Divalent Cation Binding to the High- and Low-Affinity Sites on G-Actin†

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ABSTRACT: Metal binding to skeletal muscle G-actin has been assessed by equilibrium dialysis using $^{45}Ca^{2+}$ and by kinetic measurements of the increase in the fluorescence of *N*-acetyl-*N'*-(5-sulfo-1-naphthyl)-ethylenediamine-labeled actin. Two classes of cation binding sites were found on G-actin which could be separated on the basis of their Ca^{2+} affinity: a single high-affinity site with a K_d considerably less than 1 μM and three identical moderate-affinity binding sites with a K_d of 18 μM. The data for the Mg^{2+}-induced fluorescence enhancement of actin labeled with *N*-acetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine support a previously suggested mechanism [Frieden, C. (1982) *J. Biol. Chem. 257*, 2882–2886] in which Ca^{2+} is replaced by Mg^{2+} at the moderate affinity sites(s), followed by a slow actin isomerization. This isomerization occurs independently of Ca^{2+} release from the high-affinity site. The fluorescence data do not support a mechanism in which this isomerization is directly related to Ca^{2+} release from the high-affinity site. Fluorescence changes of labeled actin associated with adding metal chelators are complex and do not reflect the same change induced by Mg^{2+} addition. Fluorescence changes in the labeled actin have also been observed for the addition of Cd^{2+} or Mn^{2+} instead of Mg^{2+}. It is proposed actin may undergo a host of subtle conformational changes dependent on the divalent cation bound. We have also developed a method by which progress curves of a given reaction can be analyzed by nonlinear regression fitting of kinetic simulations to experimental reaction time courses. This method allows testing of different kinetic mechanisms by statistical methods and has been used in the fitting of actin isomerization data.

Monomeric G-actin from rabbit muscle is known to have both a single high-affinity and multiple moderate-affinity sites for cations (Barany et al., 1962; Martonosi et al., 1964; Carlier et al., 1986). Several species of divalent, as well as trivalent, cations appear able to bind at the single high-affinity site, as well as at the moderate-affinity sites (Strzelecka-Golaszewska et al., 1978; Strzelecka-Golaszewska, 1973; Martonosi et al., 1964; Barden & Remedios, 1984). However, determining the dissociation constants for the divalent cation binding sites on actin is complicated by the presence of ATP since ATP also complexes these cations and its presence is necessary to prevent actin denaturation. While all recent schemes for the polymerization of actin (Frieden, 1983; Pantaloni et al., 1985; Cooper et al., 1983; Wegner & Savko, 1982) point to the importance of bound divalent cations on both the rate and extent of the G- to F-actin[1] transformation, distinct roles for the moderate- and high-affinity cation binding sites are not clear.

In recent years, fluorescently labeled actins have been found to yield both a sensitive and accurate measurement of actin conformation. Actin labeled at Cys-374 with the fluorescent dye AEDANS was found to undergo an ATP-dependent pseudo-first-order fluorescence change upon Mg^{2+} exchange for Ca^{2+} (Frieden et al., 1980; Frieden & Patane, 1985). Derived from data obtained by using AEDANS-actin, Frieden and co-workers (Frieden et al., 1980; Frieden, 1982; Zimmerle & Frieden, 1986) proposed a class of binding sites for which Mg^{2+} displaced bound Ca^{2+} and induced a conformational change. The calcium affinity was calculated to be about 10^{-5} M at pH 8. This site was assumed to be the high-affinity Ca^{2+} binding site as previous studies using radiolabeled Ca^{2+} suggested a dissociation constant for the high-affinity site nearly identical with this dissociation constant (Barany et al., 1962; Martonosi et al., 1964; Waechter & Engel, 1975). Recently, however, on the basis of Ca^{2+} binding measurements using the fluorescent Ca^{2+} indicator Quin-2 (Gersham et al., 1986), changes in AEDANS-actin upon addition of EGTA and magnesium (Carlier et al., 1986), and chemical modification experiments (Konno & Morales, 1985), the high-affinity Ca^{2+} site was proposed to have a dissociation constant of about 10^{-8} M at pH 8.

As a consequence of the recent Ca^{2+} binding measurements which demonstrated a considerably higher Ca^{2+} affinity than previously determined, two recent reports reexamined the fluorescence increase of AEDANS-actin attributed to the Mg^{2+}-induced conformational change. Estes et al. (1987) proposed it was release of the tightly bound Ca^{2+} ($K_d \simeq 10^{-8}$ M) which induced G-actin isomerization rather than Mg^{2+} binding. Furthermore, Ca^{2+} release from this site was both necessary and rate limiting in the Mg^{2+}-induced polymerization of actin. This idea was in contrast to the conclusions reached by Carlier et al. (1986), who proposed a class of low-affinity sites ($K_d \simeq 10^{-4}$ M), whose occupancy was important in inducing polymerization, and one high-affinity site ($K_d \simeq 10^{-8}$ M), whose occupancy appeared to affect the ATP hydrolysis rate. While Mg^{2+} binding at the high-affinity Ca^{2+} site induced the actin conformational change detected by increased fluorescence, they concluded this isomerization was not a necessary step in the Mg^{2+}-induced polymerization.

These recent proposals (Gersham et al., 1986; Estes et al., 1987; Carlier et al., 1986) and the original proposal of Frieden (1982) differed in their conclusions. Furthermore, Estes et

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[1] Abbreviations: Ca–G-actin, monomeric actin with bound Ca^{2+}; F-actin, polymerized filamentous actin; AEDANS-actin, actin reacted with *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; Quin-2, a methoxyquinoline derivative of BAPTA; CDTA, *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid; SSQ, sum of squares.

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al. (1987) utilized high concentrations of BAPTA, a calcium chelator, while Carlier et al. (1986) utilized low concentrations of EGTA. In light of recent data which suggest possible protein–chelator interactions (Chiancone et al., 1986; Mitani et al., 1986) and in order to test each of the proposed mechanisms, we have attempted in this paper to correlate the fluorescence change of AEDANS-actin with Ca^{2+} loss, to examine the fluorescence change induced by addition of metal chelators, and to fit the observed time course of fluorescence changes to each of the proposed mechanisms. We conclude that fluorescence enhancement induced by metal chelators is not specifically linked to Ca^{2+} release and that actin isomerization linked with Mg^{2+} binding is separate from Ca^{2+} release at the high-affinity site.

Materials and Methods

Chemicals. ATP (disodium salt), EGTA, EDTA, CDTA, and Quin-2 were purchased from Sigma, while all other reagents were analytical grade. Dowex (A50W-X8) was purchased from Bio-Rad, 1,5-IAEDANS from Molecular Probes, and a standardized 0.1 M calcium solution from Fisher.

Protein Purification and Modification. Rabbit muscle G-actin was purified by the method of Spudich and Watt (1971), modified as described previously (Frieden et al., 1980). AEDANS-actin was made by covalent modification of Cys-374 (Kouyama & Mihashi, 1980) by a procedure similar to that of Tawada et al. (1978), modified slightly (Frieden et al., 1980). The molar ratio of dye/actin was between 0.8 and 0.99. If the actin was not immediately used, 2 mg/mL sucrose was added per milligram of actin and the resultant solution lyophilized and stored at −20 °C. To prepare Ca–G-actin, the lyophilized powder was dissolved and dialyzed for at least 15 h against G buffer (2 mM Tris-HCl, pH 8, 200 μM Ca^{2+}, and 200 μM ATP). Protein concentrations were determined spectrophotometrically by using $A = 0.63$ at 290 nm (Houk & Ue, 1974) or by the method of Bradford (1976) using G-actin as a standard. Prior to use, all actin solutions were centrifuged at 100000g for 1 h.

Fluorescence Experiments. Static fluorescence studies were performed on either a Spex or an SLM spectrofluorometer in a mode which corrects for signal fluctuations as a result of light intensity changes. To measure AEDANS-actin fluorescence, excitation and emission wavelengths of 340 and 460 nm, respectively, were used. To measure Quin-2 fluorescence, excitation and emission wavelengths were 340 and 495 nm, respectively. Stopped-flow fluorescence measurements were obtained by using a Durrum stopped-flow apparatus in the fluorescence mode with an excitation wavelength of 340 nm and slit width of 3 mm. A Corning 0-52 filter placed before the photomultiplier absorbed scattered incident light while fully transmitting fluorescent light at wavelengths greater than 400 nm. Data from both stopped-flow and static fluorescence studies were collected continuously and stored in the digital mode for later recall.

Calcium Measurements. For experiments requiring radiolabeling, actin was dialyzed against solutions containing a trace amount of $^{45}Ca^{2+}$ along with the appropriate calcium and ATP concentrations. To remove loosely bound and free Ca^{2+}, this actin was mixed with 10% by volume of Dowex-50 and stirred for 10 min at 4 °C. The Dowex-50 was pretreated first by washing with 4 volumes of 1 N HCl, followed by extensive washing with water until the pH was above 4, and then equilibrated with 2 mM Tris, pH 8. The actin–Dowex slurry was spun in a microfuge for 5 min. The amount of Ca^{2+} bound to G-actin was calculated from measurements of $^{45}Ca^{2+}$ present in the supernatant. Dowex-50 removes all free calcium from solution but does not remove Ca^{2+} which is tightly bound to actin.

For experiments examining Ca^{2+} removal from the high-affinity site, G-actin was first treated with Dowex-50 as just described. Either EDTA or EGTA was then added to the Dowex-50-treated G-actin and the solution transferred immediately to an Amicon Centrifree micropartition system. These were centrifuged in a Sorvall table-top centrifuge for 2 min. Both the filtrate, which contained only free Ca^{2+}, and the retentate, which contained both free and bound calcium, were counted for $^{45}Ca^{2+}$. Protein concentrations were determined by the Bradford method to ensure that no loss of protein occurred during this procedure. In these experiments, the final actin concentration was 23 μM, while the ATP concentration varied between 50 and 500 μM.

For experiments in which tightly bound Ca^{2+} was removed with Quin-2, 30–60 μM Quin-2 was used. These concentrations of Quin-2 were sufficiently low to avoid inner filter effects. The intensity of Quin-2 fluorescence was calibrated to the free calcium concentration by addition of several standard $CaCl_2$ solutions. To measure the total amount of bound Ca^{2+} to G-actin by Quin-2, it was necessary to add 150 μM $MgCl_2$ to ensure complete Ca^{2+} removal. This concentration of $MgCl_2$ did not interfere with calcium measurements by Quin-2. In the presence of AEDANS-actin, Quin-2 fluorescence cannot be directly measured due to the overlapping of the emission spectra. Therefore, changes in the Quin-2 fluorescence were corrected for the fluorescence contribution attributed to the AEDANS-actin.

Data Analysis. Stopped-flow data for the addition of Quin-2 to actin, or the addition of metal chelator (EDTA or CDTA) to AEDANS-actin, were analyzed by using a nonlinear least-squares fit to a first-order rate equation. Analyses of fluorescence changes by computer simulation of the kinetic mechanisms described in this paper were performed by the program FITSIM (version 1.1) on a Digital Electronics Corp. MicroVAX II. FITSIM incorporates the program KINSIM (Barshop et al., 1983) as a subroutine and allows nonlinear fitting of progress curve data to a given kinetic mechanism by numerical integration.

Results

Bound Calcium Measured by $^{45}Ca^{2+}$. After equilibrium dialysis of actin against 200 μM ATP and 200 μM Ca^{2+} in 2 mM Tris-HCl buffer, pH 8, with a trace amount of $^{45}Ca^{2+}$, approximately 1 mol of Ca^{2+}/mol of actin remains bound. Subsequent treatment of this actin with Dowex-50 did not remove this bound calcium. This result is in good agreement with previous studies and demonstrates the presence of one high-affinity calcium binding site on actin (Barany et al., 1962; Martinosi et al., 1964; Carlier et al., 1986).

Since ATP has moderate affinity for Ca^{2+} under the above conditions, the presence of Ca^{2+} sites with affinity weaker than approximately 1 μM cannot be properly determined in the presence of ATP. Equilibrium dialysis experiments containing trace amounts of free ATP showed a total of four Ca^{2+} binding sites (Figure 1). Three of these sites appear identical and have moderate affinity for Ca^{2+} with a K_d of about 18 μM. The K_d for the remaining site was not determined from the data. However, it must have higher Ca^{2+} affinity since Ca^{2+} remains bound to this site after treatment with Dowex or in the presence of high concentrations of ATP.

Accurate determination of the dissociation constant for the high-affinity Ca^{2+} binding site cannot be obtained by equilibrium dialysis due to the very low actin concentrations (and

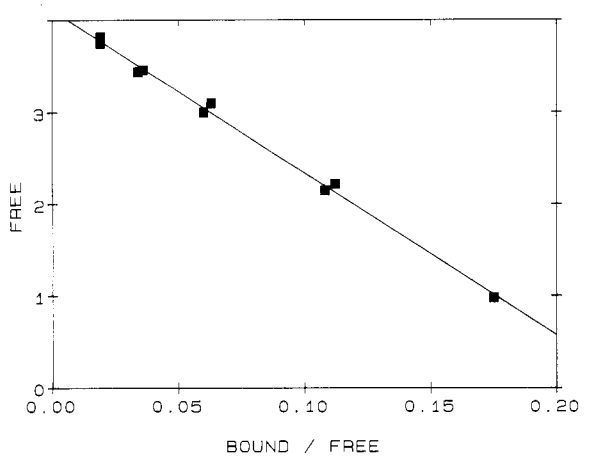

FIGURE 1: Scatchard plot of calcium binding to 23 μM G-actin at pH 8 in the presence of only a trace amount of ATP. The solid line through the points is a linear regression of the data. Although four Ca^{2+} sites are indicated, only the binding affinities of three sites are given by the data. The dissociation constant for Ca^{2+} was calculated to be 18 μM for these sites. Although the affinity of the fourth site for Ca^{2+} was not tested by this experiment, it is known to be less than 1 μM (see Results).

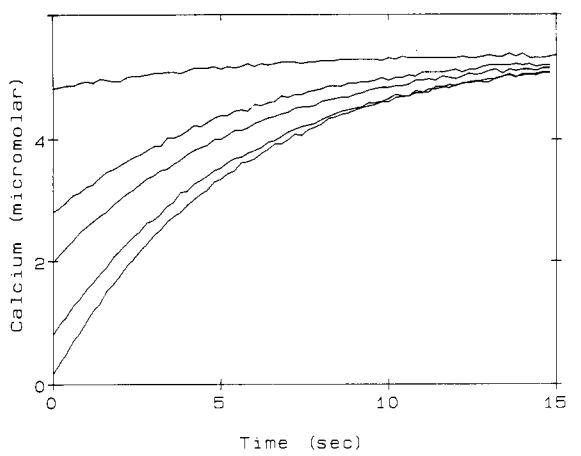

FIGURE 2: Ca^{2+} release as a function of Mg^{2+} preincubation and subsequent Quin-2 addition. The stopped-flow time courses represent the addition of Quin-2 at time zero to 5 μM G-actin preincubated for 5 min with various concentrations of Mg^{2+}. The G-actin had been dialyzed first against 200 μM ATP and 200 μM Ca^{2+} in 2 mM Tris-HCl buffer, pH 8, and subsequently against two changes of buffer containing 50 μM Mg, 100 μM ATP, and 2 mM Tris-HCl at pH 8. The instantaneous fluorescence increase corresponds to calcium not bound to the actin. A standard curve of Quin-2 fluorescence vs a calibrated stock solution of Ca^{2+} was used to quantitate the amount of "free" Ca^{2+} present. From top to bottom, the concentrations of Mg^{2+} preincubated for 5 min against this G-actin were 10 mM, 1 mM, 500 μM, 200 μM, and 50 μM.

therefore small signal over background) required for these measurements. Therefore, competition between actin and a Ca^{2+} chelator was used to approximate the Ca^{2+} affinity of this site. With $^{45}Ca^{2+}$-labeled actin treated with Dowex-50 to remove any Ca^{2+} at the lower affinity sites, the stoichiometric addition of EGTA removed approximately 60% of the remaining bound calcium. Increasing the EGTA concentration 4-fold over the actin concentration removed approximately 90% of the $^{45}Ca^{2+}$ from actin (data not shown). These results were nearly independent of the ATP concentration between 50 and 500 μM, although increasing the ATP concentration appeared to increase the Ca^{2+} affinity slightly. Identical results were found when EDTA was substituted for EGTA in these experiments.

To estimate the Ca^{2+} dissociation constant for the high-affinity site on actin, the various equilibria involved were solved simultaneously (Storrer & Cornish-Bowden, 1976). The dissociation constant of either EGTA or EDTA for calcium under these conditions is approximately 3 nM (Smith et al., 1984; Dawson et al., 1986) while the K_d for Ca^{2+}·ATP is approximately 5 μM (C. Frieden, unpublished results). The K_d required for the high-affinity site on actin to retain the amounts of bound calcium found experimentally was calculated to be between 10 and 50 nM.

Bound Calcium Measured by Quin-2. The release of calcium from actin was directly measured by Quin-2, a chelator which increases fluorescence upon calcium binding (Tsein, 1980; Tsein et al., 1982). Quin-2 can measure both the amount of free Ca^{2+} and also the amount of Ca^{2+} tightly bound to the actin. As the measured K_d for Ca^{2+}–Quin-2 under the conditions here is approximately 60 nM (Tsein, 1980), any free or loosely bound Ca^{2+} will bind immediately to Quin-2, resulting in an instantaneous increase (<2 ms) in Quin-2 fluorescence. Ca^{2+} tightly bound to the actin is removed slowly, resulting in a time-dependent Quin-2 fluorescence increase.

When either native actin or AEDANS-actin was dialyzed against G buffer, and subsequently against two changes of 2 mM Tris-HCl buffer containing 50 μM Mg^{2+} and 100 μM ATP, 1 mol of Ca^{2+}/mol of actin is still retained, as determined by the extent of the slow Quin-2 fluorescence increase (Figure 2, bottom curve), and by measurements of $^{45}Ca^{2+}$-labeled actin (data not shown). The fact that identical results were found for native actin and AEDANS-actin suggests the reaction of Cys-374 with AEDANS does not significantly change the affinity of the tight-binding Ca^{2+} site.

An experiment to measure the free and bound Ca^{2+} on unlabeled actin is shown in Figure 2 as a function of Mg^{2+} concentration. In one syringe of the stopped-flow apparatus, different concentrations of Mg^{2+} were preincubated with actin for 5 min. At time zero, the actin was mixed with an equal volume of a solution containing 120 μM Quin-2, and both the fast (i.e., the instantaneous change over background) and slow fluorescence changes associated with Quin-2 addition were measured. Since the amount of Quin-2 fluorescence increase associated with the slow phase is due to the removal of tightly bound Ca^{2+} to the actin, the removal of this Ca^{2+} by Mg^{2+} preincubation results in a greater proportion of the Quin-2 fluorescence change being instantaneous. The Mg^{2+} concentrations required to remove this tightly bound calcium were found, using FITSIM, to correspond to Mg^{2+} competing for this site with an affinity approximately 70-fold less than Ca^{2+}. This suggests that Mg^{2+} competes poorly for the high-affinity Ca^{2+} binding site.

Mg^{2+}-Induced AEDANS-actin Fluorescence Changes. In agreement with Carlier et al. (1986), there is a rapid (<2 ms) increase in fluorescence when Mg^{2+} (>100 μM) is added to AEDANS-actin. This is followed by a second phase which was previously characterized as the time-dependent Mg^{2+}-induced conformational change (Frieden, 1982; Zimmerle & Frieden, 1986). Both phases are saturable as a function of Mg^{2+} concentration, suggesting the fluorescence increase in each phase is a result of conformational changes associated with specific cation binding. Furthermore, addition of either Ca^{2+}, Na^+, or K^+ results only in a rapid fluorescence change, a result also in agreement with Carlier et al. (1986).

Figure 3 shows typical data for the time-dependent increase seen in the AEDANS-actin fluorescence induced by high or low concentrations of Mg^{2+} in the presence of high or low Ca^{2+} concentrations. The curves all appear first order, a fact used previously to analyze these data (Frieden et al., 1980; Frieden, 1982; Carlier et al., 1986; Zimmerle & Frieden, 1986). We

Table I: Analysis of the Mg^{2+}-Induced Conformational Change at pH 8 and 20 °C by FITSIM[a]

scheme	k_{+Mg} (μM^{-1} s^{-1})	k_{-Mg} (s^{-1})	k_{+Ca} (μM^{-1} s^{-1})	k_{-Ca} (s^{-1})	k_{+1} (μM^{-1} s^{-1})	k_{-1} (s^{-1})	sum of squares
I	0.9 ± 20	0.01[b]	27 ± 591	0.09 ± 0.004			32
II	10[b]	12000 ± 3000	10[b]	190 ± 40	0.17 ± 0.002	0.01[b]	0.7
II	0.1[b]	117 ± 20	10[b]	183 ± 33	0.17 ± 0.002	0.009	0.7

[a] FITSIM parameters: Marquardt algorithm, no weighting of data points, convergence occurred at changes less than 1%. [b] Value fixed for this analysis.

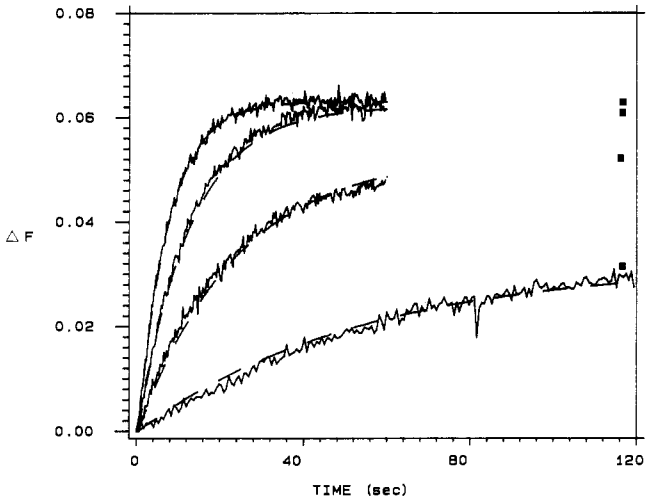

FIGURE 3: Time-dependent change at 20 °C and pH 8 induced by Mg^{2+} addition to 2.3 μM G-actin incubated overnight against 50 μM Ca^{2+} and 50 μM ATP in 2 mM Tris buffer, pH 8. From left to right, the final concentrations of Mg^{2+} were 4, 10, 0.5, and 1 mM while the final concentrations of Ca^{2+} were 0.9, 200, 0.9, and 200 μM. The square symbols at the end of each curve represent the final end point obtained. The smooth lines through the points were the best fit of the data using Scheme II as determined by FITSIM. The rate constants and FITSIM options used for determining the simulated curves are found in Table II.

attempted to fit these curves directly to simulated curves calculated using two distinct kinetic mechanisms. Scheme I is a mechanism in which the fluorescence increase is directly related to Ca^{2+} loss at the high-affinity site. Scheme II is a mechanism in which the fluorescence increase is the result of an isomerization of actin due to Mg^{2+} binding at a site distinct from the high-affinity site (i.e., unrelated to Ca^{2+} release from the high-affinity site). In Scheme I, A is G-actin with no metal bound, while ACa and AMg represent G-actin with Ca^{2+} or Mg^{2+} bound, respectively. In Scheme II, A is G-actin already containing Ca^{2+} at the high-affinity site, ACa represents Ca^{2+} bound at the moderate-affinity sites, and A′Mg is a conformationally distinct form of AMg.

Scheme I

$$A + Ca^{2+} \underset{k_{-Ca}}{\overset{k_{+Ca}}{\rightleftharpoons}} ACa$$

$$A + Mg^{2+} \underset{k_{-Mg}}{\overset{k_{+Mg}}{\rightleftharpoons}} AMg$$

Scheme II

$$A + Ca^{2+} \underset{k_{-Ca}}{\overset{k_{+Ca}}{\rightleftharpoons}} ACa$$

$$A + Mg^{2+} \underset{k_{-Mg}}{\overset{k_{+Mg}}{\rightleftharpoons}} AMg \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} A'Mg$$

In order to fit the data using Scheme I, the AEDANS-actin species ACa must have decreased fluorescence compared to A or AMg, while in order to fit the data using Scheme II, the AEDANS-actin species A′Mg must have a higher fluorescence yield than the other actin species. As shown in Table I, no satisfactory fits of the data were obtained with Scheme I since the standard deviations obtained with the fitting program were larger than the values of the rate constants. The smooth lines through the data points in Figure 3 represent the best fits of the data using Scheme II. Two different analyses using Scheme II gave identical fits of simulated to experimental data. The analyses differed only by their assumption for the values of the rate constants k_{+Mg} and k_{+Ca}. In one analysis, the rate constants were assumed close to the diffusion rate, while in the other analysis, the rate constant k_{+Mg} was chosen to be lower by a factor of 100 as assumed by Estes et al. (1987).

Actin Isomerization Occurs Independently of the Release of Tightly Bound Ca^{2+}. The analysis of the AEDANS-actin fluorescence change induced by Mg^{2+} (shown in Figure 3) suggests a considerably lower Ca^{2+} affinity of actin than that determined with Quin-2 (Figure 2). Furthermore, the Ca^{2+} dissociation constant obtained by analysis of the AEDANS-actin fluorescence change is nearly identical with that obtained for the moderate-affinity calcium sites determined by equilibrium dialysis (Figure 1). Both results suggest the Mg^{2+}-induced isomerization of actin is not related to Ca^{2+} release from the high-affinity site.

To test this hypothesis, AEDANS-actin was dialyzed against 100 μM Mg^{2+} and 200 μM ATP in the absence of Ca^{2+}. AEDANS-actin dialyzed in this manner was found to still contain approximately 1 mol of tightly bound calcium per mole of actin, as determined by the extent of the time-dependent Quin-2 fluorescence change upon Quin-2 addition and confirmed by $^{45}Ca^{2+}$ measurements. Further addition of Mg^{2+} to this actin induced no time-dependent fluorescence change, although a small instantaneous fluorescence increase was recorded. Addition of 2 mM Ca^{2+} brought about a slow 15% decrease in the AEDANS-actin fluorescence, as reported previously (Frieden et al., 1980; Zimmerle & Frieden, 1986), at a rate consistent with reversal of the Mg^{2+}-induced conformational change ($\simeq$0.01 s^{-1}). These results suggest the actin had already undergone isomerization as a result of dialysis against Mg^{2+} even though the tightly bound Ca^{2+} remained. The above results, taken together, prove the Mg^{2+}-induced conformational change can take place independent of calcium release at the tight-binding site, and the two processes are not directly related.

Metal Chelator Induced AEDANS-actin Fluorescence Changes. When a metal chelating agent (EGTA, EDTA, or CDTA) is added to AEDANS-actin, there is a time-dependent fluorescence increase. This fluorescence increase of AEDANS-actin is reversible upon readdition of calcium, suggesting a process associated with chelator addition and/or calcium removal rather than irreversible actin denaturation. This result is consistent with the BAPTA-induced fluorescence change in AEDANS-actin found by Estes et al. (1987). As shown in Table II, the rate constant for the CDTA-induced fluorescence change appears nearly independent of the CDTA concentration added. However, the extent of the fluorescence change increases with increasing CDTA concentration. The CDTA concentration required to induce the maximal fluorescence change in the presence of 200 μM ATP is about

Table II: Observed First-Order Rate Constants and Extent of Fluorescence Change Induced by CDTA Addition[a]

[CDTA] (mM)	k_{obsd}[b]	fluorescence enhancement	[CDTA]/[actin]
0		1.00	0
0.005	0.08	1.03	2
0.02	0.07	1.04	9
0.1	0.09	1.07	45
0.5	0.08	1.10	220
2.5	0.08	1.13	1100

[a] Conditions were 500 μM ATP and 2 mM Tris-CH buffer, pH 8 at 20 °C. [b] Observed first-order rate constant.

1000-fold over the actin concentration. As shown above by $^{45}Ca^{2+}$ measurements, a 4-fold excess of EGTA (with a Ca^{2+} dissociation constant similar to CDTA) removes over 90% of the tightly bound Ca^{2+}. Thus, the fluorescence change induced by metal chelator does not appear directly related to Ca^{2+} removal.

The extent of the fluorescence increase induced by metal chelator addition is dependent on the ATP concentration. At a constant metal chelator concentration, the fluorescence increase decreases as the ATP concentration is increased, and at very low ATP concentrations, the fluorescence increase is followed by a decrease. Subsequent addition of either ATP, Ca^{2+}, or Mg^{2+} fails to reverse this fluorescence decrease, suggesting an irreversible conformational change related to denaturation is being measured here.

Addition of Other Divalent Cations to AEDANS-actin. It is well documented that Ca^{2+} can be displaced by other divalent cations besides Mg^{2+} (Curmi et al., 1982; Kasai & Oosawa, 1968; Strzelecka-Golaszewska & Drabikowski, 1968; Strzelecka-Golaszewska, 1973). To test whether the fluorescence of AEDANS-actin might also be sensitive to the exchange of Ca^{2+} for other cations besides Mg^{2+}, the divalent cations Mn^{2+} and Cd^{2+} were added to AEDANS-labeled Ca^{2+}–actin. Addition of Mn^{2+} induces fluorescence changes similar to Mg^{2+} addition. There is a first-order fluorescence increase and, depending on the concentration, an instantaneous fluorescence increase as well. Indeed, the addition of Mn^{2+} to Ca^{2+}–actin yields results qualitatively identical with Mg^{2+} addition to Ca^{2+}–actin. The addition of Ca^{2+} to Mn^{2+}–actin, as it does for Mg^{2+}–actin, yields a first-order fluorescence decrease. The addition of Mg^{2+} to Mn^{2+}–actin induces no further fluorescence changes.

Concentrations of free Cd^{2+} below approximately 20 μM also result in a time-dependent increase in the AEDANS-actin fluorescence qualitatively similar to either Mg^{2+} or Mn^{2+} addition to Ca^{2+}–actin. However, at free Cd^{2+} concentrations greater than approximately 20 μM, there is a rapid time-dependent fluorescence change which is completed in approximately 1 s in addition to the fast fluorescence phase completed within 2 ms. As shown by Figure 4, when a low concentration of Cd^{2+} was added with a large excess of Mg^{2+}, the rate for the time-dependent fluorescence change is considerably slower than that found for Cd^{2+} alone, but considerably faster than that found for Mg^{2+}. When a low Cd^{2+} concentration was added with excess Ca^{2+}, a fast time-dependent response was seen, a result not seen when calcium alone was added.

Discussion

We propose here the existence of at least two classes of cation binding sites on the actin monomer which can be separated on the basis of their calcium affinity: a single high-affinity site and three sites with moderate affinity. The three identical calcium binding sites of moderate affinity are identified on the basis of equilibrium dialysis experiments while the high-affinity site is identified on the basis of 1 mol of Ca^{2+}/mol of actin remaining after extensive dialysis of actin against Ca^{2+}-free buffers or after Dowex-50 treatment. Equilibrium dialysis experiments in the presence of high ATP concentrations were also consistent with this conclusion when corrections were applied for the removal of free metals by ATP. There is also the possibility of cation sites with even weaker affinity for Ca^{2+}, as described by others (Strzelecka-Golaszewska et al., 1978; Martonosi et al., 1964; Tellam, 1985).

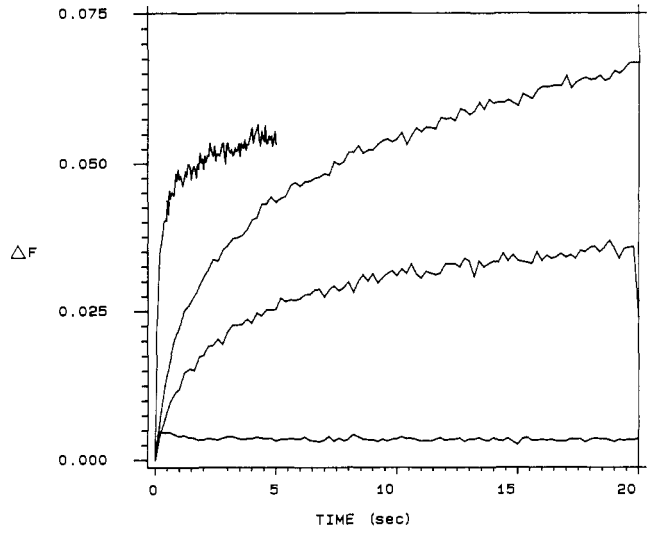

FIGURE 4: Stopped-flow time courses of the AEDANS-actin fluorescence change induced by additions of various divalent cations. From top to bottom, the final concentrations of added cations were (A) 500 μM $CdCl_2$, (B) 2 mM $MgCl_2$, and 100 μM $CdCl_2$, (C) 2 mM $CaCl_2$ and 100 μM $CdCl_2$, and (D) 2 mM $CaCl_2$. The final concentration of AEDANS-actin was 2.3 μM, and both syringes before mixing contained 200 μM ATP in 2 mM Tris-HCl buffer, pH 8 at 20 °C.

These results partly agree and partly disagree with previous reports. While the presence of a calcium not removed by Dowex treatment is well documented (Barany et al., 1962; Martonosi et al., 1964; Carlier et al., 1986), there are several values reported for both the number and affinity of the weak calcium binding sites. Strzelecka-Golaszewska (1978), using ultracentrifugation, reported a single class of five weak calcium binding sites with a K_d of 200 μM. Tellam (1985) by modeling Ca^{2+}-induced polymerization reported five calcium binding sites with a K_d of 500 μM. Carlier et al. (1986) recently reported a K_d of 150 μM for an undetermined number of sites, using the fluorescent enhancement of AEDANS-actin. All of these values are approximately an order of magnitude greater than the value we find. An explanation for this difference could be that the equilibrium experiments reported here directly measured free and protein-bound cation concentrations while the previously reported methods utilized indirect calcium binding measurements.

Estes et al. (1987) recently demonstrated addition of the metal chelator BAPTA induces a time-dependent fluorescence change in AEDANS-actin. We have confirmed with EGTA, EDTA, and CDTA that metal chelator induced fluorescence increases in AEDANS-actin do occur and that the apparent first-order rate constants for these fluorescence changes are independent, while the maximal fluorescence extents are dependent, on the metal chelator concentration. Such a result suggests that the fluorescence changes induced upon Mg^{2+} addition may simply be due to calcium release, and not a Mg^{2+}-induced isomerization as postulated by Frieden (1982)

and Carlier et al. (1986). Alternatively, as discussed below, the chelator may induce actin conformational changes independent of Ca^{2+} release.

First, while experiments with $^{45}Ca^{2+}$ demonstrated a 4-fold excess of chelator over actin was sufficient to remove nearly all tightly bound calcium, a considerably higher chelator concentration is necessary to induce the maximal AEDANS-actin fluorescence increase possible. This suggests that the fluorescence increase associated with chelator addition is not directly related to calcium removal. Second, Chiacone et al. (1986) recently proposed BAPTA may directly alter the conformation of many calcium binding proteins, resulting in erroneous calcium release rates. Third, although no direct binding of EDTA to actin has previously been detected (Strzelecka-Golaszewska et al., 1974; Strohman & Samorodin, 1962), indirect effects of EGTA and EDTA on proteins, as found recently for α-lactalbumin, may also induce protein conformational changes (Mitani et al., 1986). Finally, a direct effector-like action of EDTA on actin was postulated by Waechter and Engel (1977) on the basis of a large increase in the nucleotide dissociation step upon excess EDTA addition.

Using the program FITSIM, we fit the experimentally obtained progress curves directly to the kinetic mechanism described by either Scheme I or Scheme II. Scheme I assumes AEDANS fluorescence changes occur as a result of Ca^{2+} release, as proposed by Estes et al. (1987), while Scheme II assumes AEDANS fluorescence changes are a function of Mg^{2+} binding, as first proposed by Friden et al. (1980). This fitting procedure not only allows for the determination of individual rate constants in each mechanism but also can distinguish which kinetic mechanism better describes the observed data.

As demonstrated in Table I, even the best obtainable fits of the data to Scheme I were quite poor. In contrast, good fits of the data using Scheme II were obtained regardless of whether k_{+Mg} and k_{+Ca} were assumed to be identical or significantly different. The first analysis set both k_{+Ca} and k_{+Mg} to 10 μM^{-1} s^{-1}, a constant approximately equal to the expected diffusion limit. As suggested by Estes et al. (1987), the water dissociation rate constants for the hydration shell around Mg^{2+} were used for k_{+Mg} in the second analysis. Such an assumption forces k_{+Mg} to be significantly lower than k_{+Ca}. By the criteria of an equal sum of squares (SSQ) between the two fits, no significant difference between these analyses could be determined.

Although the best kinetic model found is not always identical with the actual kinetic mechanism, other experimental evidence also supports Scheme II over Scheme I. We have demonstrated here that actin with 1 mol of tightly bound Ca^{2+} appears to undergo the Mg^{2+}-induced conformational change independent of Ca^{2+} release at the high-affinity site. Additionally, simulations using Scheme I showed two properties which were not observed in the experimental data. First, the AEDANS-actin fluorescence change would be expected to decrease with decreasing chelator concentration and the observed first-order rate constant for this process increase. As shown in Table II, the observed first-order rate constant remains approximately constant over the 500-fold range in CDTA concentration tested. Second, if changes in AEDANS-actin fluorescence were due to calcium release, the observed first-order rate upon Mg^{2+} addition would show little increase with increasing Mg^{2+} concentration. In contrast, the data found previously by us (Frieden, 1982; Zimmerle & Frieden, 1986) and Carlier et al. (1986) show the observed first-order rate is dependent on the Mg^{2+} concentration.

Since metal chelators appear to directly induce actin conformational changes and Mg^{2+}-induced fluorescence changes can occur independent of Ca^{2+} release at the high-affinity site, any experiment in the presence of both Mg^{2+} and metal chelators may be difficult to interpret and should be avoided. Thus, direct comparisons of Mg^{2+} displacement of Ca^{2+} with that of Ca^{2+} removal by chelators are not possible, and the determination that the Ca–G-actin conformation is sensitive to BAPTA addition as found by Estes et al. (1987) does not differentiate between whether the fluorescence change is the result of calcium release (Scheme I) or a Mg^{2+}-induced isomerization (Scheme II).

The kinetic mechanism given by Scheme II was recently favored by Carlier et al. (1986) as well. However, their results suggested rate constants for the conformational change (k_{+1} and k_{-1} of Scheme II) 2–3-fold lower than the values reported here, and by us earlier (Zimmerle & Frieden, 1986; Frieden, 1982). Furthermore, they reported calcium affinity between 10^{-8} and 10^{-9} M for the cation site associated with the Mg^{2+}-induced conformational change. While we agree there is a high-affinity calcium binding site, we propose that the occupany of this site does not have a direct bearing on the conformational change induced by Mg^{2+}. Attempts to fit the data to Scheme II, and forcing the calcium affinity to be in the range of 10^{-8} M, resulted in a large decrease in the quality of the fit between simulated and experimental data. Thus, there appear to be differences in the data obtained here and by Carlier et al. (1986) which cannot be obviously explained at this time. It should be noted their experiments were carried out in the presence of 10 μM EGTA, a condition which may alter the kinetic parameters obtained.

While most experiments reported here used the nonselective metal chelators EDTA and CDTA, the effect of the more Ca^{2+}-specific chelator EGTA was also tested. EGTA appeared to induce AEDANS-actin fluorescence changes at slightly lower concentrations than either EDTA or CDTA. However, since trace quantities of Mg^{2+} were found in our buffers (<70 nM by atomic absorption), selective chelation of Ca^{2+} by EGTA was generally avoided due to the possibility of trace amounts of Mg^{2+}, or other metals in actin solutions, binding rapidly to Ca^{2+}-free actin. This possibility is supported by reports what show EGTA addition promotes actin polymerization while EDTA and CDTA addition inhibits it (Avissar et al., 1979; Maruyama, 1981a,b).

We have also shown that an actin conformational change occurs upon binding of metal cations other than Mg^{2+} or Ca^{2+}. The binding of Mn^{2+} to Ca–G-actin induces AEDANS-actin fluorescence changes similar to Mg^{2+} addition, while Cd^{2+} addition induces fluorescence increases which are dramatically different. The Cd^{2+}-induced AEDANS-actin changes could be explained by Cd^{2+} binding to a site distinct and separate from either the Mg^{2+} or the Ca^{2+} binding sites. This seems unlikely, however, since cadmium has the same charge and nearly the same size as calcium. It differs from calcium mainly in preferring 6-coordination geometry over 7- and 8-coordination and in possessing cadmium-oxygen bonds having more covalent character (Martin, 1984). Furthermore, cadmium has been used previously as a spectroscopic probe of calcium binding sites in NMR experiments [e.g., see Vogel et al. (1985) and Drakenberg et al. (1987)].

Differences between the fluorescence changes induced by different cations suggest the possibility of unique actin conformations which are dependent on the species of bound cation. This is not a novel suggestion since reports of subtle variations in metalloprotein conformation with the ligand preference and

ionic radius of the cation have been made for other proteins, including actin (Strzelecka-Golaszewska, 1973). For example, Kwan et al. (1975) correlated differences in pyruvate kinase activity upon the extent to which each particular cation "adjusted" the protein conformation. Similar mechanisms have been proposed for α-lactalbumin and S100 proteins (Musci & Berliner, 1985; Baudier & Gerard, 1983). It is proposed a host of subtle changes in actin conformation, dependent on the nature of the cation exchange, may occur.

The results of cadmium, while difficult to entirely interpret at this time, do suggest interactions between the metal binding sites of actin. This is not surprising since detailed cation binding studies for other Ca^{2+} binding proteins point to complex kinetic mechanisms and binding isotherms. Experiments with α-lactalbumin suggest mutually nonexclusive metal binding to a "calcium site" and to a "zinc site" simultaneously, and at least three conformers of the protein (Musci & Berliner, 1985). In addition, complex interactions between cation binding sites have been reported for troponin C, concanavalin A, calmodulin, and porcine intestinal binding proteins (Grabarek et al., 1986; Palmer et al., 1980; Milos et al., 1986; Vogel et al., 1985). We propose the possibility calcium binding and exchange on actin may be similarly complex. The experiments presented here suggest not only a minimum of two different G-actin conformers but also the possibility of at least two distinctly different cation binding sites. How this may affect the kinetic mechanism of actin polymerization is, at this point, unclear.

In conclusion, we propose there are at least two classes of cation binding sites on the actin monomer which are distinguishable by their calcium affinities. The time-dependent fluorescence change in AEDANS-actin appears associated with the binding sites of moderate affinity toward Ca^{2+} (10^{-5} M) and not with the high-affinity calcium binding site (10^{-8} M). Furthermore, as the AEDANS-actin fluorescence change induced by metal chelator addition and the one induced by Mg^{2+} are not directly comparable, fluorescence changes in the presence of both are difficult to interpret. Finally, actin appears to be conformationally sensitive to the binding of different metals, and there may be complex interactions between metal binding sites on the actin monomer.

APPENDIX

Analysis of Full Time Course Reactions. Barshop et al. (1983) have published a user-friendly kinetic simulation program, KINSIM, which can calculate the full time course of nearly any given kinetic mechanism. While KINSIM proved a powerful kinetic simulator, analysis of experimental progress curves could only be accomplished by tedious manual manipulation of the rate constants. Furthermore, no knowledge of the uniqueness of the fit or the kinetic parameter errors could readily be obtained by such manual optimization methods. Thus, the evaluation of the goodness of fit and the reliability of the kinetic constants estimated could not be easily obtained.

We describe here a program, FITSIM, which by pairing nonlinear regression algorithms with the efficient numerical methods and flexibility of KINSIM allows relatively quick analysis of several full time course data sets to specific kinetic mechanisms. FITSIM is designed to be highly flexible and to allow for the fitting of widely different kinetic mechanisms to experimental data. Thus, rival kinetic mechanisms can be evaluated and individual rate constants obtained for each reaction mechanism. This potential has been demonstrated in this paper by fitting AEDANS-actin fluorescence changes induced by Mg^{2+} to two distinct mechanisms.

For each set of experimental data, the fitting of experimental data to a given kinetic mechanism requires four essential steps:

(1) Mechanism entry and compilation. Mechanism entry and compilation are identical with that described previously in more detail for KINSIM (Barshop et al., 1983). The user can type a representation of a chemical scheme using conventional chemical formulation and output equation calculations which monitor one or more of the species concentrations within the chemical scheme.

(2) Entry of data parameters. For each data set, or experimentally observed reaction time course, the kinetic parameters and reactant concentrations pertinent to that data set must be stored in a format readable by FITSIM. This includes all reactant concentrations, output scales, and the initial values for the kinetic constants.

(3) Entry of regression options and parameters. A separate interactive program prompts for all additional required input and gives various options available for regression analysis of the experimental data. Specific rate constants in a mechanism can also be linked together. This allows the best fit to be obtained while maintaining user-entered ratios between specific rate constants.

(4) Fitting. Both the data files and parameters are automatically entered, and an initial SSQ is calculated by using the initial kinetic constants. The chosen regression algorithm calculates new values for the kinetic constants, the resultant SSQ is calculated, and iteration proceeds until either the convergence parameters are met or the net reduction in the SSQ reaches some minimum value. When iteration terminates, various statistics concerning the analysis of variance are output, including the standard deviation of the data from the simulated fit, a multiple R^2 value which allows a numerical measure of the fit obtained, and the mean squared error. An example of the final fits of simulated to experimental data calculated by using FITSIM is shown in Figure 3.

These programs were developed on a Digital Equipment Corp. microVAX II computer, and program implementation is similar to that described by Barshop et al. (1983) for KINSIM. It should be pointed out that FITSIM is not a replacement of the original KINSIM program. Rather, FITSIM is a powerful extension of that program. Indeed, initial crude estimates of the kinetic rate constants should be obtained using KINSIM. Final estimates of the kinetic rate constants can also be resimulated and displayed, listed, or plotted using KINSIM. The description of FITSIM here concerns version 1.1, and both complete documentation and the program itself are available.[2]

Registry No. Ca, 7440-70-2; Mg, 7439-95-4; Cd, 7440-43-9; Mn, 7439-96-5.

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[2] Documentation and programs available from Carl Frieden, Department of Biological Chemistry, Washington University School of Medicine.

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