Mechanism of Action of Phalloidin on the Polymerization of Muscle Actin†

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ABSTRACT: Under conditions where muscle actin only partially polymerizes, or where it does not polymerize at all, a significant enhancement of polymerization was observed if equimolar phalloidin was also present. The increased extent of polymerization in the presence of phalloidin can be explained by the reduced critical actin concentration of partially polymerized populations at equilibrium. Under such conditions, the rate of polymerization, as judged by the length of time to reach half the viscosity plateau, was found to be essentially independent of the phalloidin concentration. Moreover, the initial rate of polymerization of actin was also found to be independent of phalloidin concentration. However, phalloidin apparently causes a reduction in the magnitude of the reverse rates in the polymerization reaction, as was demonstrated by the lack of depolymerization of phalloidin-treated actin polymers. This effect of phalloidin is also supported by the identification of actin nuclei and short polymers in populations of G-actin incubated with phalloidin in the absence of added KCl. Our conclusion, then, is that phalloidin influences the polymerization of actin by stabilizing nuclei and polymers as they are formed.

It has been reported that the presence of equimolar concentrations of the mushroom toxin phalloidin will prevent a drop in the viscosity of muscle F-actin when it is treated with the destabilizing agent potassium iodide and, in addition, inhibit the ATP hydrolysis activity of F-actin during ultrasonic vibration (Dancker et al., 1975). Indeed, since the discovery that phalloidin binds mainly to filamentous actin in liver membrane preparations (Govindan et al., 1973), the stabilizing effect of phalloidin on muscle F-actin has been demonstrated by electron microscopy (Lengsfeld et al., 1974), by the reversal of the destabilization of F-actin by cytochalasin B (Low et al., 1975), by the protection of actin from heat and alkali denaturation (deVries et al., 1976), and by the prevention of the proteolysis of F-actin by subtilisin (deVries & Wieland, 1978). In addition to stabilizing F-actin, phalloidin reportedly accelerates the rate of formation of F-actin from G-actin (Dancker et al., 1975).

The polymerization of actin is considered to be a sequence of reversible steps initiated by the addition of neutral salt to G-actin, which induces G-actin to undergo a conformational change to form the polymerization intermediate F-monomer (Rich & Estes, 1976). When the concentration of F-monomer in the population reaches the critical actin concentration, the F-monomer units slowly aggregate into F-actin nuclei in a rate-limiting reaction which has a third- or fourth-order dependence on actin concentration, implying that three or four monomers must join to form an actin nucleus (Kasai et al., 1962). Polymerization then proceeds with the rapid, first-order addition of F-monomers onto nuclei or growing polymers. The theory of polymerization of linear polymers developed by Oosawa & Asakura (1975) indicates that the dispersed monomer concentration, c_1, changes at a rate given by $dc_1/dt = -k_+c_1m + k_-m$, where k_+ and k_- are overall rate constants of polymerization and depolymerization, respectively, and m is the number concentration of polymers. The monomer concentration at equilibrium (when $dc_1/dt = 0$) is simply equal to k_-/k_+. Whether phalloidin alters either or both of these rate constants has not been established, but the accelerating effect of phalloidin on actin polymerization could occur by an increase in k_+, while the stabilizing effect of phalloidin on F-actin could mean k_- is reduced.

To investigate these possibilities, we determined the influence of phalloidin on actin under conditions of partial polymerization, where any effects on the polymerization mechanism would be expected to be most evident. Our findings show that the presence of equimolar phalloidin greatly increases the extent of polymerization and significantly reduces the critical actin concentration. The time to reach half the final viscosity plateau was found to be essentially independent of phalloidin concentration, as was the initial rate of polymerization of actin, and, thus, phalloidin does not appear to directly influence the forward rate of polymerization, or k_+. Rather, phalloidin apparently acts by blocking the reverse steps in the polymerization reaction, or reduces k_-, as demonstrated by its inhibition of depolymerization under conditions where nuclei and polymers will depolymerize in the absence of phalloidin, and by the identification of nuclei and oligomers in populations of G-actin incubated with phalloidin in the absence of added KCl.

Methods

Actin was extracted from rabbit acetone powder (Szent-Gyorgyi, 1951) at 0 °C for 20 min with 25 volumes of 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.5 mM dithiothreitol, and 2 mM Tris, pH 7.8. After polymerization of the crude extract in 0.1 M KCl, the actin was made 0.8 M in KCl and incubated at 0 °C for 90 min before centrifugation at 170000g for 2 h (Spudich & Watt, 1971). The resulting pellet was homogenized in 150 mL of 0.1 M KCl, 0.02 mM $CaCl_2$, and 2 mM Tris, pH 7.8, centrifuged again, and then homogenized in and dialyzed against 0.1 mM ATP, 0.02 mM $CaCl_2$, and 2 mM Tris, pH 7.8, for three or four changes at 12-h intervals. Following three 10-s periods of ultrasonic vibration at 0 °C, the resulting G-actin was centrifuged at 170000g for 30 min and then passed through a column of Sephadex G-100. This step in the purification procedure was found to be necessary to ensure the removal of any denatured, aggregated, or nu-

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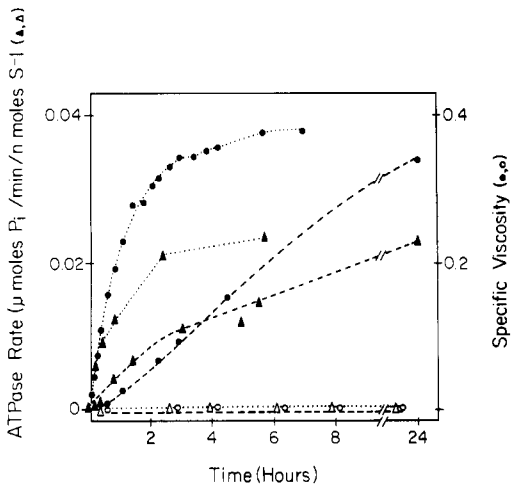

FIGURE 1: Effect of phalloidin on the polymerization of 10 μM actin in 10 mM KCl (dotted lines) or 5 mM KCl (dashed lines). Equimolar phalloidin (●,▲) or buffer (○,△) was added at $t = 0$. Specific viscosities (●,○); ATPase activity of S-1 (2 μM final concentration) in the presence of portions of the actin samples (▲,△). Conditions: 0.1 mM ATP, 0.02 mM $CaCl_2$, 0.01 mM $MgCl_2$, and 2 mM Tris, pH 7.8, 25 °C.

cleated actin, which eluted from the column before the major (second) peak of pure G-actin. Finally, the purified G-actin was filtered through 0.45-μm filters into a sterile vessel to prevent bacterial growth and stored at 4 °C until used. Heavy meromyosin subfragment 1 (S-1) was prepared by the method of Lowey et al. (1969) directly from myosin (Kielley & Harrington, 1960). For calculation purposes, molecular weights of 43 000 for actin and 110 000 for S-1 were used. Phalloidin and subtilisin (subtilisin BPN) were purchased from Sigma Chemical Co.

Viscosity measurements were performed by using 2 mL capacity Ostwald viscometers having outflow times for water of about 20 s. The rates of digestion by subtilisin of portions of an actin sample were measured as previously described (Rich & Estes, 1976). The ability of actin populations to activate the myosin (S-1) ATPase activity was determined by pH-stat titration at 25 °C (Eisenberg & Moos, 1967). Except for the analysis of the column chromatography fractions, the activation of S-1 ATPase activity was determined from the increase in the ATPase rate when an aliquot of an actin sample was added to a mixture of S-1 and Mg^{2+}-ATP whose base-line rate had been established. For analysis of the ATPase-activating ability of the column chromatography fractions (Figure 5), an aliquot containing 0.5 mg of S-1 was added to 2.0 mL of the column fraction and the ATPase activity measured.

Results

The enhancement of the polymerization of 10 μM actin by equimolar phalloidin in 10 mM KCl or in 5 mM KCl is shown in Figure 1. Whether monitored by viscosity or the ability of the samples to activate the S-1 ATPase, essentially full polymerization of the actin occurred in about 6 h with 10 mM KCl present and in about 24 h in the presence of 5 mM KCl. In the absence of phalloidin, however, actin did not polymerize during this time period, as demonstrated by the lack of any detectable change in the viscosity or in the ATPase-activating ability of these solutions. This lack of polymerization at these KCl concentrations and without phalloidin suggests that the presence of polymer in the samples with phalloidin may be due to a lower critical actin concentration.

For examination of this possibility, various concentrations of actin were polymerized with 10 mM KCl in the presence

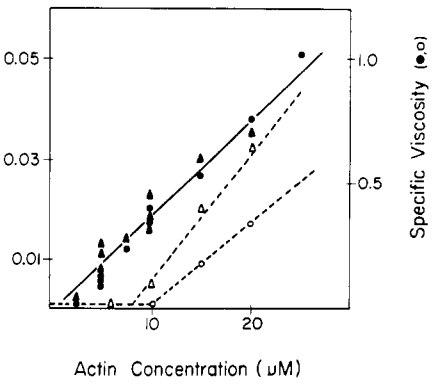

FIGURE 2: Effect of phalloidin on the critical actin concentration. Various concentrations of actin were polymerized to equilibrium without phalloidin (open symbols) or with phalloidin added to a concentration equimolar with actin (filled symbols). Specific viscosities (●,○); ATPase activity of S-1 (2 μM final concentration) in the presence of aliquots of the various actin samples (▲,△). Conditions: 10 mM KCl, 0.02 mM $CaCl_2$, 0.1 mM ATP, 0.01 mM $MgCl_2$, and 2 mM Tris, pH 7.8, 25 °C.

and absence of equimolar phalloidin (Figure 2). Under the conditions employed, the ATP hydrolysis due to reversible monomer–polymer cycling consumed less than 10% of the total ATP present and thus was judged not significant. At equilibrium, the critical actin concentration was determined by measuring both the viscosity of the solutions and their ability to activate the S-1 ATPase activity. The critical actin concentration under these conditions in the absence of phalloidin was in the range 8–10 μM while in the presence of phalloidin both measurements yielded a value of about 1 μM. This observation that phalloidin lowers the critical actin concentration, or the ratio k_-/k_+, in partially polymerized actin populations is similar to its reported effect under conditions of full polymerization (Faulstich et al., 1977) and could be due to a reduction in k_- or an increase in k_+, or both.

If phalloidin directly influences the foward rates in the actin polymerization reaction by causing an increase in k_+, then the rate of polymerization of actin would be expected to be dependent on the phalloidin concentration. To ascertain if this is the case, the experiments shown in Figure 3A,B were conducted with varying amounts of phalloidin added at zero time. In Figure 3A, all of the samples took essentially the same time to reach half the final viscosity plateau (mean = 69 ± 8 min), with the exception of the 50 μM phalloidin condition. We are presently uncertain why the polymerization rate was enhanced so much more at the phalloidin/actin ratio of 5:1, but it is apparent that in the region of phalloidin/actin ratios between 1:5 and 2:1 phalloidin does not have a significant direct influence on the forward polymerization rate constants. In the experiment shown in Figure 3B, 0.1 mL of 20 μM F-actin, fully polymerized in 0.4 M KCl, was added to 1.9 mL of 10 μM G-actin (final KCl concentration 20 mM) and the viscosity measured. When phalloidin was also present, equal volumes of the appropriate concentration of phalloidin in 0.4 M KCl were first mixed with 40 μM fully polymerized actin in 0.4 M KCl immediately prior to adding 0.1 mL of the mixture to G-actin. Again, it is apparent from the similarity of the initial rates of polymerization that within a 10% experimental variation phalloidin does not increase the forward rate constant of polymerization, k_+.

How, then, does phalloidin accelerate the polymerization of actin? The results shown in Figure 4 provide an answer: Phalloidin greatly reduces the magnitude of the rate constant of depolymerization in the polymerization reaction, or k_-. For a demonstration of this, fully polymerized actin in 20 mM KCl

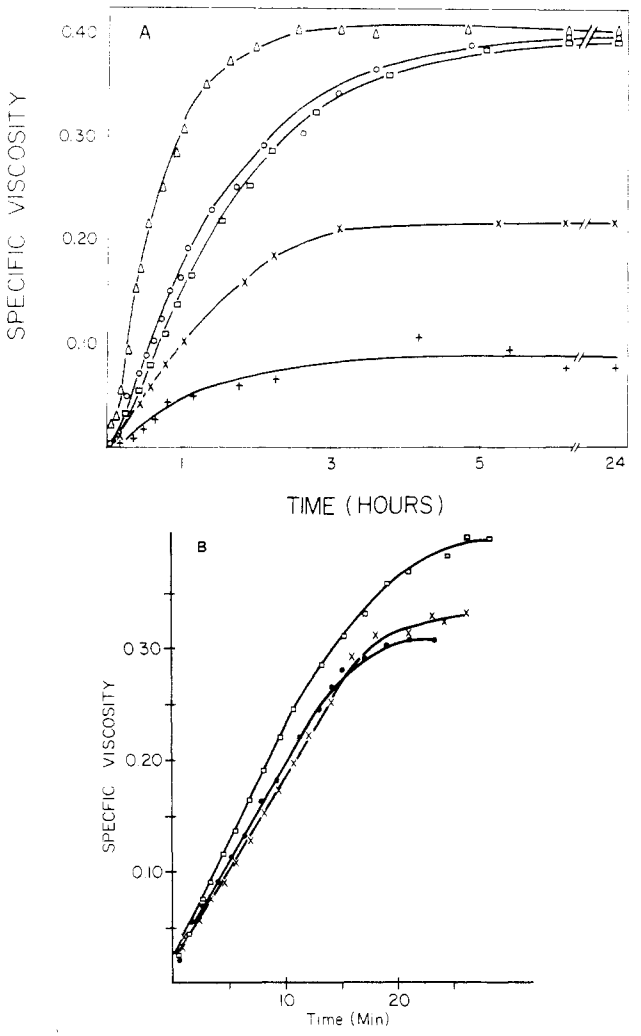

FIGURE 3: (A) Dependence of polymerization on phalloidin concentration. Various phalloidin concentrations were added at $t = 0$ to 10 μM actin in 10 mM KCl, 0.1 mM ATP, 0.02 mM $CaCl_2$, and 2 mM Tris, pH 7.8, at 25 °C. Phalloidin concentration (μM): 2 (+); 5 (×); 10 (□); 20 (○); 50 (△). (B) Dependence of initial polymerization on phalloidin concentration. To 1.9 mL of 10 μM G-actin in 0.2 mM ATP, 0.1 mM $CaCl_2$, and 2 mM Tris, pH 7.8, was added 0.1 mL of fully polymerized 20 μM F-actin at $t = 0$. Where phalloidin was also present, the F-actin in 0.4 M KCl was mixed with an equal volume of 4 or 400 μM phalloidin also in 0.4 M KCl immediately before 0.1 mL of the mixture was added to the G-actin. Phalloidin concentrations (μM): 0 (●); 0.1 (×); 10 (□).

and with or without 20 μM phalloidin was diluted at zero time to final concentrations of 2 μM actin and 2 mM KCl, and with or without 2 μM phalloidin. In the absence of phalloidin, both the viscosity of the actin samples and their ability to activate the S-1 ATPase dropped abruptly as depolymerization occurred while the rates of digestion of portions of these samples were observed to increase from the low values for F-actin to higher values similar to those obtained for mixtures of G-actin and F-monomer (Estes & Gershman, 1978). In the presence of phalloidin, however, no detectable change for as long as 3 h after dilution, was observed in the viscosity values, in the ability of the actin to activate the S-1 ATPase, or in the digestibility of the actin populations. In other experiments not shown here, essentially no change in the viscosity value or in the ability of the phalloidin actin populations to activate the S-1 ATPase could be detected for period of time as long as 24 h after dilution. The polymer state must therefore be extremely stable in the presence of phalloidin, and because of this, the depolymerization of polymers and nuclei, or k_-, must be somehow blocked.

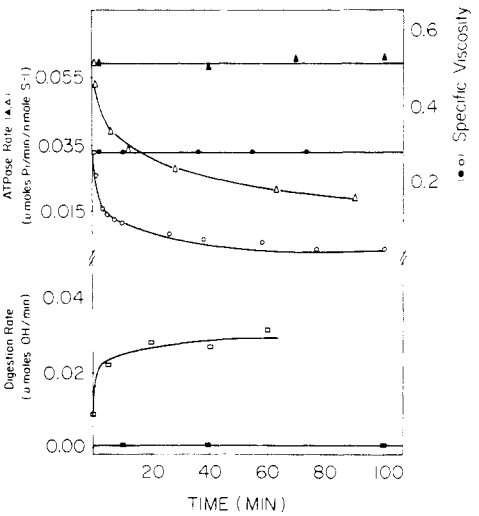

FIGURE 4: Depolymerization of F-actin by dilution in the presence (filled symbols) or absence (open symbols) of phalloidin. F-actin at a concentration of 20 μM in 20 mM KCl and with or without 20 μM phalloidin was diluted 10-fold at $t = 0$. Samples were removed at various times for viscosity measurements (●,○), for determination of the ATPase activity of S-1 (2 μM final concentration) in the presence of the actin samples (▲,△), and for measurement of the rate of digestion of the actin by 40 μg/mL subtilisin (final concentration) (■,□).

Phalloidin reportedly does not bind to G-actin (Wieland et al., 1975; Wieland, 1977). We reasoned, however, that even without the addition of KCl to a population of G-actin molecules, whose tertiary structures are constantly undergoing change, the transient formation of some F-monomer conformations should occasionally occur, and these F-monomers might spontaneously form dimers, trimers, or nuclei, which would rapidly break apart. The presence of phalloidin would stabilize any nuclei formed if the effect of phalloidin is to reduce k_-. Column-purified G-actin (100 μM) was incubated with equimolar phalloidin at 4 °C in the absence of added KCl (contaminant KCl concentration was less than 1 mM). Samples were removed from the population at various times and chromatographed on Sephadex G-100. The complete separation of G-actin and phalloidin immediately after the two components were mixed at time zero is seen in the optical density record from the column monitor (Figure 5A). The actual time of separation of G-actin from phalloidin in the column matrix is estimated to be about 5 min while elution of the entire sample took around 4 h. Figure 5B shows the concentrations of actin and phalloidin in the two peaks as calculated from the ultraviolet absorption spectra of fractions of the column effluent. This calculation of the concentrations of actin and phalloidin from the ultraviolet absorption spectra was accomplished by the method of Loring (1955) by using molar extinction coefficients for actin of 2.75×10^4 at 290 nm (Rich & Estes, 1976) and 5.01×10^3 determined at 300 mm and for phalloidin of 1.45×10^4 determined at 290 nm and 1.18×10^4 at 300 nm (Wieland et al., 1975). The inability to detect the presence of phalloidin in the G-actin peak verifies the suggested lack of binding of phalloidin to G-actin. At these low actin concentrations, a very low viscosity and no detectable increase in the base line S-1 ATPase activity were found for each of the fractions. These characteristics indicate that the zero-time mixture is truly monomeric actin (Estes & Gershman, 1978).

After incubation of the mixture for 15 h at 4 °C, the column chromatographic separation of the components was again performed. The optical density profile shows three peaks (Figure 5C), and the concentrations of actin and phalloidin

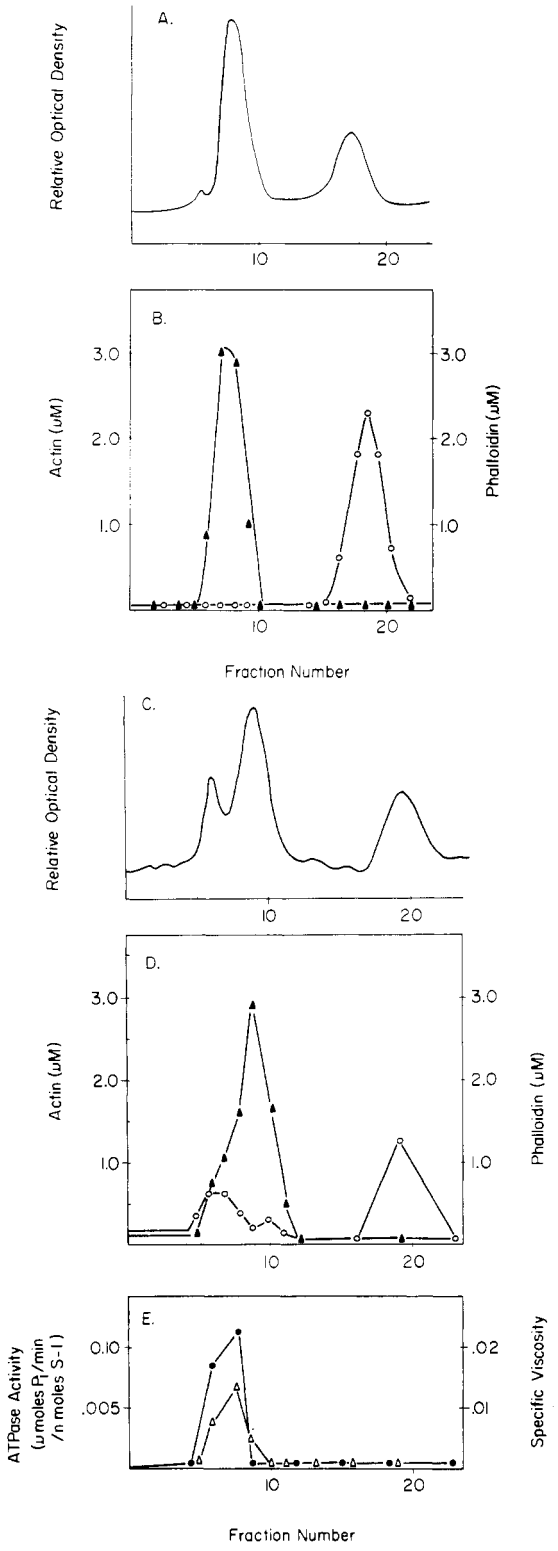

FIGURE 5: Sephadex G-100 column chromatography of 100 μM G-actin populations incubated with equimolar phalloidin. Conditions: no added KCl, 0.1 mM ATP, 0.02 mM $CaCl_2$, 2 mM Tris, pH 7.8, 5 °C. Incubation time: t = 0 (A and B); t = 15 h (C–E). (A and C) Column monitor record at 280 nm; (B and D) concentrations of actin (▲) and phalloidin (○) calculated from the ultraviolet absorption spectra of the fractions collected; (E) specific viscosity of each fraction (●) and ATPase activity of S-1 (2 μM final concentration) (△) added to each fraction.

calculated from the absorption spectra of the fractions (Figure 5D) indicate that the leading peak is a mixture of actin and phalloidin while peak two is essentially pure actin, and peak three contains phalloidin only. Both the viscosity values of these fractions and their abilities to activate the S-1 ATPase activity are shown in Figure 5E. The increase in viscosity and in ATPase-activating ability is associated with peak one, and therefore, this peak must contain nuclei and oligomers which are stabilized by bound phalloidin. This identity of the actin species in peak one as nuclei, oligomers, and polymers and in peak two as monomeric actin is consistent with the molecular exclusion limit of Sephadex G-100. The small amount of phalloidin consistently detected in peak two (Figure 5D) may be free in solution or bound to F-monomer, but we have no evidence at present to establish if this possibility is the case. Thus, phalloidin apparently stabilizes the nuclei and oligomers formed even in the absence of KCl. This chromatographic analysis of populations of G-actin with phalloidin has also been performed with longer periods of incubation, and with more time, both the size of peak one and its phalloidin content continually increase, as do its viscosity and ability to activate the S-1 ATPase activity. In the absence of phalloidin, none of these changes are observed. In the presence of phalloidin with small amounts of added KCl (in the range 2–10 mM), the same progression of polymerization stages as just described occurs with no added KCl, but much more quickly. These data strongly support the previous conclusion that the mechanism of action of phalloidin in increasing the rate of polymerization of actin is not to increase the forward rate constant of polymerization, k_+, but rather to reduce the rate constant of depolymerization, k_-.

Discussion

The enhancement of the polymerization of actin by phalloidin (Figure 1), which can be accounted for by the lowered critical actin concentration under partially polymerized conditions in the presence of phalloidin (Figure 2), is consistent with the reported protection of F-actin by phalloidin against depolymerization by KI (Dancker et al., 1975; Wieland & Faulstich, 1978). However, the enhanced rate of polymerization does not appear to be due to a direct effect of phalloidin on the foward rate constant of polymerization, k_+ (Figure 3A,B), but rather is apparently due to a significant decrease in the magnitude of the reverse rate constant, k_-, or breakdown of nuclei and polymers, in the polymerization reaction (Figure 4).

Because a linear increase in plateau viscosity was observed with increasing phalloidin concentration (Figure 3A), phalloidin apparently stabilizes the concentration of polymers formed up to the magnitude of its concentration present. With the viscometers employed, the viscosity of fully polymerized 10 μM actin has a value of 0.4, and this accounts for the constant upper plateau at concentrations of phalloidin equimolar with actin or higher. Moreover, the time to reach polymerization equilibrium is reciprocally related to the sum of the forward and reverse rate constants, k_+ and k_- (Oosawa & Asakura, 1975). The observed similarity in this time for various phalloidin/actin ratios appears to be inconsistent with the conclusion that phalloidin decreases the critical actin concentration, or the magnitude of k_-. However, under the conditions employed, the magnitude of k_+ is so much greater than k_- that a decrease in k_- by phalloidin could not be experimentally detected in the value of the reciprocal of their sum.

The results presented here are also in agreement with the notion that phalloidin cannot bind to G-actin. However, the presence of a small peak of phalloidin in the second or monomer actin peak in Figure 5D suggests that phalloidin may bind to the F-monomer conformation. If this is the case, it could have the effect of increasing the size of the F-monomer population, which would then provide more actin monomers

with the appropriate conformation to form nuclei or to add onto growing polymers. This possibility was examined in other experiments not shown here in which monomeric actin was incubated with equimolar phalloidin and various concentrations of KCl up to 20 mM. If the binding to F-monomer occurred, then the total amount of phalloidin in the phalloidin only peak (cf. Figure 5B,D) should decrease in proportion to the amount of F-monomer present. The data showed that the area of the phalloidin only peak remained essentially constant regardless of how much F-monomer was present. It is more likely then that the only conformation of actin which will bind phalloidin is the conformation formed in the polymerization reaction after F-monomer itself has undergone a conformational change to form nuclei or has been added onto a polymer (Higashi & Oosawa, 1965; Stone et al., 1970; Estes, 1974, 1975). Information is not available from the techniques employed in this study, or from the results of other laboratories, to ascertain if phalloidin binds to the F-monomer species.

The mechanism of the ability of phalloidin to decrease k_-, and thus stabilize nuclei and polymers, remains uncertain at present. One possibility is that phalloidin induces a conformational change in the subunits in an actin nucleus or a polymer, causing the monomer units to adhere more tightly to each other. While the interaction of phalloidin and F-actin does produce an ultraviolet difference spectrum, it was not possible to detect a conformational change in F-actin–phalloidin mixtures by the method of susceptibility to proteolytic digestion because such mixtures do not show a measurable rate of digestion. This lack of digestbility of phalloidin-stabilized actin, compared with the measurable rate of digestion of F-actin in the absence of phalloidin (Figure 4; Rich & Estes, 1976), has been also reported by other investigators (deVries & Wieland, 1978) using different enzymes (Pollender & Gruda, 1979). These findings could be evidence that a conformational change occurs in actin upon binding phalloidin. However, these data can be equally well explained by the possibility that the actin polymer, with or without phalloidin present, is essentially indigestible and that the low rate of digestion observed with normal F-actin is actually the rate of digestion of the F-monomer and G-actin species in equilibrium with the polymer. It is not possible with the data available to establish which possibility is correct, but in any event, the binding of phalloidin to nuclei and polymers, and the inhibition of their breakdown, appears to be its mechanism of action on the polymerization of actin.

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