Cytochalasin D and Platelet Gelsolin Accelerate Actin Polymer Formation. A Model for Regulation of the Extent of Actin Polymer Formation in Vivo†

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ABSTRACT: The fluorescence enhancement of actin labeled with *N*-(1-pyrenyl)iodoacetamide was used to monitor the rate of incorporation of monomeric G-actin into actin polymer. When such experiments are performed in undisturbed solutions, both the rate of polymerization and the extent of polymer formation can be quantitatively measured. Under all conditions, cytochalasin D accelerates polymerization as reflected by an enhanced rate of incorporation of monomer into polymer and/or a decreased polymerization lag time. However, the presence of cytochalasin D decreases the extent of polymer formation. The amount of polymer formed in the presence of cytochalasin D is more strongly dependent on the conditions under which polymerization occurs, i.e., the Mg^{2+} concentration and the temperature, than is the amount of polymer formed in the absence of cytochalasin D. A general observation is that in the presence of cytochalasin D, conditions that give slow rates of polymerization incorporate much less monomeric actin into polymer than conditions that give rapid rates of polymerization. The results are interpreted in terms of the nucleation–elongation model for actin polymerization and the supposition that cytochalasin binds preferentially to one end of a growing polymer. However, not all the observations may fit this simple model. We have isolated an actin binding protein from platelets that shows effects similar to those of cytochalasin D. This protein appears to correspond to macrophage gelsolin. It is proposed that preferential binding of such proteins to one end of a growing polymer may influence both the rate and extent of polymerization. If these parameters are altered by physiological conditions, certain actin binding proteins could regulate the concentration of free G-actin in vivo.

Actin, a protein found in all eucaryotic cells (Pollard & Weihing, 1974; Korn, 1978), has been demonstrated to be intimately involved in many forms of cellular motility ranging from cell movement in *Acanthamoeba* (Weihing & Korn, 1971) to the filopodial projections and clot retraction of platelets (Crawford, 1976; Nachmias, 1980; van Deurs & Behnke, 1980). This protein undergoes a polymerization from the monomeric form (G-actin) to a long helical double-stranded polymer (F-actin) under the influence of physiological ionic strength (typically 0.1 M KCl) or by the addition of divalent cations (typically magnesium at millimolar concentrations). Polymerization is characterized as nucleation followed by an elongation process representing the addition of monomers to the ends of the filament (Kasai, 1969; Kasai et al., 1962; Oosawa & Kasai, 1962, 1971).

The cytochalasins, a group of cellular motility inhibitors of fungal origin, have been characterized as inhibitors of polymerization and are found to bind tightly to F-actin filaments (Flanagan & Lin, 1980; Lin et al., 1980; Brown & Spudich, 1981; Hartwig & Stossel, 1979). A general model for the mode of action of these toxins envisages them "capping" F-actin filaments at the rapidly growing end and consequently blocking further elongation of the filament from that end (Brenner & Korn, 1979; Lin et al., 1980; Flanagan & Lin, 1980). This model accounts for the apparent inhibitory activity of cytochalasins at substoichiometric concentrations of the toxin although there have been reports of an activation of the initial rate of polymerization which have been interpreted as enhanced nucleation (Brenner & Korn, 1980; Selden et al., 1980; MacLean-Fletcher & Pollard, 1980a; Wieland & Löw, 1980; Löw et al., 1979). However, the activation of the initial rate is frequently not observed or is observed only under certain conditions (Brenner & Korn, 1980, 1979; Dancker & Löw, 1979). Invariably, high shear viscosity has been used as a monitor of the polymerization process during these studies. Data obtained by this technique are difficult to interpret quantitatively because viscosity is a complex function of filament length, flexibility, concentration, and intermolecular interactions. In addition, the viscosity of F-actin solutions depends strongly on the shear rate (Maruyama et al., 1974), and high rates of shear disrupt filaments and accelerate polymerization. Further, many previous studies have used non-gel-filtered actin containing contaminating proteins that may influence the polymerization process (MacLean-Fletcher & Pollard, 1980b). Because of these reasons, previous studies probably have not given a fully consistent picture of the effect of cytochalasins on actin polymerization. We have therefore used a fluorescent derivative of actin that provides a quantitative measure of the incorporation of monomer into polymer independently of filament length, or concentration, and in the absence of any applied shear stress.

We report here the effects of cytochalasin D on actin filament formation under a variety of conditions. We demonstrate that this toxin always activates the polymerization process but influences the extent of polymerization such that less polymer is formed. Further, the effect of conditions altering the polymerization extent is much more marked in the presence of cytochalasin D than in its absence. This latter effect has not been previously reported and may partially explain the wide variation in results observed by others. We have followed the kinetics of actin polymerization with a sensitive fluorescently labeled actin [*N*-(1-pyrenyl)iodoacetamide-labeled actin] (Kouyama & Mihashi, 1981), which is a quantitative monitor of this process.

The effect of several actin binding proteins at substoichiometric concentrations (e.g., fragmin, villin, and gelsolin) on actin polymerization in vitro may be analogous to that of

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cytochalasins (Isenberg et al., 1980; Hasegawa et al., 1980; Craig & Powell, 1980; Glenney et al., 1981; Southwick & Stossel, 1981; Wang & Bryan, 1981; Yin et al., 1981; Schliwa, 1981), reinforcing the need for fully understanding the influence of toxins like cytochalasin on actin filament formation. We have purified a platelet protein similar to macrophage gelsolin and shown that its influence on actin polymerization is qualitatively similar to cytochalasin D.

Experimental Procedures

All reagents were of analytical grade with ATP being purchased from Sigma Chemical Co. Cytochalasin D was from Aldrich Chemical Co. and *N*-(1-pyrenyl)iodoacetamide from Molecular Probes Inc. "Ultrapure $MgSO_4$" was obtained from Ventron Corp., Alfa Division. All solutions were made from deionized distilled water.

Actin Purification. Gel-filtered skeletal muscle actin was isolated and purified according to the method of Spudich & Watt (1971) with the gel filtration (Sephadex G-150) modification of MacLean-Fletcher & Pollard (1980b). Unless used immediately after gel filtration, the actin was stored lyophilized in the presence of sucrose (2 mg/mg of actin) at −20 °C. This actin was dissolved at the required concentration (always <120 μM) in 2 mM Tris, 200 μM ATP, 200 μM $CaCl_2$, and 1.5 mM NaN_3, pH 8.0 (G buffer),[1] and dialyzed exhaustively against the same buffer over a 2-day period at 4 °C.

Protein Determination. Actin concentrations were measured spectrophotometrically by using the value $E^{1mg/mL} = 0.63$ at 290 nm (Houk & Ue, 1974) and a M_r of 42 300 (Elzinga et al., 1973). Low concentrations of actin and all platelet gelsolin concentrations were measured by using the Bradford (1976) assay with G-actin as a standard.

Fluorescent Labeling of Actin. Gel-filtered G-actin (~24 μM), dialyzed against G buffer in the absence of reducing agents, was polymerized by the introduction of Mg^{2+} to a final concentration of 2 mM at room temperature (23 °C). The actin was reacted with an equimolar amount of *N*-(1-pyrenyl)iodoacetamide according to the procedure of Kouyama & Mihashi (1981), except that the probe was initially dissolved in *N*,*N*-dimethylformamide (DMF). The final concentration of solvent in the reaction was <0.1%. The reaction was allowed to continue for 24 h at room temperature, whereupon the centrifuged (68 000 rpm, 1 h, 4 °C, 75 Ti rotor) F-actin pellet was dissolved in G buffer and depolymerized by extensive dialysis vs. this buffer at 4 °C. This *N*-(1-pyrenyl)iodoacetamide-labeled actin (pyrene–actin) was stored in the same manner as unlabeled actin. Typically, this actin was labeled to an extent of 0.7–0.8 mol/mol of actin, as measured by using a molar extinction coefficient for the protein–dye complex equal to 2.2×10^4 M^{-1} cm^{-1} at 344 nm (Kouyama & Mihashi, 1981) and quantitating the actin concentration by using the Bradford technique (1976). The probe is attached to the highly reactive cysteine group (Cys-373) (Kouyama & Mihashi, 1981).

Fluorescent Monitoring of Polymerization Process. All actin solutions were centrifuged before use with an air-driven Beckman airfuge in an A-100 (30°) rotor (180000*g* for 30 min). Trace quantities of pyrene–actin (typically 10 μg/mL or 0.24 μM) were used in conjunction with unlabeled actin to measure the fluorescence enhancement (excitation at 365 nm and emission at 386 nm) upon polymerization (in G buffer) induced by magnesium. The wavelengths chosen gave the maximal enhancement of the fluorescence upon polymerization, which has a partial contribution from an absorption change at the excitation wavelength. Complete polymerization results in an enhancement of the fluorescence of 22–26-fold. Since the excitation and emission wavelengths are reasonably close together, a check was made for light scattering effects with unlabeled actin polymerized with 2 mM Mg^{2+}. No increase in the emission signal upon polymerization of this unlabeled actin was detectable under our conditions. Correction of the final fluorescence extent for the critical concentration of G-actin at each condition allowed calculation of the expected fluorescence extent if all of the actin was in the filamentous state. The fluorescence change during polymerization was normalized to this calculated extent. The critical concentration of G-actin at each condition was measured either directly by sedimentation (see below) or by comparison of the fluorescence extent to that associated with a known F-actin concentration. The large fluorescence change upon polymerization allows one to make very sensitive quantitative measurements of the time course of the polymerization. There was no change in the fluorescence intensity of pyrene–actin at concentrations below the critical concentration for filament formation after the introduction of 2 mM Mg^{2+}. Polymerization was induced by adding magnesium to the actin solution (2.5 mL), which was subsequently mixed for 25 s or less with a rotating magnetic stirrer in the bottom of the fluorescence cuvette. Since continual mixing resulted in an acceleration of polymerization (Tait & Frieden, 1982), it was important that the mixing time be as short as possible. The fluorescence intensity was measured with a Spex Fluorolog fluorometer in a ratio mode, which compensates for fluctuations in the lamp intensity. Unless otherwise stated, cytochalasin D was added simultaneously with the addition of Mg^{2+} to the actin solution.

It should be noted that one must distinguish between the effects of cytochalasin D and that of its solvent, DMF. Concentrations of this solvent as low as 1% can cause a discernible slowing of the time course of fluorescence enhancement upon polymerization (unpublished observations). Therefore, in our experiments, the concentration of DMF was always kept less than 0.1%, where its effects are insignificant.

Although great care was taken to be consistent in the preparation of actin, different batches of the protein did not behave identically with regard to the half-time for polymerization, the characteristic lag time, or the maximal rate of polymerization. The lag time was determined by extrapolation of the line drawn to represent the maximal rate of change of fluorescence upon polymerization (maximal rate of polymerization) to zero fluorescence change. However, under a given condition, these parameters never varied by more than 20% between batches, and all qualitative characteristics of the kinetics of the enhancement were fully reproducible. However, it is stressed that actin from the same batch must be used for direct comparative purposes.

Measurement of Critical Concentrations of Polymerized Actin Solutions. The critical concentration can be operationally defined as the concentration of supernatant protein left after centrifugation to remove F-actin. Polymerized actin samples (200 μL, triplicates) were centrfuged in a Beckman airfuge (180000*g*) for 30 min. A small sample of the supernatant was carefully removed so as not to disturb the pellet. Subsequently, the protein concentration in this sample was measured by using a Bradford (1976) protein determination

[1] Abbreviations: G buffer, 2 mM Tris, 200 μM ATP, 200 μM $CaCl_2$, and 1.5 mM NaN_3, pH 8.0; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; NBD-labeled actin, 7-chloro-4-nitro-2,1,3-benzoxadiazole-labeled *N*-ethylmaleimide–actin; ATP, adenosine triphosphate; pyrene–actin, *N*-(1-pyrenyl)iodoacetamide-labeled actin; DMF, *N*,*N*-dimethylformamide; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate.

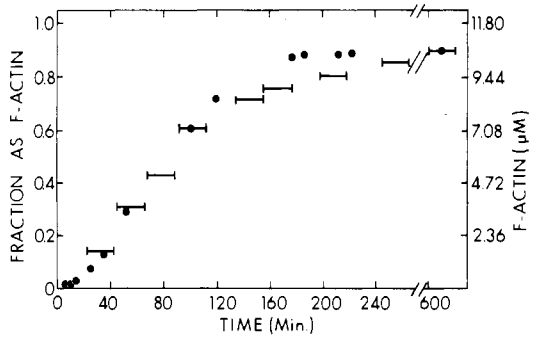

FIGURE 1: Correlation of time courses for fluorescence enhancement and formation of F-actin mass upon polymerization. Actin (12 μM) containing 0.24 μM pyrene–actin was induced to polymerize with 1 mM Mg^{2+} at 25 °C. The fluorescence enhancement (●) has been normalized to the extent expected for 100% F-actin. The concentration of F-actin at various times during a corresponding polymerization (bars) was measured via high-speed centrifugation. The error bars denote the duration of the centrifugation procedure (20 min).

with G-actin as a standard. When following the time course for the increase in F-actin mass upon polymerization (see Figure 1), the supernatant protein was obtained under similar conditions except that the sample was only centrifuged for 20 min.

Preparation of Platelet "Gelsolin". Outdated platelets were obtained from the Missouri–Illinois Red Cross as platelet-rich plasma. They were washed according to the method of Phillips & Agin (1977) and stored frozen. The thawed platelets were sonicated 3 times for 30 s in the extraction buffer used by Wang & Bryan (1981). The extract (after centrifugation) was passed over a DNase I coupled Sepharose affinity column according to the method of Wang & Bryan (1981). The protein was further purified by ion-exchange chromatography on DE-52 in a manner similar to that described by Yin & Stossel (1980) for macrophage gelsolin. The activity (i.e., the ability to reduce the gelation of F-actin) was subsequently eluted from that column at 0.2 M KCl. This activity of the protein was stable for at least 1 week in solution, and the protein could be lyophilized without destroying its activity.

After the DNase I–Sepharose column, $NaDodSO_4$–polyacrylamide gels indicated two major bands of M_r 90 000 and 42 000, the latter migrating to the same position as skeletal muscle actin. After the DE-52 column, the material migrated as a single species on $NaDodSO_4$ gels with a M_r of 90 000. The protein shows many of the characteristics of macrophage gelsolin (Yin & Stossel, 1980), including visible reduction of the gelation of F-actin at substoichiometric concentrations, a M_r of 90 000, and similar elution patterns from DE-52 and from DNase I–Sepharose by EGTA. We have therefore called this material platelet gelsolin.

Results

Correlation of Fluorescence Enhancement and Incorporation into F-Actin. Kouyama & Mihashi (1981) have shown that the fluorescence of pyrene-labeled F-actin is markedly enhanced (~25-fold) relative to the same concentration of pyrene-labeled G-actin. They also showed that the fluorescence enhancement of actin labeled with pyrene could be used to measure the critical concentration of actin. However, it is necessary to establish that trace quantities of pyrene–actin can be used to measure the time course of polymerization. In Figure 1 we show the correlation of the change in fluorescence of an actin solution during polymerization with the amount of pelletable F-actin. The latter was determined as the difference between the total actin and the amount of supernatant

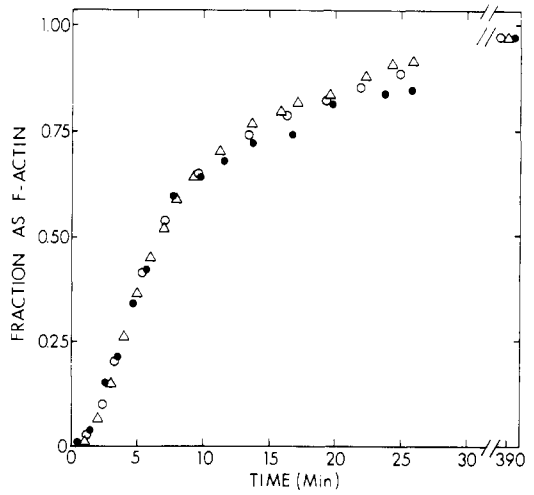

FIGURE 2: Time course of fluorescence enhancement as a monitor of actin polymerization. Trace quantities of pyrene–actin [0.05% (●), 0.50% (○), 5.0% (△)] were added to an unlabeled actin solution (total actin = 24 μM) and induced to polymerize with 2 mM Mg^{2+} at 20 °C. The fluorescence emission intensity was normalized to the extent expected if all of the actin were in the filamentous state.

protein left after high-speed centrifugation at various times during the polymerization. The fluorescence was normalized to the extent expected if all of the actin were filamentous. The horizontal error bars indicate the 20 min during which the actin solution was subjected to high-speed centrifugation. There is a good agreement between the two time courses, and the fluorescence change can therefore be used as a quantitative measure of polymer formation.[2]

Figure 2 shows that the time course of polymerization and the extent of fluorescence enhancement are identical over a 100-fold change in the ratio of pyrene-labeled to unlabeled actin when the total actin concentration is maintained constant. This result shows that there are no apparent differences in the polymerization characteristics of labeled and unlabeled actin. However, since there is a change in the absorption spectrum of the labeled actin upon polymerization, it was deemed better to use a low ratio of labeled to unlabeled actin in all experiments. The data of Figures 1 and 2 show that the use of trace amounts of pyrene-labeled actin ensures that the parameters of the polymerization process are in fact those of the unlabeled actin.

Effect of Cytochalasin D on Rate and Extent of Actin Polymerization. Figures 3–6 show that there is an acceleration of actin polymerization in the presence of cytochalasin D in all cases examined. This is reflected by a shortening or elimination of the lag period observed in the absence of cytochalasin and an increased rate of polymerization under most conditions. Further, there is a decrease in the extent of polymerization in the presence of the cytochalasin in all cases. Of most interest is that the extent of this decrease is dependent on the conditions of polymerization.

The magnesium concentration dependence of the fluorescence change with 24 μM (1 mg/mL) actin at 25 °C is shown in Figure 3. At 2 mM Mg^{2+}, cytochalasin D (2 μM) markedly enhances the initial polymerization, and the final fluorescence reading is 90% of that in the absence of cytochalasin D. This

[2] We have also observed that the same correlation holds for some other fluorescently labeled actins including rhodamine-labeled actin (Tait & Frieden, 1982) and NBD-labeled actin (Detmers et al., 1981). However, with NBD-labeled actin there is a small but significant fluorescence change during the initial lag period, which may be a consequence of a cation-induced fluorescence change.

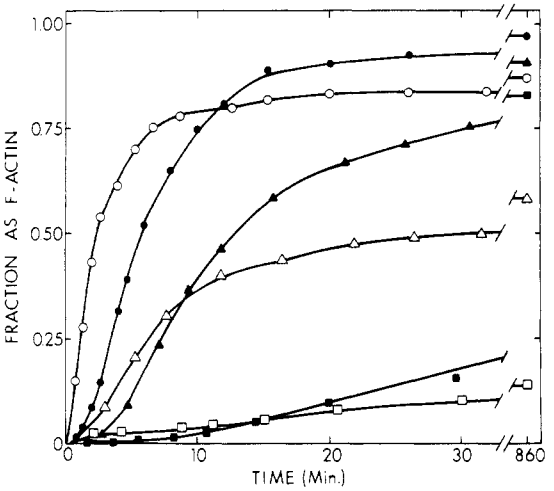

FIGURE 3: Effect of cytochalasin D upon actin polymerization at various magnesium concentrations. Actin (24 μM, 0.24 μM pyrene–actin) was polymerized at 25 °C by the addition of 2 (●, ○), 1 (▲, △), and 0.5 mM (■, □) Mg^{2+} in the absence (closed symbols) and the presence (open symbols) of 2 μM cytochalasin D. The fluorescence enhancements have been normalized to the extent expected for 100% F-actin.

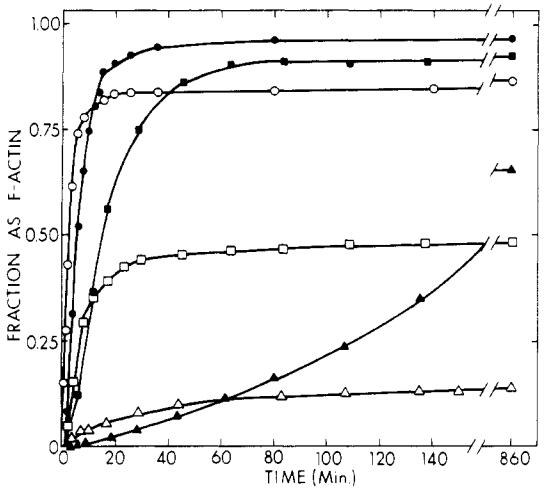

FIGURE 4: Effect of cytochalasin D on time course of fluorescence enhancement upon polymerization at various actin concentrations. Actin solutions (containing 0.24 μM pyrene–actin) were induced to polymerize with 2 mM Mg^{2+} at 25 °C in the absence (closed symbols) and presence (open symbols) of 2 μM cytochalasin D. The actin concentrations were 24 (●, ○), 9.5 (■, □), and 2.4 μM (▲, △). The results have been normalized to the extent expected for 100% F-actin at each actin concentration.

Table I: Critical Concentrations in the Presence and Absence of Cytochalasin D[a]

addition	total actin (μM)	pyrene-actin to total actin (%)	critical concn (μM)
none	2.6	10.0	0.73
2 μM cytochalasin D	2.6	10.0	2.05
none	9.7	2.5	0.62
2 μM cytochalasin D	9.7	2.5	4.11
none	24.0	1.0	0.76
2 μM cytochalasin D	24.0	1.0	3.62

[a] Actin solutions (2.6, 9.7, and 24 μM) containing 0.24 μM pyrene-actin were polymerized with 2 mM Mg^{2+} at 25 °C in the presence and absence of 2 μM cytochalasin D. After 20 h the critical concentration was measured from the protein concentration of supernatants after high-speed centrifugation.

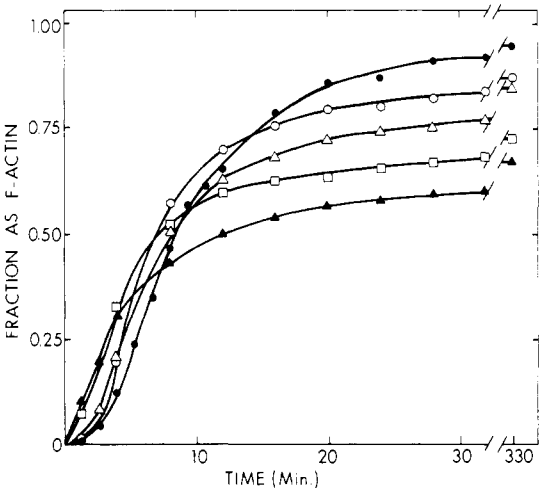

FIGURE 5: Polymerization of actin at different cytochalasin D concentrations. A 16 μM actin solution (containing 0.24 μM pyrene–actin) was polymerized with 2 mM Mg^{2+} at 25 °C with various cytochalasin D concentrations: 0 (●), 0.16 (○), 0.4 (△), 2 (□), and 8 μM (▲). The time courses were normalized to the extent expected if all of the actin were in the filamentous form.

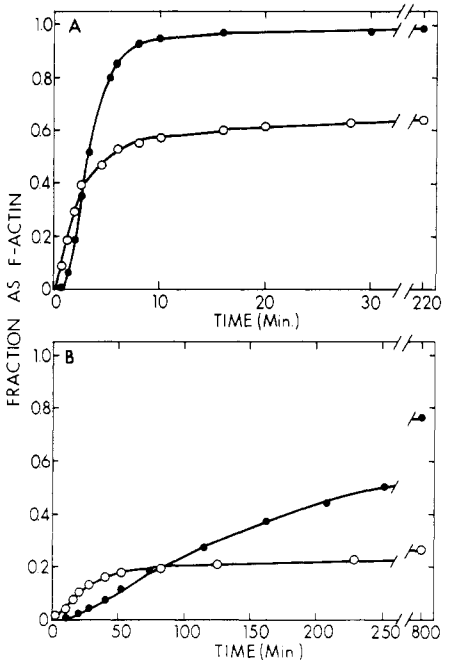

FIGURE 6: Effects of temperature on actin polymerization time course in the presence and absence of cytochalasin D. A 9.4 μM actin solution (containing 0.24 μM pyrene–actin) was induced to polymerize at temperatures of 40 (A) and 10 °C (B) in the absence (closed symbols) and presence (open symbols) of 2 μM cytochalasin D. Results at 25 °C, reported in Figure 5 for the same actin concentration, are additionally relevant. The results have been normalized to the extent expected for 100% F-actin at each temperature.

10% decrease in the final fluorescence extent is what would be expected from the change in critical concentration in the presence of the cytochalasin under these conditions (cf. Table I). In contrast, at low magnesium levels (0.5 mM), there is small but distinct initial rate enhancement in the presence of cytochalasin D but an almost complete lack of further incorporation of monomer into the polymer as measured over an additional 14-h period. The final fluorescence level is only 16% of that in the absence of cytochalasin D [final extent in the absence of cytochalasin D shown by (■)]. At intermediate magnesium levels (1 mM) in the presence of cytochalasin, the polymerization is also characterized by an initial rate en-

hancement and decrease in the final fluorescence level (67% of the extent in the absence of cytochalasin). It is clear that the influence of cytochalasin D on the final extent of polymerization appears to be greatest at the lower magnesium levels as represented by greater differences between the final extent of the control and that of the solution containing the cytochalasin.

Figure 4 shows that the rate of polymerization in the absence of cytochalasin is dependent on the actin concentration. In the presence of cytochalasin there is an enhancement of the polymerization in all cases. The critical concentration in the presence of cytochalasin is essentially independent of the actin concentration.[3] It should be noted that the data of Figure 4 have been normalized to the extent expected if all of the actin was filamentous at each actin concentration. Thus at low actin concentrations the ratio of the critical concentration to the total actin concentration is high, leading to a decreased fraction of the material as F-actin. It is of interest that, when polymer fragments (formed by shearing F-actin through a fine hypodermic needle) are added, the rate of polymerization is further enhanced but the extent of polymerization reaches exactly the same level as when no fragments are added either in the absence or in the presence of cytochalasin (data not shown).

A change in the extent of polymerization should also be measurable as an increase in the critical concentration in the presence of cytochalasin D, and Table I demonstrates this to be the case at three different actin concentrations. In all cases, polymerization was initiated by the addition of 2 mM Mg^{2+}. In agreement with previous studies (Gordon et al., 1977), the critical concentration under these conditions (2 mM Mg^{2+}, pH 8, 25 °C) is approximately 0.70 μM (0.03 mg/mL), and Brenner & Korn (1979) have shown that it increases in the presence of cytochalasin D. As expected from the results given in the previous section, there was a good correlation between the fluorescence enhancement and amount of polymer formed both in the presence and in the absence of cytochalasin D (data not shown).

The dependence of the kinetics and extent of polymerization on cytochalasin D concentration are shown in Figure 5 at an actin concentration of 16 μM (2 mM Mg^{2+}, 25 °C). Here it is shown that the accelerated polymerization (reflecting a decreased lag time and an increased polymerization rate) and decreased final fluorescence levels are both dependent on the cytochalasin concentration. The final extent of fluorescence enhancement in the presence of the cytochalasin appears to be approaching saturation under these conditions as the time course at 16 μM cytochalasin D (data not shown) is very similar to that at 8 μM cytochalasin D.

It has been previously reported that the rate of polymerization is temperature sensitive (Asakura et al., 1960). In Figure 6, normalized polymerization time courses are compared for two temperatures, 40 and 10 °C (pH adjusted to 8.0 at each temperature). The data can be compared with the previous experiments (see Figure 4) at an actin concentration of 9.4 μM at 25 °C. In agreement with the above data, cytochalasin D accelerates polymerization but decreases its extent. In this case, at a given cytochalasin concentration, smaller extents of polymerization are observed at lower temperatures, all other conditions (pH, Mg^{2+} concentration, actin concentration) being the same. It is noted that the polymerization rate is indeed very dependent on temperature and

[3] In the experiments of Figure 4 and Table I, the ratio of labeled to unlabeled actin was different for each total actin concentration. The curves were normalized by using 100% as the extent of polymerization if all the actin had been incorporated into polymer.

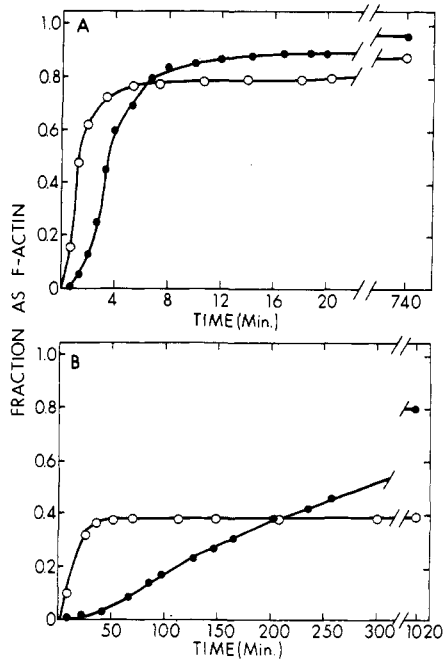

FIGURE 7: Effect of platelet gelsolin on actin polymerization. G-Actin (19.5 μM) was polymerized at 25 °C by the addition of 2 (A) or 0.38 mM (B) Mg^{2+} in the absence (closed symbols) and presence (open symbols) of 0.4 μM platelet gelsolin. The results were normalized to the extent expected if all of the actin were in the filamentous form. The buffer contained 200 μM Ca^{2+}.

is similar to that previously observed by Oosawa & Kasai (1971). After normalization of the fluorescence time courses at a particular temperature to the final extent expected if all of the actin were in the polymeric state, the results of Figure 6 are qualitatively identical with those of Figures 3 and 5.

Effect of Platelet Gelsolin on Actin Polymerization. As described under Experimental Procedures, we have isolated a protein from platelets that appears to have the characteristics of macrophage gelsolin. We have termed this protein platelet gelsolin. The protein is one of several that, at substoichiometric concentrations, markedly affect the viscosity of F-actin in vitro (Yin & Stossel, 1980; Schliwa, 1981).

The effect of a substoichiometric concentration of this protein on the Mg^{2+}-induced actin polymerization is shown in Figure 7. Gelsolin has been reported to show Ca^{2+}-dependent effects (Yin et al., 1981). The experiments reported here have been performed at saturating Ca^{2+} concentrations (200 μM). Its effects are qualitatively similar to those of cytochalasin D, in that it accelerates actin polymerization and reduces the final extent of polymerization. Further, the decrease in the extent of polymerization depends on the conditions for polymerization. At 2 mM and 0.38 mM Mg^{2+}, the final extent of polymerization in the presence of gelsolin is 97% and 51%, respectively, of that in its absence.

Discussion

The ability to measure actin polymerization in the absence of shear allows quantitative comparisons to be made under different conditions of polymerization. This is essential for the polymerization process since it is clear that frequently used methods, such as viscometry, break actin filaments (Tait & Frieden, 1982; Maruyama et al., 1974; Maruyama, 1964) and thereby provide more ends to which monomeric actin may add. Thus, the commonly used methods of measuring polymerization influence the polymerization process in some undefined way.

The data of Figure 1 (and Table I) show that the change

of fluorescence of pyrene-labeled actin is a valid and quantitative measure of the rate of incorporation of monomeric actin into polymer. Furthermore, the continuous time course is easily measured with a high degree of sensitivity. It is noteworthy that NBD-labeled actin and rhodamine-labeled actin only enhance the fluorescence upon polymerization 2.2- and 1.5-fold, respectively, compared to the 22–26-fold enhancement observed for pyrene-labeled actin.

A striking application of this technique is the influence of cytochalasin D on the polymerization process. Figures 3–6 show that in *every* case, cytochalasin accelerates the approach to the final level of polymer but decreases the extent of polymerization. The acceleration of polymerization is reflected by an enhanced rate of polymerization and/or a shortened lag time. Although some conditions have been reported in which polymerization appears to be more rapid in the presence of cytochalasin (Brenner & Korn, 1980; Seldon et al., 1980; MacLean-Fletcher & Pollard, 1980a; Wieland & Löw, 1980; Löw et al., 1979), our result differs from most of those currently in the literature, which consider the effect of cytochalasin D to be inhibitory. Such inhibition is now shown to be a decrease in the extent of polymerization. In fact, since cytochalasin D activates the rate of polymerization, it would be best if the term "inhibition" were avoided in favor of enhanced rate and decreased extent of polymerization.

A second striking result is that the effect of the conditions of polymerization in influencing the amount of F-actin formed is dramatically different in the presence of cytochalasin D compared to that in the absence of cytochalasin D. In general, conditions that result in a slow rate of polymerization in the absence of cytochalasin also result in a smaller extent of polymer formation in the presence of cytochalasin relative to that in the absence of cytochalasin. These conditions include lower Mg^{2+} concentrations, lower temperature, and lower pH (unpublished observations). This additional sensitivity of the final extent of polymer formation to the conditions of the polymerization in the presence of cytochalasin D has not been previously reported.

Cytochalasin has been reported to enhance the hydrolysis of ATP on G-actin (Brenner & Korn, 1980, 1981). We have measured the concentration of ATP remaining after both rapid (24 μM actin, 2 mM Mg^{2+}) and slow (24 μM, 0.5 mM Mg) polymerizations in the presence and absence of 2 μM cytochalasin D. Although cytochalasin D does enhance the rate of hydrolysis of ATP, at least 40 μM ATP (of the initial 200 μM) remains after 20 h in all cases studied. Further, addition of 200 μM ATP to these samples after 20 h does not increase the extent of polymerization.

Any scheme for the effect of cytochalasin on actin polymerization must encompass the results shown in Figures 3–6. Cytochalasin is known to bind preferentially to actin filaments in substoichiometric amounts relative to that of G-actin (Hartwig & Stossel, 1979; Flanagan & Lin, 1980). It is believed that it specifically binds to one end of the actin filament, the "fast-growing" end, and prevents further growth of the filament from that end (Brenner & Korn, 1979; Lin et al., 1980; Flanagan & Lin, 1980). Indeed, there is evidence that actin filaments grown in the presence of cytochalasin are shorter than those grown in its absence (Hartwig & Stossel, 1979; Maruyama et al., 1980), and fluorescence photobleaching recovery experiments suggest that actin filaments in undisturbed solutions are shorter in the presence of cytochalasin relative to those in its absence (Tait & Frieden, 1982). In simple terms, actin polymerization follows a nucleation–elongation mechanism. Nucleation is not a favored event because of either an unfavorable equilibrium between monomer and low molecular weight nuclei or a slow rate of formation of nuclei (Oosawa & Kasai, 1971). The elongation process appears to be quite rapid, occurring, as indicated above, at different rates from the two ends of the growing filament. Since cytochalasin blocks the fast-growing end and therefore inhibits elongation, the simple model suggests that the enhanced rate and decreased lag time in the presence of cytochalasin must occur at the nucleation step, i.e., either a larger number of nuclei in equilibrium with monomer or a faster rate of nuclei formation. Enhanced nucleation in the presence of cytochalasin and some actin binding proteins has been proposed by other workers (Brenner & Korn, 1980; Craig & Powell, 1980; Yin et al., 1981; Glenney et al., 1981). If one makes the reasonable assumption that monomer is in equilibrium with low molecular weight nuclei (dimer, trimer, tetramer, ...) and that cytochalasin D binds preferentially to these nuclei, then the initial enhanced rate of polymerization results from a higher concentration of nuclei even though only the "slow-growing" end is available for elongation. Since similar effects are observed for platelet gelsolin, an analogous explanation of the acceleration of actin polymerization by this protein can be made. Yin et al. (1981), using flow birefringence, have recently observed that macrophage gelsolin accelerates polymerization but decreases the extent of polymerization.

The data also show that the extent of polymerization in the presence of cytochalasin D is more dependent on the conditions of polymerization than is the extent of polymerization in the absence of cytochalasin D. It is not clear yet whether the effect represents equilibrium considerations or kinetic considerations, but it is possible to decribe this result in terms of the simple mechanism described above. Thus, lowering the Mg^{2+} concentration or temperature, which also lowers the rate of polymerization, probably changes the ability of polymer to add monomeric units at either or both the fast- and slow-growing ends of the filament; that is, the critical concentration at either or both ends is higher at lower Mg^{2+} concentration or lower temperature. When one end is blocked by cytochalasin, the final extent reflects the critical concentration of the other end under the conditions used (i.e., low Mg^{2+} or temperature).

As shown by Figure 4, cytochalasin D accelerates polymer formation at several actin cocentrations. Table I shows, however, that the critical concentration in the presence and absence of cytochalasin D is different but essentially independent of the actin concentration. Figure 5 shows that not only the rate of polymerization but also the final extent (and therefore the critical concentration) is dependent on the total cytochalasin concentration. At the actin concentration used in Figure 5 (16 μM), the effect of cytochalasin D on the extent of polymerization is complete at a concentration between 8 and 16 μM. In view of the current concepts for the mode of action of cytochalasin (tight stoichiometric binding to the fast-growing end of each filament), the observed results are difficult to explain. This suggests that the effect of cytochalasin D may be more complex than previously supposed.

The quantitative explanation of these results may involve many more aspects of the polymerization process not represented by the simple mechanism. First, electron microscopy shows that filaments grown in the presence of cytochalasin are in general shorter than those in its absence (Hartwig & Stossel, 1979). In fact, the rheological properties of these solutions are remarkably different. By simple direct observation, undisturbed polymerization in the absence of cytochalasin yields a reasonably solid gel that is difficult to pour from the container. In the presence of cytochalasin (or platelet gelsolin),

the solution is not at all gellike, even though the polymerization as measured by fluorescence change under certain conditions can be nearly the same. This difference may reflect a very different structure of the polymer, which in turn influences the critical concentration of the filament. We have shown, for example, that cytochalasin inhibits the onset of network formation (Tait & Frieden, 1982). Second, it has been shown that cytochalasin enhances the ATP hydrolysis rate of G-actin in the presence of 0.5 mM Mg^{2+}, and it was postulated that ATP on G-actin can exist in an additional "refractory state", namely, ADP·P_i (Brenner & Korn, 1981). Under different conditions, the state of the nucleotide on G-actin might be influenced by cytochalasin, which may influence the final extent of polymerization. Third, actin filaments undergo spontaneous breaking and reannealing. Each break may result in cytochalasin binding and subsequently shorter filaments. Very short filaments may have a different critical concentration than longer filaments. Since the chance of a filament breaking is larger over long periods of time (slow polymerization) relative to short times (fast polymerization), the effect of cytochalasin may depend on the conditions of the polymerization process. The spontaneous breaking and reannealing process may also relate to the issue of ATP hydrolysis during polymerization or at steady state. Fourth, it is possible that cytochalasin D binds much less tightly ($K_D \sim 1$ μM) to actin filaments than has been previously suggested. The observation that the effect of cytochalasin depends on the conditions of polymerization may result from any or all of the above reasons, and at this time there is insufficient information to distinguish the cause.

Figure 7 shows that the protein we have termed platelet gelsolin accelerates the rate but decreases the extent of polymerization.[4] Wang & Bryan (1981), using high shear viscosity as a measure of polymerization, observed a decrease in the final viscosity extent in the presence of an impure platelet "gelsolin" solution. However, their results do not clearly indicate if there is any acceleration of polymerization.

Our observation that the extent of polymerization is dependent on the conditions of polymerization in the presence of platelet gelsolin, in a manner qualitatively similar to that seen for cytochalasin D, may have implications for the in vivo regulation of actin polymerization. Gelsolin, in the presence of Ca^{2+}, and a number of other so-called capping proteins are believed to act in a manner similar to that of cytochalasin (Schliwa, 1981) in that they bind to a specific end of an actin filament, thereby preventing further growth from that end. These proteins include villin (Craig & Powell, 1980), fragmin (Hasegawa et al., 1980), and a platelet protein of M_r 65 000 (Grumet & Lin, 1980) as well as gelsolin (Yin et al., 1981). Indeed Yin et al. (1981) have reported that macrophage gelsolin may preferentially bind to two actin monomers under nonpolymerizing conditions, stabilizing them against any dissociation and thus providing nuclei. They note also the functional similarities between gelsolin, cytochalasins, villin, fragmin, and other proteins and suggest a Ca^{2+} control of filament length.

We have seen in the cytochalasin D and gelsolin studies that the extent of polymerization, and thus the amount of G-actin present after polymerization, depends on the conditions of polymerization. It is known that in nonmuscle cells there can be a considerable pool of G-actin (Bray & Thomas, 1976; Gordon et al., 1977). It should now be clear that the pool of G-actin depends on the presence of capping proteins like gelsolin as well as on the physiological conditions that induce polymerization. In vivo, very low concentrations of these proteins could provide a highly sensitive regulation of the state of actin as distributed between G and F forms. Large G-actin pools have also been attributed to the presence of a protein, profilin, that specifically binds stoichiometrically with G-actin (Carlsson et al., 1977), but it now seems clear that there are other possible mechanisms for this control. Such control is of particular interest with respect to gelsolin since the effect of this protein is Ca^{2+} sensitive due to the fact that its binding to actin is Ca^{2+} dependent (Wang & Bryan, 1981). It has been shown, for example, that there is a transient increase in Ca^{2+} concentration in platelets following activation (Massini & Luscher, 1976). It is interesting to speculate that proteins like gelsolin may magnify the effect of different physiological conditions in terms of the pool of available G-actin in a manner similar to that shown in this paper for the effect of cytochalasin D.

[4] The use of trace amounts of pyrene-labeled actin and substoichiometric amounts of gelsolin or cytochalasin D makes it unlikely that the chemical modification of the actin interferes with the binding of gelsolin or cytochalasin D to the actin.

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Reactivity of Sarcoplasmic Reticulum Adenosinetriphosphatase with Iodoacetamide Spin-Label: Evidence for Two Conformational States of the Substrate Binding Site†

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ABSTRACT: The labeling kinetics of sarcoplasmic reticulum ATPase with the iodoacetamide spin probe *N*-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide were followed under conditions designed to selectively label all reactive groups. Approximately 1 mol of spin-label reacted per one 100 000-dalton ATPase chain, indicating only one residue on the enzyme had been labeled. One uniform rate of labeling was observed in the presence of Ca^{2+}. When substrate was then added, approximately one-half of the residues showed a 10-fold increase in labeling rate while the remaining residues reacted at the initial, slower rate. Sequential labeling experiments further established that the two labeling rates correspond to the coexistence of two conformational states of the enzyme. Both Ca^{2+} and substrate are required to obtain an equal distribution between states, and the effect is completely reversed when substrate is removed. The iodoacetamide spin probe is known to be highly sensitive to the conformation of the ATPase binding pocket, and the residue labeled here is the one which generates broadening in the electron paramagnetic resonance spectrum on substrate binding. Due to the unique selectivity of the labeling reaction, it is suggested that when both substrate and Ca^{2+} are bound to the enzyme, conditions which are precursory to enzyme phosphorylation, two specific conformations of the binding pocket exist in approximately a 50:50 ratio.

It has been established for some time that sarcoplasmic reticulum (SR)[1] ATPase can be selectively labeled with the iodoacetamide spin probe ISL without loss of activity (Landgraf & Inesi, 1969). The spectrum of the labeled enzyme exhibits a high degree of sensitivity to substrate binding and is, in turn, regulated by the presence of Ca^{2+} (Inesi & Landgraf, 1970; Coan & Inesi, 1977).

Since the labeled enzyme remains fully functional, this technique has produced an independent means of monitoring the allosteric effect of Ca^{2+} on the substrate binding site. This has aided the interpretation of Ca^{2+} binding studies involving high-affinity sites which are precursory to enzyme activation (Inesi et al., 1980), and it has also allowed Ca^{2+} regulation of the substrate site to be followed directly during active turnover (Coan et al., 1979).

The continued use of the iodoacetamide label has made it increasingly important to ascertain the number and nature of the labeled residues on the ATPase. Kinetic labeling studies

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[1] Abbreviations: SR, sarcoplasmic reticulum; ISL, *N*-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide; Mops, 4-morpholinepropanesulfonate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*-*N*′,*N*′-tetraacetate; AMP-P(NH)P, adenyl-5′-yl imidodiphosphate; [SnoPP(NH)P]$_2$, disulfide of thioinosine 5′-(β,γ-methylenetriphosphate); NaDodSO$_4$, sodium dodecyl sulfate.

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