffer just supplemented with 20 mM-KCl. Viscosity measurements were performed 2 min after the addition of the sonicated sample. Sonication was performed as described in Fig. 4 legend.

Expt. no.	Initial concn. of G-actin (μ M)	Preincubation time in 7.5 mM-KCl (min)	Sonicated actin added (μ M)	Specific viscosity after 2 min incubation in 20 mM-KCl
1	14.0	0	—	0.00
2	14.0	100	—	0.03
3	14.0	360	—	0.09
4	14.0	—	0.5	0.22
5	14.0	—	1.0	0.30

G-actin in 20 mM-KCl increases with the time of preincubation in 7.5 mM-KCl, and that the apparent rate constant of the phenomenon depends on the initial concentration of actin.

Barden *et al.* (1982) have detected directly by liquid chromatography the accumulation of nuclei under experimental conditions very similar to our own. The ‘ATP-F-monomer’ first described by Rich & Estes (1976) shares the same properties as the nuclei that are formed in our conditions, even though these authors did not recognize the formation of the F-actin monomer as an association reaction. The ‘G*-monomer’ described by Rouayrenc & Travers (1981), on the other hand, does not seem to be immediately related to the nuclei that are formed in our conditions. Furthermore, it is difficult to assess the relationship between the spectral changes described, which occur in the time of the manual mixing of the reagents, and the polymerization process. Moreover, the concentration of KCl needed to elicit the half spectral change peculiar to the ‘G*-monomer’ is significantly larger than the concentration needed to accumulate nuclei from G-actin.

The fragments that are formed when either F-actin or G-actin is submitted to sonication are characterized by the same ability to trigger the

Fig. 6. Induction of the 'explosive-polymerization'-triggering activity and the hydrolysis of the ATP in sonicated G-actin

ATP-G-actin (14 μ M), dissolved in the actin buffer, was supplemented with 10.5 mM-KCl, and immediately thereafter sonicated at 22°C. At time intervals samples were taken for the determination of the ability to trigger the 'explosive polymerization' (O) and of the P_i production (●). To measure the former 0.05 ml of the sonicated samples was added to 0.8 ml of 14 μ M-G-actin solution just supplemented with 20 mM-KCl. Specific viscosity was measured after 2 min. The dotted line on the Figure indicates the final value of the specific viscosity. P_i was measured by the method of Tashima & Yoshimura (1975) after precipitation of the protein with 5% (w/v) trichloroacetic acid (final concn.).

'explosive polymerization' and by the same half-life. The fragments are certainly different from the nuclei formed spontaneously in actin solutions. These have a half-life of more than 2 h even in 1.5 mM-KCl; the former have a half-life of only about 2 min independently of the concentration of KCl in which they are kept. The higher rate of polymerization of a solution of G-actin supplemented with the fragments as compared with that of a solution of actin containing the nuclei spontaneously formed in 7.5 mM-KCl cannot thus be due to the presence of a larger concentration of the same kind of priming species in the first solution. In this case, in fact, the decrease of the rate of polymerization as a function of the time should occur with the same rate constant.

We propose that the nuclei that form spontaneously in 7.5 mM-KCl are not directly susceptible

to elongation. They must first be converted into activated nuclei, which exist in low concentration at the steady state. The activated nuclei are directly susceptible to elongation, they have a short life and they decay rapidly into the ground state unless the elongation reaction occurs.

The problem is now to decide whether the sonication acts merely by breaking the F-actin filaments and thus exposing the interacting surfaces of the actin monomers: these are presumed to be in the right conformation to react 'explosively' with the free monomers in the medium. Alternatively, sonication, by a suitable energy transfer, could displace the equilibrium between the ground state and the activated nuclei in favour of the latter, thus increasing the rate of the polymerization.

By sonicating G-actin we have found that maximal priming activity is obtained when only 40% of the ATP bound to actin has been dephosphorylated, and that this percentage is independent of the initial concentration of actin. This means that most of the actin is present as mixed oligomers of ATP-G-actin and ADP-G-actin. It seems therefore that, starting from G-actin, the formation of the species able to prime the 'explosive polymerization' proceeds through the formation of oligomers, which need to be very short, as indicated by their large ATP content. The effect of sonication could be to promote the helical winding of the oligomers, which would rapidly collapse again unless elongation takes place.

This work was supported by grants from the Italian Consiglio Nazionale delle Ricerche and by the Ministero della Pubblica Istruzione.

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