Sonic Vibration Induces the Nucleation of Actin in the Absence of Magnesium Ions and Cytochalasins Inhibit the Elongation of the Nuclei*

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Polymerization of actin was completely inhibited by metal chelating agents, such as EDTA or diaminocyclohexanetetraacetic acid, even in the presence of 0.1 mM KCl and excess ATP. Addition of 1 mM MgCl₂, but not CaCl₂, caused full polymerization in the presence of 10 mM diaminocyclohexanetetraacetic acid, suggesting that micromolar concentrations of Mg ions are required for the polymerization of actin. However, it turned out that sonic vibration induced a very rapid polymerization of actin in the absence of Mg ions. The sonication-induced polymerization of actin was greatly inhibited by cytochalasins D and B, and partially by β-actinin. This can be explained by assuming that the fast growing ends of the formed nuclei are blocked by the cytochalasins, and the slowly growing ends with β-actinin. The elongation of added F-actin fragments was also similarly inhibited by the cytochalasins and β-actinin in the absence of Mg ions. It appears that sonic vibration enhances nuclei formation of actin monomers by mechanical agitation in the absence of Mg ions, which stabilize formed nuclei.

Several reports have revealed that micromolar concentrations of Ca²⁺ retard the polymerization of actin, when the polymerization is started by the addition of a few tenths of millimolar concentrations of Mg²⁺ (1-3). Furthermore, it has been claimed that micromolar concentrations of Mg²⁺ are required for the nucleation of actin at low ionic strength (4). Since the discovery by Straub (5) of the globular-filamentous transformation of actin, it has been well established that 0.05 to 0.1 mM KCl causes a rapid polymerization of actin. Therefore, in the present study, it was intended to test whether or not trace amounts of Mg²⁺ are required for the formation of actin nuclei, when polymerized by KCl. Apparently, the results obtained showed Mg²⁺ but not Ca²⁺ is necessary for nucleation at a moderate ionic strength.

On the other hand, it has been observed that sonication induces a rapid nucleation in the absence of Mg²⁺, e.g., in the presence of DCTA, a strong chelating agent. Cytochalasins D and B remarkably inhibited the polymerization of actin evoked by sonic vibration. β-Actinin, an ending factor of the actin filaments in situ, also retarded the elongation process. The present work clearly demonstrates that the nucleation of actin is greatly dependent on mechanical agitation regardless of ionic conditions.

MATERIALS AND METHODS

Actin was prepared from an acetone powder of rabbit skeletal muscle and purified according to Spudich and Watt (6). CaCl₂ was omitted from the dialysis solution. β-Actinin was purified from rabbit skeletal muscle as described before (7), and its inhibition of the reannealing of actin filaments was tested before its use (7).

Flow birefringence was measured in an Edsall-type apparatus (Rao Instrument Co., New York) as an index of filament formation, as described elsewhere (8). The measurements were carried out at a velocity gradient of 100 s⁻¹ at 20°C. Sonic vibration was performed at a setting of 3 in a Tomy sonifier (model UR-20P; 20KC).

Chelating agents, DCTA, EDTA, EGTA, and NTA were purchased from Dojin Chemicals, Kumamoto, and cytochalasins D and B were obtained from Sigma.

RESULTS

In order to test the requirement of trace amounts of Mg²⁺ for polymerization of actin, several chelating agents were used (cf. Ref. 9). Since these chelators quickly inactivated G-actin by removal of the bound calcium, addition of a rather high concentration of ATP was needed (10, 11). In some of the present experiments, 10 to 20 mM DCTA was present in the incubation medium, and 10 mM ATP had to be added to protect G-actin from inactivation during polymerization for up to 1 h at pH 8.0 and 20°C. Even in the presence of 10 mM ATP, some 20% denaturation of G-actin occurred after a 1-h incubation with 20 mM DCTA. As shown in Fig. 1, EGTA markedly enhanced the formation of F-actin at all of the concentrations tested (10⁻⁴ to 10⁻² M) in the presence of approximately 0.1 mM KCl. NTA only slightly elevated the level of flow birefringence due to oriented actin filaments. On the other hand, EDTA and DCTA enhanced the polymerization at low concentrations, but inhibited it at higher concentrations. The inhibitory action by DCTA was 10 times more effective than by EDTA (Fig. 1). The stability constants of DCTA, EDTA, EGTA, and NTA for Mg²⁺ are approximately 10⁻¹⁴, 8.7, 5.4, and 5.4, respectively, and those for Ca²⁺ are 12.5, 10.6, 10.7, and 6.4, respectively (12). From these stability constants, the present results strongly suggest that submicromolar concentrations of Mg²⁺ are necessary for KCl-induced polymerization of actin (cf. Ref. 4). In support of this conclusion, addition of 1 mM MgCl₂, but not CaCl₂, resulted in full polymerization of actin in the presence of 10 mM DCTA, as demonstrated in Fig. 2.

During the course of the study of the inhibitory action of DCTA, it was noticed that the extent of inhibition varied with shaking the test tubes and also with the speed of injecting the test solution, through a syringe, into the cell of the flow birefringence apparatus. Inhibition was most notable when the test tube was not shaken and the solution was put into the cell as slowly as possibly. In this case, almost no polymerization was detected after a 30-min incubation with 20 mM diaminocyclohexanetetraacetic acid; G-actin, globular actin; F-actin, filamentous actin; EGTA, ethylene glycol bis(β-aminoethyl ether)N,N,N',N⁴-tetraacetic acid; NTA, nitrilotriacetic acid.
process and further sonication for cations. The reaction mixture was as in Fig. within a few minutes full polymerization was achieved (Fig. 20)

A, mixture was polymerization process in the absence of added DCTA (control) about 10 times and to 30-min incubation with sonic vibration induced a very rapid polymerization after a 15-
cated; sonic vibration for 10

actin filaments, approximately 1 mol/400 mol of actin monomers (14), and that these inhibit the elongation of preformed nuclei at the rapidly growing end (15-18). The inhibitory

DCTA. However, when the tube was frequently shaken, some 20% polymerization occurred, as presented in Fig. 3. Strikingly, sonic vibration for 10 s induced an immediate polymerization; within a few minutes full polymerization was achieved (Fig. 3). There was no significant change in the rate of the polymerization accelerated by sonication between with and without 20 mM DCTA. Sonication enhanced the rate of polymerization in the absence of added DCTA (control) about 10 times and sonic vibration induced a very rapid polymerization after a 15- to 30-min incubation with 20 mM DCTA (Fig. 3).

The effect of the duration of sonic vibration on the onset of the fast polymerization of actin in the absence of Mg2+ is presented in Fig. 4. Sonication for 10 s gave the maximal effect. Sonic vibration for 2 s slightly initiated the polymerization and further sonication for 2 s after 5 min was more effective than 4 s of continuous sonication. This was presumably due to the formation of short fragments from the preformed filaments by the second sonication (19).

Recent investigations show that cytochalasins are bound to actin filaments, approximately 1 mol/400 mol of actin monomers (14), and that these inhibit the elongation of preformed nuclei at the rapidly growing end (15-18). The inhibitory action of cytochalasins D and B was tested on the sonication-induced polymerization of actin in the absence of Mg2+. As shown in Fig. 5, 20 μM cytochalasin B lowered the rate of polymerization to about 1/40, and 1 μM cytochalasin D to 1/100. A slow but steady polymerization in the presence of cytochalasins might be ascribed to the slow elongation of the nuclei at their other ends, to which cytochalasins did not bind. A similar experiment was performed using short F-actin fragments produced by sonication. Addition of F-actin fragments to G-actin with DCTA led to an instant polymerization as rapidly as that induced by sonication. Cytochalasins also markedly retarded the rate of polymerization, but to a lesser extent than in sonication-induced polymerization. Very probably, the binding of the cytochalasins to the preformed nuclei was rate-limiting.

In contrast to the cytochalasins, β-actinin, an ending factor of actin filaments at the free end (opposite direction to Z-lines) (7), only partially inhibited the elongation process of nuclei formed by sonic vibration (Fig. 5) or of added fragments.

This was evidently due to the binding of β-actinin to the slowly growing end of the nuclei (7). The polymerization of actin was very slow, if it occurred at all, in the presence of

DCTA present. 1, left standing; 2, sonicated for 2 s; 3, sonicated for 4 s; 4, sonicated for 10 s, first sonicated for 2 s, and then sonicated for 2 s after 5 min.

FIG. 5 (right). Effect of cytochalasins B and D and β-actinin on the sonication-induced polymerization of actin in the absence of divalent cations. The reaction mixture was as in Fig. 1 except for 20 mM DCTA present. Sonication occurred for 10 s at 0 time. ○, control; ●, 20 μM cytochalasin B; △, 1 μM cytochalasin D; Δ, β-actinin, 0.01 mg/ml; ○, 1 μM cytochalasin D and β-actinin, 0.01 mg/ml.
both β-actinin and cytochalasin D (Fig. 5). It should be noted that the amount of β-actinin was enough to inhibit the reannealing of sonicated F-actin fragments completely (7).

**DISCUSSION**

Oosawa’s school has established that the polymerization of actin consists of the two steps: nucleation and the elongation of the formed nuclei into filaments (19). Usually, the former is the rate-limiting step of polymerization. We have experimentally verified this theory by measuring the changes in the length distribution of actin filaments formed during polymerization (13). In addition, we have reported that the elongation process is not dependent on micromolar concentrations of Mg2+ or Ca2+ (4).

The present work has revealed two new findings on the process of actin polymerization. First, submicromolar concentrations of Mg2+ stabilize nuclei formed at the initial step of actin polymerization induced by ~0.1 mM KC1. In the presence of 10 to 20 mM DCTA or EDTA, there is practically no polymerization at all, and an addition of 1 mM Mg2+, but not Ca2+, induces a rapid polymerization. Second, Mg2+ ions are, however, not absolutely necessary for nuclei formation in the process of actin polymerization. Mechanical shaking accelerates nuclei formation in the absence of Mg2+. Especially sonic vibration for a short time is most effective in inducing a very rapid polymerization. The fast rate evoked by sonication is the rate-limiting step of polymerization. We have experimentally verified this theory by measuring the changes in the viscosity as F-actin consists of the two steps: nucleation and the elongation of polymerization at low KC1 concentrations, where the calcium bound to F-actin can be removed in 10 mM DCTA or EDTA, there is practically no polymerization (4). They showed that actin could be fully polymerized in the presence of 15 mM KC1, when the sodium salt of EDTA (4 mM) was added while in the sonic field (22).

It remains, however, obscure how sonic vibration induces a rapid formation of actin nuclei under the conditions where spontaneous polymerization is negligible. One can exclude the effect of heat production by sonication, because a brief incubation at 30 or 40°C did not result in any polymerization of actin in the present experiments. Very probably, mechanical agitation enhances the formation of actin nuclei, since a mere shaking of the test tube accelerated the polymerization of actin in the presence of a metal chelator (cf. Fig. 3).

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