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Mechanism of $CaCl_2$-Induced Actin Polymerization

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ABSTRACT: The $CaCl_2$ concentration dependence of the rate of actin filament elongation and of the actin monomer concentration at steady state with actin polymer (the critical actin concentration) has been investigated. A relative rate of actin filament elongation from actin polymer intermolecularly cross-linked with *N,N'-p*-phenylenebis(maleimide) showed a sigmoidal dependence on the concentration of $CaCl_2$ used to induce actin polymerization. This result is shown to be consistent with a model in which only actin monomer containing five equivalently bound Ca^{2+} ions (K_a = 2 mM^{-1}) is capable of addition to actin polymer. A relative dissociation rate constant for actin monomer removal from polymer was calculated from the product of the critical actin concentration and the relative elongation rate constant and was found to be virtually independent of $CaCl_2$ concentration. The relationship between Ca^{2+} binding sites on actin and the $CaCl_2$ concentration dependence of the kinetics of actin filament elongation is discussed.

Actin is a major protein of eukaryotic cells and has essential roles in cell structure and motility and an involvement in a wide range of other cellular activities [see reviews by Clarke & Spudich (1977), Pollard (1981), and Korn (1982)]. This protein reversibly polymerizes from a monomer or G-actin to long double-helical actin filaments or F-actin containing many hundreds of actin protomers. Many actin-related activities of nonmuscle cells may require the disassembly and reassembly of actin filaments at specific sites and times within a cell. The degree and rate of actin polymerization in a cell are probably influenced by factors such as pH, bivalent cations, ionic strength, temperature, adenine nucleotides, and actin binding proteins, all of which affect the in vitro polymerization properties of actin [reviewed by Korn (1982)]. Thus, an understanding of cellular activities involving this protein may first require detailed knowledge of the influence of these factors on the state of actin in vitro.

G-Actin can be induced to polymerize into F-actin by millimolar concentrations of bivalent cations and/or physiological ionic strengths. The overall rate of actin polymerization is remarkably sensitive to the concentration of bivalent cations required to induce polymerization (Kasai et al., 1962; Kasai, 1971; Oosawa & Kasai, 1971; Pollard & Mooseker, 1981; Rouayrenc & Travers, 1981; Frieden, 1983; Tobacman & Korn, 1983; Lal et al., 1984). Although this sensitivity has been well documented, its origin remains obscure.

The present study reports the $CaCl_2$ concentration dependence of the kinetics of actin filament elongation from actin polymer intermolecularly cross-linked with *N,N'-p*-phenylenebis(maleimide) and of the concentration of actin monomer at steady state with actin polymer (the critical actin concentration). A simple model is proposed which describes the $CaCl_2$ concentration dependence of these results.

Experimental Procedures

Rabbit skeletal muscle actin was isolated and purified according to the procedure described by Pardee & Spudich (1982) with the gel filtration (Sephadex G-150) modification of MacLean-Fletcher & Pollard (1980). G-Actin was in a buffer consisting of 2 mM tris(hydroxymethyl)aminomethane (Tris),[1] 200 μM ATP, 200 μM $CaCl_2$, and 1.5 mM NaN_3, pH 8.0 (G buffer), and used within 4 days of G-150 Sephadex chromatography. Before all experiments, the G-actin was centrifuged (3 h, 4 °C, 96000*g*) to remove any actin oligomers. Actin concentrations were measured spectrophotometrically at 290 nm using an extinction coefficient of 0.63 $mL \cdot mg^{-1}$ (Houk & Ue, 1974) and a molecular weight of 42300 (Elzinga et al., 1973).

N-Pyrenylactin was prepared as previously described (Tellam & Frieden, 1982) and dialyzed exhaustively against G buffer at 4 °C. This chemically modified G-actin was centrifuged (178000*g*, 30 psi, Beckman airfuge) before use. The concentration of *N*-pyrenylactin was determined by using the Bradford (1976) protein assay with unmodified G-actin as a standard. The ratio of dye to protein was approximately 0.70–0.85.

All fluorescence measurements were made in a Perkin-Elmer Model LS-5 spectrofluorometer at 25 °C. Incorporation of *N*-pyrenylactin into actin polymer results in appoximately a 25–26-fold fluorescence enhancement of the labeled actin at an emission wavelength of 407 nm and an excitation wavelength of 365 nm (Kouyama & Mihashi, 1981). The time course of fluorescence increase of trace levels (<10% of the

[1] Abbreviations: G buffer, 2 mM Tris, 200 μM ATP, 200 μM $CaCl_2$, and 1.5 mM NaN_3, pH 8.0; *N*-pyrenylactin, (*N*-pyrenylcarboxamidomethyl)actin; Tris, tris(hydroxymethyl)aminomethane.

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total actin content) of *N*-pyrenylactin in the presence of unlabeled actin under polymerizing conditions is a very sensitive means of following the incorporation of actin monomer into polymer, i.e., the kinetics of actin polymerization (Tellam & Frieden, 1982). There was no difference between modified and unmodified actins in their ability to polymerize under these conditions. The fluorescence intensity was generally measured intermittently to avoid photobleaching of the *N*-pyrenylactin. However, there was less than a 2% decrease in the fluorescence of *N*-pyrenylactin monomer in G buffer after 3 h of continuous fluorescence monitoring. The polymerization of actin was initiated by the addition (with mixing; three cuvette inversions) of a small volume of a concentrated $CaCl_2$ solution to a 3-mL cuvette containing G-actin in G buffer preequilibrated to 25 °C. The $CaCl_2$ concentration used to induce actin polymerization is that added in excess of the 200 μM $CaCl_2$ already present in G buffer. The latter is required to maintain Ca^{2+} at the "high-affinity" bivalent cation binding site (K_a = 143 mM^{-1}; Frieden, 1982) on actin monomer without which G-actin readily denatures. In addition, the majority of $CaCl_2$ present in G buffer will be complexed with ATP ($K_a^{Ca\text{-}ATP}$ = 167 mM^{-1}; O'Sullivan & Perrin, 1964; Phillips et al., 1966). Thus, the free Ca^{2+} ion concentration in G buffer is negligible compared with the $CaCl_2$ concentrations (>1 mM) generally required to induce actin polymerization.

Critical actin concentrations were determined by two methods. First, G-actin (32 μM unlabeled actin and 0.34 μM *N*-pyrenylactin) in G buffer was polymerized by making the solution 1 or 5 mM in $CaCl_2$ at 25 °C. The polymerized actin samples were then diluted with G buffer containing the same $CaCl_2$ concentrations to several lower actin concentrations, and the steady-state fluorescence was measured after 10 h at 25 °C. Plots of the steady-state fluorescence vs. total actin concentration at each $CaCl_2$ concentration were extrapolated to intercept with a similar plot but in the absence of additional $CaCl_2$. The total actin concentration at this intercept is the critical actin concentration (Oosawa & Kasai, 1971). Further, these results allowed calculation of the concentration of F-actin (and therefore the concentration of polymerized *N*-pyrenylactin) corresponding to a particular change of fluorescence upon polymerization. Second, critical actin concentrations at several $CaCl_2$ concentrations were measured according to the method described by Tobacman et al. (1983). G-Actin (32 μM unlabeled actin and 1.6 μM *N*-pyrenylactin) in G buffer was first polymerized by making the solution 2.0 mM in $CaCl_2$ at 25 °C. This polymerized actin was sonicated (~5 s) and diluted with G buffer to 5 μM concentrations in separate samples containing 1–10 mM added $CaCl_2$ at 25 °C. The steady-state fluorescence was measured after 6 h at 25 °C. Knowledge of the fluorescence change associated with the formation of a known concentration of polymeric *N*-pyrenyl actin (see above) allowed calculation of the F-actin concentration at each $CaCl_2$ concentration. The difference between the total actin concentration and the F-actin concentration gave the critical actin monomer concentration. In addition, similar experiments were performed with higher initial actin concentrations and lower initial $CaCl_2$ concentrations to allow measurement of critical actin concentrations at $CaCl_2$ concentrations less than 1 mM.

F-Actin was cross-linked intermolecularly with *N,N'-p*-phenylenebis(maleimide) by the method described by Knight & Offer (1978). This cross-linked actin did not fully depolymerize after exhaustive dialysis against G buffer, i.e., under conditions where F-actin would fully depolymerize. It has been demonstrated that this cross-linked actin polymer is able to elongate by the reversible addition of actin monomers under polymerizing conditions (Mockrin & Korn, 1983). The concentration of cross-linked actin polymer was determined by using the method described by Bradford (1976) with G-actin as a standard.

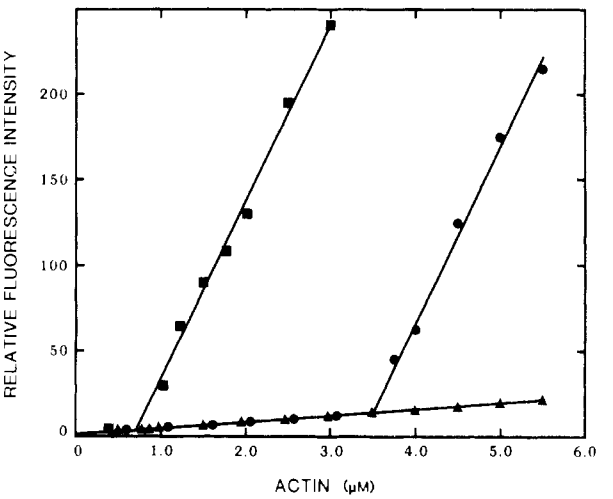

FIGURE 1: Determination of the critical actin concentration in 1 and 5 mM $CaCl_2$. G-Actin (32 μM, 0.34 μM *N*-pyrenylactin) in G buffer at 25 °C was polymerized with 1 or 5 mM $CaCl_2$. Each polymerized actin sample was then diluted to several lower actin concentrations with G buffer containing the same $CaCl_2$ concentration used to initially induce polymerization. The steady-state fluorescence (excitation, 365 nm; emission, 407 nm) was measured after 10 h at 25 °C. The $CaCl_2$ concentration was that added to actin solutions in excess of the 200 μM $CaCl_2$ already present in G buffer. Controls (▲) were performed in the absence of additional $CaCl_2$; (●) 1 mM $CaCl_2$; (■) 5 mM $CaCl_2$.

RESULTS

Effect of Ca^{2+} on the Critical Actin Concentration. Actin polymerization is characterized by a critical actin concentration, A_1^∞ (Oosawa & Kasai, 1971). At actin concentrations below A_1^∞, virtually no polymer is formed, and actin in excess of A_1^∞ directly becomes F-actin. The critical actin concentration is the concentration of actin monomer present at steady state with regard to the F-actin concentration and in terms of the nucleation–elongation model (Oosawa & Kasai, 1971) for actin polymerization, $A_1^\infty = k_-/k_+$, where k_- is the rate constant for the removal of an actin monomer from an actin filament and k_+ is the rate constant for the addition of actin monomer to an actin filament. In reality, each of these rate constants is the sum of the relevant rate constants from the two separate ends of an actin filament. Figure 1 shows the relationship between the steady-state fluorescence of actin containing 1.1% *N*-pyrenylactin and the total actin concentration for polymerizations induced by 1 and 5 mM $CaCl_2$. At concentrations of actin below the critical actin concentration, the fluorescence is identical with that for G-actin. Actin polymerized with 1 or 5 mM $CaCl_2$ has critical actin concentrations (A_1^∞) of 3.5 and 0.7 μM, respectively. The difference between the total actin concentration and A_1^∞ allowed calculation of the F-actin concentration and therefore the concentration of polymerized *N*-pyrenylactin associated with a particular change of fluorescence For all further results, a given fluorescence change was directly converted to the corresponding F-actin concentration.

The dependence of the critical actin concentration on the added concentration of $CaCl_2$ is shown in Figure 2. Clearly, the magnitude of A_1^∞ is strongly dependent on the $CaCl_2$ concentration; i.e., A_1^∞ increases from 1.4 μM at 2 mM $CaCl_2$ to 4.6 μM at 0.9 mM $CaCl_2$. However, the magnitude of A_1^∞ is relatively constant at about 0.6–0.7 μM for $CaCl_2$ concen-

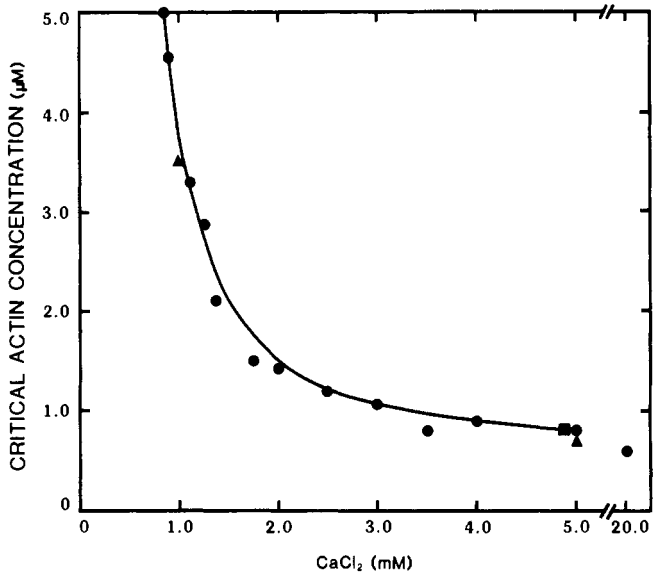

FIGURE 2: Effect of $CaCl_2$ on the critical actin concentration. G-Actin (32 μM, 1.6 μM *N*-pyrenylactin) in G buffer at 25 °C was polymerized with 2.0 mM $CaCl_2$ and then diluted with G buffer to 5.0 μM actin in a series of samples containing 1–10 mM added $CaCl_2$. The steady-state fluorescence (excitation, 365 nm; emission, 407 nm) was measured after 6 h at 25 °C. The data from Figure 1 allowed calculation of the polymerized *N*-pyrenylactin concentration (and therefore the F-actin concentration) associated with a particular fluorescence change. The critical actin concentration (●) was determined from the difference between the total actin concentration and the F-actin concentration. The sensitivity of these measurements was extended to higher and lower $CaCl_2$ concentrations by dilutions to lower and higher final actin concentrations, respectively (see Experimental Procedures). The critical actin concentration has been shown as a function of the added $CaCl_2$ concentration. The critical actin concentrations determined from Figure 1 (▲) and Figure 4 (■) are also included.

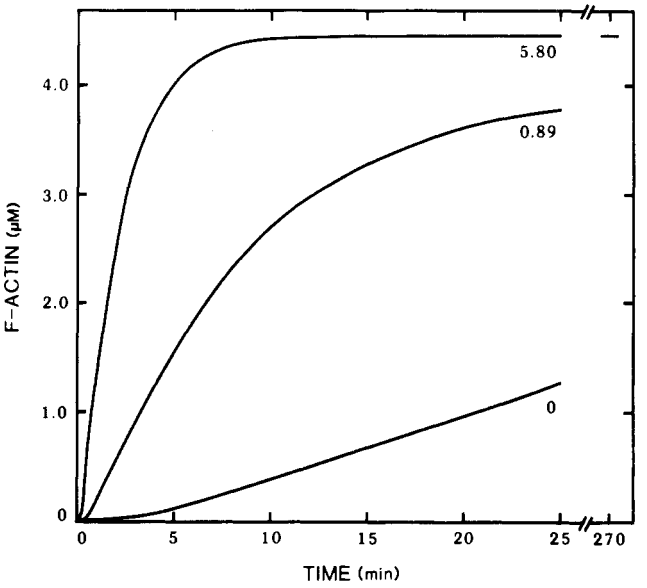

FIGURE 3: Effect of the addition of cross-linked actin polymer on the time course of actin polymer formation. G-Actin (5 μM, 0.15 μM *N*-pyrenylactin) in G buffer at 25 °C was polymerized with 5.0 mM $CaCl_2$ in the absence and presence of 0.89 and 5.80 μg/mL covalently cross-linked actin polymer. The fluorescence change at a particular time was converted to the corresponding F-actin concentration by using the data of Figure 1. The final extents of polymer formation were identical.

trations greater than 5 mM. The value of A_1^∞ increases greatly at low $CaCl_2$ concentrations (<2 mM) and apparently tends toward an asymptote at approximately 0.5 mM $CaCl_2$.

Dependence of the Rate of Actin Filament Elongation on $CaCl_2$ Concentration. The kinetics of actin polymerization have been shown to be consistent with a nucleation–elongation mechanism in which a small actin oligomer or nucleus (consisting of three to four actin monomers) is initially formed which then rapidly elongates to finally produce an actin filament. The rate of formation of nuclei is rate limiting in this process, thereby accounting for the characteristic initial lag period in the kinetics of polymerization from actin monomer (Oosawa & Kasai, 1971).

F-Actin intermolecularly cross-linked with *N,N'-p*-phenylenebis(maleimide) is resistant to full depolymerization under conditions where F-actin depolymerizes. Addition of only a small quantity of this cross-linked actin polymer to an actin polymerization markedly accelerates the overall time course of polymerization and virtually eliminates the initial lag period of the polymerization (Figure 3). Close examination of the time course of actin polymerizations in the presence of cross-linked actin does reveal a small (<50 s) lag peirod which has been previously reported by Gilbert & Frieden (1983) and Lal et al. (1984). The latter study showed that for a given ionic condition, the length of this small lag phase was independent of the cross-linked actin polymer concentration, monomer concentration, or preincubation of monomer or cross-linked polymer in polymerizing buffer. Examination of the time courses of actin polymer formation (in the presence of cross-linked actin polymer) induced by a range of $CaCl_2$ concentrations revealed that the size of this small lag period was largely unaffected by the $CaCl_2$ concentration. The origin of this lag period is unknown. The results shown in Figure 3 suggest that the addition of cross-linked actin polymer to an actin polymerization bypasses a rate-limiting step in the kinetics of actin polymerization from actin monomer, namely, nucleation (Oosawa & Kasai, 1971). Careful choice of actin, $CaCl_2$, and cross-linked actin polymer concentrations allowed investigation of actin polymer elongation without the effects of spontaneous nucleation; i.e., actin polymer formation in the presence of cross-linked actin polymer could be measured during a time interval when there was little or no polymerization in the absence of the cross-linked actin polymer. Semilogarithmic plots of the time courses of actin polymer formation in the presence of cross-linked actin polymer were linear after the initial lag period of ~50 s. Some full-time courses, especially at low $CaCl_2$ concentrations, were quite slow; therefore, the maximum rate (v) of actin polymer formation as a measure of the rate of actin filament elongation was measured.

Figure 4A shows the relationship between v and the total actin monomer concentration at a constant concentration of cross-linked actin polymer. Actin polymer formation was induced with 5.0 mM $CaCl_2$. There is linear increase of v with increasing actin monomer concentration for actin concentrations greater than 0.80 μM. The magnitude of v approaches zero at concentrations of actin monomer less than 0.8 μM. This concentration is in good agreement with the steady-state value of A_1^∞ (0.70 μM) as determined from Figure 1 at the same $CaCl_2$ concentration. Further, there was a direct proportionality between v and the concentration of cross-linked actin polymer (between 0.89 and 5.80 μg/mL) at a fixed initial actin monomer concentration of 5.15 μM (Figure 4B). Nonlinearity of either of the plots in Figure 4 was usually indicative of conditions which promoted rapid polymer formation in both the presence and absence of cross-linked actin polymer. Presumably, significant spontaneous nucleation was occurring in these solutions concomitant with elongation from the cross-linked actin polymer [cf. Pollard (1983)]. In terms

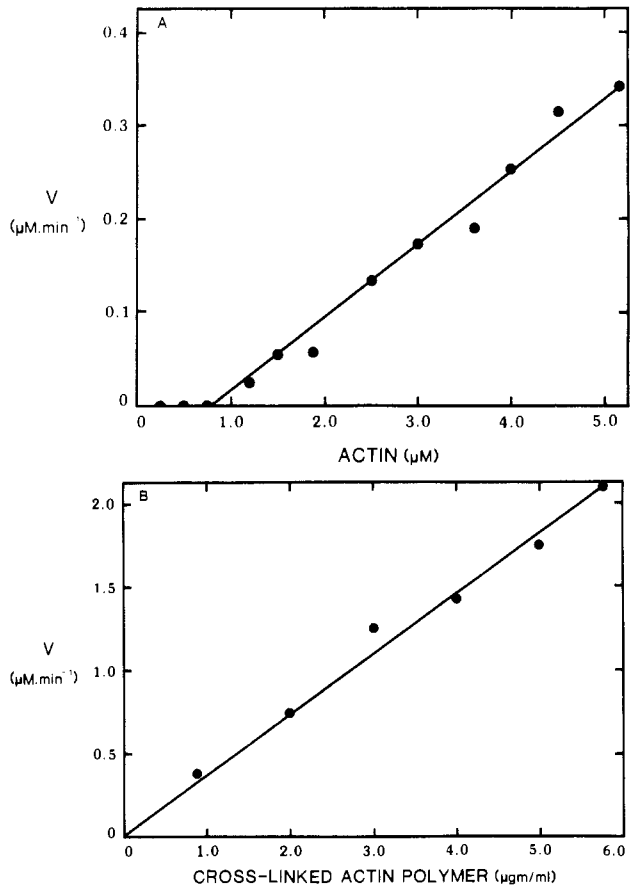

FIGURE 4: Maximum rate of F-actin formation from cross-linked actin polymer as a function of (A) the initial actin monomer concentration and (B) the concentration of cross-linked actin polymer. (A) G-Actin (5% *N*-pyrenylactin) at various concentrations and in G buffer was polymerized with 5 mM $CaCl_2$ in the presence of 0.89 μg/mL cross-linked actin polymer. (B) G-Actin (5 μM, 0.15 μM *N*-pyrenylactin) was polymerized with 5 mM $CaCl_2$ in the presence of a range of concentrations of cross-linked actin polymer. In both (A) and (B), the maximum rate of fluorescence change (at 25 °C) was directly converted to the maximum rate of formation of F-actin from the data of Figure 1.

of a nucleation–elongation model for actin polymerization, the rate of formation of F-actin protomers, dA_F/dt, from a fixed concentration of added actin nuclei (N) in the absence of spontaneous nucleation is

$$\frac{dA_F}{dt} = k_+NA_1 - k_-N = Nk_+(A_1 - A_1^\infty) \qquad (1)$$

where A_F is the F-actin protomer concentration, A_1 is the actin monomer concentration, k_+ and k_- are the forward and reverse rate constants, respectively, describing the elongation reaction, and $A_1^\infty = k_-/k_+$ (Oosawa & Kasai, 1971; Tobacman & Korn, 1983). The data presented in Figure 4 are well described by eq 1 in which v approximates to dA_F/dt at $t = 0$. Thus, it can be concluded that measurement of the maximum rate of F-actin formation from cross-linked actin polymer is a good measure of the rate of actin filament elongation [see also Lal et al. (1984)]. However, it should be noted that absolute rate constants cannot be obtained as the number concentration of cross-linked actin polymer is unknown.

The direct proportionality between v and the actin monomer concentration in excess of A_1^∞ for experiments in the presence of cross-linked actin polymer allowed the total actin concentration to be varied so as to obtain convenient rates of actin elongation with little or no spontaneous actin nucleation. The values of v so obtained were divided by $A_1 - A_1^\infty$ to normalize

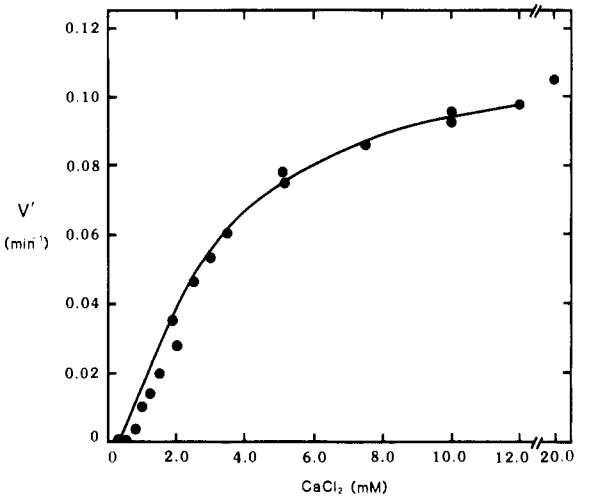

FIGURE 5: $CaCl_2$ concentration dependence of the normalized maximum rate of F-actin production from cross-linked actin polymer. Experiments similar to those described in the caption to Figure 4 were performed over a wide range of $CaCl_2$ and actin concentrations with a fixed concentration (0.89 μg/mL) of cross-linked actin polymer. The actin concentrations (0.5–140 μM; 1–5% *N*-pyrenylactin) were chosen to allow measurement of covenient rates of actin polymer formation and also to ensure that there was a direct proportionality between the maximum rate of F-actin formation (v) and the actin concentration in excess of the critical actin concentration. v' is the maximum rate of F-actin formation normalized by the steady-state F-actin concentration. The latter was determined from the difference between the total actin concentration and the steady-state critical actin concentration (Figure 2). The curve through the data has been calculated from eq 5 with $n = 5$, $k_{app} = 0.12$ min^{-1}, and $K = 2$ mM^{-1}.

Scheme I

$$A + C \overset{K}{\rightleftharpoons} A(C)_1$$
$$A(C)_1 + C \overset{K}{\rightleftharpoons} A(C)_2$$
$$\vdots$$
$$A(C)_{n-1} + C \overset{K}{\rightleftharpoons} A(C)_n$$
$$A(C)_n + N_{p-1} \underset{k'_-}{\overset{k'_+}{\rightleftharpoons}} N_p$$

the data at each actin concentration. Figure 5 shows the dependence of the normalized rate of actin filament elongation (v') on the added $CaCl_2$ concentration. At concentrations of $CaCl_2$ greater than 8–10 mM, the magnitude of v' approaches a limit indicating saturation of the actin with Ca^{2+}. One of the noteworthy features of Figure 5 is the sigmoidality of the plot, reflecting the fact that there is relatively little or no actin polymer formation at $CaCl_2$ concentrations less than approximately 0.6 mM.

A simple scheme that accounts for the sigmoidality of the plot in Figure 5 is one in which only actin monomer with a specific number (n) of bound Ca^{2+} ions is capable of addition to cross-linked actin polymer or F-actin filaments (Scheme I). In Scheme I, A is the actin monomer, n is the number of Ca^{2+} ions (C) required to bind to A before it can be incorporated into actin polymer, and N_p is the actin polymer containing p protomers (or simply cross-linked actin polymer, N). In this scheme, the binding of Ca^{2+} to multiple equivalent sites on actin monomer (site binding constant of K) is assumed to be rapid compared with the addition of $A(C)_n$ to N_{p-1}. The rate constant for actin filament elongation (k_+') and the rate constant for the dissociation (k_-') of actin monomer from polymer are independent of the Ca^{2+} concentration. In these circumstances

$$\frac{dA_F}{dt} = Nk_+'[A(C)_n - A(C)_n^\infty] \qquad (2)$$

where dA_F/dt is the rate of formation of F-actin protomers, N is the fixed number concentration of actin nuclei, and $A(C)_n^\infty = k_-'/k_+'$. [It should be noted that the Ca^{2+} binding sites in this model bear no relationship to the so-called high-affinity Ca^{2+} binding site on actin monomer (K = 143 mM^{-1}; Frieden, 1982) which in G buffer would be already fully occupied before polymerization is induced with millimolar concentrations of $CaCl_2$. Thus, the Ca^{2+} binding sites on the monomer represented in Scheme I are only those postulated to be directly involved in the actin polymerization process.]

The concentration of $A(C)_n$ is given by

$$A(C)_n = A_I\left(\frac{KC}{1 + KC}\right)^n \quad (3)$$

where A_I is the total actin monomer concentration and C is the free Ca^{2+} ion concentration (assumed to be equal to the added Ca^{2+} ion concentration used to induce actin polymer formation). Substitution for $A(C)_n$ into eq 2 yields

$$\frac{dA_F}{dt} = Nk_+'\left(\frac{KC}{1 + KC}\right)^n (A_I - A_I^\infty) \quad (4)$$

where $A(C)_n^\infty = A_I^\infty[KC/(1 + KC)]^n$. Measurement of the initial rate (v) of F-actin protomer formation allows eq 4 to be rewritten as

$$v' = \frac{v}{A_I - A_I^\infty} = k_{app}\left(\frac{KC}{1 + KC}\right)^n \quad (5)$$

where $k_{app} = Nk_+'$. Figure 5 shows a simulated curve calculated from eq 5 that has been fitted to the normalized experimental data by a nonlinear least-squares regression analysis with k_{app} = 0.12 min^{-1}, K = 2 mM^{-1}, and n = 5. The analysis was first performed by allowing all three parameters to vary, thereby defining a value of n, and second, by then setting n equal to the nearest integer and only allowing k_{app} and K to vary. Reasonable fits to the experimental data could also be obtained with n = 4 (K = 1.6 mM^{-1}) and n = 6 (K = 2.4 mM^{-1}), both with k_{app} = 0.12 min^{-1}. Values of n greater than 6 or less than 4 did not fit the experimental data at both high and low $CaCl_2$ concentrations for any values of k_{app} and K. Simulations of more complex mechanisms may also fit the experimental data; however, the mechanism proposed is the simplest that can account for the results (see Discussion).

Relative rate constants for the dissociation of actin monomer from F-actin (k_-) can be calculated from the product of A_I^∞ (Figure 2) and v' (Figure 5) at each $CaCl_2$ concentration. In contrast to a 5.4-fold increase of v' from 1.0 to 3.0 mM $CaCl_2$, k_- changes by only 1.6-fold. This result reinforces the assumption made in Scheme I that k_-' is independent of $CaCl_2$ concentration. Thus, the $CaCl_2$ concentration dependence of A_I^∞ (Figure 2) is primarily a reflection of the dependence of k_+ on Ca^{2+}.

Discussion

The strong bivalent cation concentration dependence of the kinetics of actin polymerization has been well documented although little is known about the relationship between metal ion binding to actin and the rate and extent of actin polymerization (Kasai et al., 1962; Kasai, 1969; Oosawa & Kasai, 1971; Pollard & Mooseker, 1981; Rouayrenc & Travers, 1981; Pardee & Spudich, 1982b; Frieden, 1983; Tobacman & Korn, 1983; Lal et al., 1984). The majority of those studies were performed with G-actin preequilibrated with $CaCl_2$ and polymerized with $MgCl_2$, KCl, or $MgCl_2$ and KCl. In the present study, the use of $CaCl_2$ for G-actin preequilibration and for inducement of polymer formation has a number of advantages. First, G-actin should initially have its high-affinity bivalent cation binding site (K_a^{Ca} = 143 mM^{-1}; Frieden, 1982) fully saturated with Ca^{2+} in G buffer. This ensures that the Ca^{2+} dependence of the kinetics of actin polymer formation is a reflection of the binding of Ca^{2+} to sites on actin monomer other than this high-affinity site. Indeed, actin is thought to have a number of poorly defined low-affinity bivalent cation binding sites which are directly involved in actin polymerization (Martonosi et al., 1964; Strzelecka-Golaszewska et al., 1978a,b). Second, studies of the kinetics of actin polymerization are complicated by monomer "activation" when actin is preequilibrated with Ca^{2+} and polymerized with Mg^{2+} (Cooper et al., 1983; Frieden, 1983; Tobacman & Korn, 1983; Gershman et al., 1984). Third, in G buffer, actin has its high-affinity Ca^{2+} binding site fully saturated but has little or no tendency for polymerization below an actin concentration of about 200 μM; i.e., there is a good separation of the Ca^{2+} titration curve of the high-affinity site from the titration curve for those sites directly involved in actin polymerization. This separation is not observed with Mg^{2+} (Frieden, 1983).

A model for the mechanism of Ca^{2+}-induced actin polymer elongation has been presented in which only actin monomer with five bound Ca^{2+} ions at low-affinity bivalent cation binding sites is capable of addition to actin polymer. More complex mechanisms may also fit the experimental data; e.g., more than one actin–$(Ca)_i$ species may be able to incorporate into polymer. However, it is unlikely that F-actin protomers contain varying calcium contents as this would imply a heterogeneity of these protomers and of the structure of actin filaments in general. The latter are highly ordered and regular structures with no obvious evidence of actin protomer heterogeneity. Further, the relative rate constant for actin monomer dissociation from F-actin is independent of $CaCl_2$ concentration, directly suggesting the presence of only one actin–$(Ca)_i$ species within F-actin. It is also possible that there is an interdependence of the Ca^{2+} binding sites on actin monomer. However, no evidence for this is observed in direct binding studies (Strzelecka-Golaszewska et al., 1978b).

The number of Ca^{2+} ion binding sites (five) that are required to be occupied before monomer can be incorporated into polymer in the proposed model (Scheme I) is similar to the number of low-affinity bivalent cation binding sites (four to eight) on actin which have been determined by direct binding measurements (Martonosi et al., 1964; Strzelecka-Golaszewska et al., 1978b). Those studies also indicated that full actin polymerization, as determined by viscosity measurements, occurred when about four to six of these low-affinity sites were occupied by bivalent cations. It is possible that a specific number of Ca^{2+} ions are required to bind to actin monomer to neutralize repulsive negatively charged amino acids on the surface of actin before actin self-association can occur (Rouayrenc & Travers, 1981). In addition, the magnitude of the association constant (K = 2 mM^{-1}) describing the binding of Ca^{2+} ions to actin monomer in the proposed model is similar to the binding affinity of bivalent cations to low-affinity sites on actin, K_a = 1–6 mM^{-1} (Martonosi et al., 1964; Loscalzo & Reed, 1976; Strzelecka-Golaszewska, 1978a,b). However, the specific affinity of Ca^{2+} for these low-affinity sites on actin, as measured by those direct binding studies, is not clear.

It is noteworthy that the $MgCl_2$ concentration dependence of the critical actin concentration has qualitatively similar features to that described herein for $CaCl_2$ (Tobacman et al., 1983; unpublished observations). Since the rate constant for

actin monomer dissociation from actin polymer is independent of $MgCl_2$ concentration (Tobacman & Korn, 1983), then it is also likely that there is a sigmoidal Mg^{2+} ion concentration dependence of the rate of actin filament elongation. Thus, the model described in Scheme I may also be applicable to Mg^{2+}-induced "nucleated" actin polymerizations. Frieden (1983) has concluded from studies of the full time course of actin polymerizations (from monomer) induced by 1.5, 3.0, and 5.05 mM Mg^{2+} that a single site (or class of sites) with a Mg^{2+} binding constant of 0.2 mM^{-1} is directly responsible for actin polymerization. However, a sigmoidal $MgCl_2$ concentration dependence of actin polymer formation has been demonstrated for actin solutions nucleated by the presence of the serum protein brevin (Doi & Frieden, 1984).

In summary, by studying the $CaCl_2$ concentration dependence of the critical actin concentration and the rate of actin filament elongation, it is concluded that five Ca^{2+} ions must first bind to low-affinity sites on actin monomer before that monomer can add to actin polymer.

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