pH-Induced Changes in G-Actin Conformation and Metal Affinity†

Chris T. Zimmerle‡ and Carl Frieden*

Department of Biological Chemistry, Washington University School of Medicine, St. Louis, Missouri 63110
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ABSTRACT: Metal-induced conformational changes in actin at 20 °C have been investigated as a function of pH using actin labeled at Cys-374 with *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine. At pH 8, the addition of a high Ca^{2+} concentration (2 mM) to G-actin gives an instantaneous fluorescence increase while the addition of a high Mg^{2+} concentration gives both an instantaneous and a slow fluorescence increase. The instantaneous increase is interpreted as divalent cation binding to low-affinity, relatively nonspecific sites, while the slow response is attributed to Mg^{2+} binding to specific sites of moderate affinity [Zimmerle, C. T., Patane, K., & Frieden, C. (1987) *Biochemistry 26*, 6545–6552]. The magnitudes of both the instantaneous and slow fluorescence increases associated with Mg^{2+} addition to G-actin are shown here to decrease as the pH is lowered while the fluorescence of labeled G-actin in the presence of low or moderate Ca^{2+} concentrations (<200 μM) increases. The pH-dependent data suggest that protonation of a single class of residues with an approximate p*K* of 6.8 alters the immediate environment of the label differently depending upon the cation bound at the moderate-affinity site. The pH-dependent changes in the magnitude of the slow fluorescence response upon Mg^{2+} addition to Ca^{2+}–actin are not associated with changes in the Mg^{2+} affinity at the moderate-affinity site but result from protonation altering the fluorescence response to Mg^{2+} binding. Protonation of this same class of residues is proposed to induce an actin conformation similar to that induced by cation binding at the low-affinity sites. Ca^{2+} binding at a single high-affinity ($\sim 10^{-9}$ M) site remains quite tight, with the rate constant of release decreasing approximately 10-fold as the pH is lowered from 8 to 6.

It is well established that monomeric G-actin contains a single site with high affinity for Ca^{2+} and multiple binding sites of lower affinity for Ca^{2+} and other divalent cations (Barany et al., 1962; Martonosi et al., 1964; Carlier et al., 1986; Zimmerle et al., 1987; Tellam, 1985). Conformational differences in actin, related to the specific site occupied and the species of metal cation bound, have been proposed to exist in both the monomeric (Frieden et al., 1980; Barden & dos Remedios, 1985; Estes et al., 1986; Zimmerle et al., 1987; Carlier et al., 1986; Cooper et al., 1983) and polymeric (Mihashi et al. 1979; Miki et al., 1982) states. These conformational changes induced by the exchange of one metal by another may have importance in the in vivo regulation and extent of actin polymerization.

Although it is generally agreed upon that actin labeled at Cys-374 with the fluorescent dye *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (IAEDANS)[1] undergoes a pseudo-first-order fluorescence change upon Mg^{2+}–Ca^{2+} exchange, there have been conflicting interpretations about its meaning. The change has been attributed to actin isomerization upon Mg^{2+} binding to either a moderate-affinity ($\sim 10^{-5}$ M) Ca^{2+} site (Frieden, 1982; Frieden & Patane, 1985; Zimmerle & Frieden, 1986) or a high-affinity (10^{-9} M) Ca^{2+} site (Carlier et al., 1986), as well as simply to the release of Ca^{2+} from the high-affinity site (Estes et al., 1987). Recently, we demonstrated that addition of a low concentration of Mg^{2+} induces the AEDANS fluorescence change independent of release of a tightly bound Ca^{2+} and thus is a consequence of binding to moderate-affinity sites (Zimmerle et al., 1987).

Regardless of the mechanism used for describing the AEDANS-actin fluorescence change, neither the considerably slower Ca^{2+} release at lower pH found by Estes et al. (1987) for the high-affinity site nor the apparent weaker Mg^{2+} binding found by Frieden (1982) for the moderate-affinity site appears consistent with the fact that the rate of polymerization is increased at lower pH (Zimmerle & Frieden, 1988; Oosawa & Kasai, 1971). Therefore, in the present study, both the Ca^{2+} affinity of actin and the conformational changes in actin induced by cation binding, as measured by fluorescence changes of AEDANS-actin, were reexamined as a function of pH.

We find that a single Ca^{2+} remains tightly bound to actin between pH 8 and 6 and that the release of this Ca^{2+} is approximaely 10-fold slower at pH 6 than at pH 8. We conclude that, as at pH 8, Mg^{2+}–Ca^{2+} exchange at the moderate-affinity sites between pH 6 and 8 induces a conformational change. However, the rate of this conformational change becomes slower with decreasing pH, and the magnitude of the fluorescence change associated with the conformational change is altered by the protonation of a class of residues with a p*K* of 6.8. Protonation of this same class of residues also appears to induce a conformer similar to that of the salt-induced conformer found on metal binding to the low-affinity sites. The above information has been incorporated into a mechanism which we believe to explain the results. In addition, we demonstrate that alterations in the fluorescence magnitude associated with a binding process as a function of pH are not

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* Address correspondence to this author at Box 8094, Washington University School of Medicine, 660 S. Euclid, St. Louis, MO 63110.

‡ Present address: Miles Inc., P.O. Box 70, Elkhart, IN 46515.

[1] Abbreviations: Ca–G-actin, monomeric actin with bound Ca^{2+}; F-actin, polymerized filamentous actin; AEDANS-actin, actin labeled with *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid; Quin-2, a methoxyquinoline derivative of BAPTA.

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necessarily correlated with a change in ligand affinity.

Materials and Methods

Chemicals. ATP (disodium salt) and Quin-2 were purchased from Sigma, Dowex (A50W-X8) was purchased from Bio-Rad, and IAEDANS was from Molecular Probes. $CaCl_2$ (Puratronic) and "Ultrapure" $MgSO_4$ were obtained from Ventron Corp., Alpha Division; $^{45}CaCl_2$ was from Amersham, while a standardized 0.1 M calcium solution, used in calibrating the Quin-2 fluorescence increase associated with Ca^{2+} addition, was from Orion. All other materials used were reagent grade.

Protein Purification and Modification. Rabbit muscle G-actin was purified by a modified (Frieden et al., 1980) procedure of Spudich and Watt (1971), utilizing a final Sephadex G-150 gel filtration step as described by MacLean-Fletcher and Pollard (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+}, 0.5 mM DTE, 1.5 mM NaN_3, and 200 μM ATP) at 4 °C. Protein concentrations were determined spectrophotometrically by using $E_{1mg/mL}$ = 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 for 100000*g* 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 of 340 and 495 nm, respectively, were used. 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 digital mode for later recall.

Calcium Measurements. Calcium measurements utilized both radiolabeling and Quin-2, a chelator with fluorescence sensitivity toward calcium binding and whose Ca^{2+} affinity is relatively insensitive to pH (Tsein, 1980; Tsein et al., 1982). For measurements using radiolabeling, actin was dialyzed against solutions containing a trace amount of $^{45}Ca^{2+}$ along with various calcium and ATP concentrations. For measurements by Quin-2, the fluorescence change upon the addition of 5 μM actin to 30–60 μM Quin-2 was measured and correlated to a standard curve obtained by incremental addition of a standard $CaCl_2$ solution. To ensure complete removal of any bound Ca^{2+}, 150 μM Mg^{2+} was routinely added. This concentration of Mg^{2+} did not interfere with calcium measurements by Quin-2. Prior to Ca^{2+} determination in some experiments, loosely bound Ca^{2+} and free Ca^{2+} were removed by Dowex-50 treatment in the same manner as reported previously (Zimmerle et al., 1987).

pH Titrations. For the titrations of AEDANS-actin from high to low pH, 2–20-μL increments of either 0.1 M NaOAc buffer, pH 5.0, or 0.2 M MES were added to a cuvette containing 0.23 μM AEDANS-actin (10 μg/mL) in 1 mM Tris-HCl buffer and 200–500 μM ATP. For titrations from low to high pH, 2–20-μL increments of 0.1 or 1 M Tris were added to a cuvette containing the above AEDANS-actin concentration in 1 mM MES buffer, pH 6, and 500 μM ATP. The concentrations of Ca^{2+} and Mg^{2+} used varied, depending upon the experiment. This actin concentration was low enough so that no polymerization occurred. In all titrations, after each buffer increment the resultant pH and fluorescence were measured. Fluorescence values were corrected for the small amount of dilution which occurred with each addition. The results of the titrations did not depend upon the buffer system used to raise or lower the pH. Under these conditions, complete reversibility of pH-induced AEDANS-actin fluorescence changes could be achieved.

Stopped-Flow pH Jumps. For pH jump experiments in which the pH was lowered from 8 to 6, a solution containing 2.3 μM AEDANS-actin (0.1 mg/mL) and 500 μM ATP in 1 mM Tris buffer, pH 8, was mixed at time zero with a solution containing 4 mM MES buffer, pH 5.8. For pH jump experiments in which the pH was raised from 6 to 8, a solution containing 2.3 μM AEDANS-actin and 500 μM ATP in 1 mM MES buffer, pH 6, was mixed at time zero with a solution containing 4 mM Tris-HCl buffer, pH 8.2. For all pH jump experiments, the final pH was measured to ensure the desired value had been obtained. Depending upon the experiment, Ca^{2+} or Mg^{2+} was preincubated with the actin or added at time zero.

Data Analysis. Quin-2 fluorescence changes were analyzed by using a nonlinear least-squares fit to a first-order rate equation. Analyses of the data to the kinetic mechanisms described in this paper were performed by the program FITSIM (version 1.51) on a Digital Electronics Corp. MicroVAX II. For analysis using FITSIM, the options chosen were equal weighting of the data points and data files, regression using the Marquardt algorithm, convergence of parameters at 1%, and the convergence by sum of squares reduction set at 0.01%. When convergence values were set at smaller values, no significant changes either in the fit or in the parameters were observed. The program FITSIM has recently been briefly described elsewhere (Zimmerle et al., 1987) and allows nonlinear regression fitting of kinetic simulations calculated by the program KINSIM (Barshop et al., 1983) to experimentally obtained progress curve data. KINSIM, modified to simulate pH titration curves (version 3.41), was used for the simulations presented in Figure 6. RS/1 was used to fit the pH titration to the equation $F = (pH)F_{max}/(pK + pH)$ where F is the fluorescence increase observed and F_{max} is the maximum fluorescence observed.

Results

pH and the Stoichiometry of Tightly Bound Calcium. Although several Ca^{2+} binding sites exist, only one site appears to have affinity for calcium. Calcium remains bound at this site after treatment with ion-exchange resins (Baraney et al., 1962; Zimmerle et al., 1987; Gersham et al., 1986; Carlier et al., 1986). Two methods, equilibrium dialysis using $^{45}Ca^{2+}$ and changes in Quin-2 fluorescence, confirm that there is a single high-affinity binding site for Ca^{2+} at all pH values used. The rate of Ca^{2+} release, as measured by an increase in Quin-2 fluorescence, appeared first order but was approximately 10-fold slower at pH 6 than at pH 8. The measured Ca^{2+} release rates at pH 7 and pH 8 are in good aggrement with the rates found previously by Estes et al. (1987) using the same method of measurement. These off rates are shown in Table I. If

Table I: Comparison of the Rate of Calcium Release (k_{-Ca}) As Measured by Quin-2 and the Maximum Rate of the AEDANS Fluorescence Change (k_{+iso})

pH	k_{-Ca}	k_{+iso}
6.0	0.0065 ± 0.001	ND[a]
7.0	0.013 ± 0.001	0.018 ± 0.002
8.0	0.056 ± 0.005	0.170 ± 0.02

[a] Not determined. The fluorescence change is too small to get a reliable rate constant.

we assume the on rate for calcium binding is insensitive to pH, Ca^{2+} binding becomes 10-fold tighter at the high-affinity site as the pH is decreased from 8 to 6.

Effect of pH on AEDANS-actin Fluorescence in the Presence of Ca^{2+}. Addition of 2 mM Ca^{2+} to 10 μg/mL AEDANS-actin in 200 μM ATP, 200 μM Ca^{2+}, and 1 mM Tris-HCl buffer, pH 8, caused a rapid (less than 5 ms) 13–15% fluorescence increase. In agreement with Carlier et al. (1986), the extent of this rapid fluorescence change followed a normal saturation curve with a K_d of 200 μM. This rapid fluorescence increase can be reversed by the addition of various metal chelators or excess ATP (data not shown), presumably due to the lowering of the free Ca^{2+} concentration by these reagents. In agreement with the conclusion of Carlier et al. (1986) and Frieden et al. (1980), these instantaneous fluorescence changes are related to cation binding at the low-affinity sites of actin. Since rapid phases are also observed upon the addition of the divalent cations cadmium, manganese, and zinc or the monovalent cations sodium and potassium (Frieden et al., 1980; C. T. Zimmerle, unpublished results), the rapid fluorescence increase induced by Ca^{2+} addition appears to be a general salt-induced effect.

The fact that either monovalent or divalent cations can induce this rapid fluorescence change demonstrates the nonspecificity of these sites. Such nonspecificity is in agreement with the proposal that actin polymerization may proceed by a salt-induced conformational change brought about by the neutralization of negative charges on the actin molecule (Martonosi et al., 1964; Rich & Estes, 1976; Strzelecka-Golaszewska et al., 1978). As proton binding will also neutralize negative charges on actin, lowering the pH may be expected to induce a response similar to that of salt addition.

This hypothesis is consistent with pH titrations of AEDANS-actin in buffer containing 500 μM ATP and 10 μM Ca^{2+}. AEDANS-actin shows an approximately 13–15% increase in fluorescence on lowering the pH from 8 to 5.9 under these conditions (Figure 1). This fluorescence change appears to be due to the titration of a single class of ionizable groups since the data can be fit to a single titration curve with a pK of 6.8. When the low-affinity sites are saturated with Ca^{2+}, lowering the pH results in only a slight increase (3%) in fluorescence, the same as the small change noted with 1,5-IAEDANS reacted with dithiothreitol (data not shown). This result suggests that similar actin conformations are induced either by protonation of a class of residues with pK of 6.7 or upon metal binding to the low-affinity sites.

Stopped-flow studies in which the pH was rapidly changed were carried out to examine further the pH-induced AEDANS-actin fluorescence changes. Under conditions where the pH was rapidly changed from pH 8 to 6.0 or 6.5, the fluorescence increase occurred within 5 ms, the dead time of our stopped-flow apparatus. The results suggest a nearly instantaneous change occurs in the environment of AEDANS covalently attached to actin when the pH is changed. This is consistent with a rapid conformational change upon proton binding to actin.

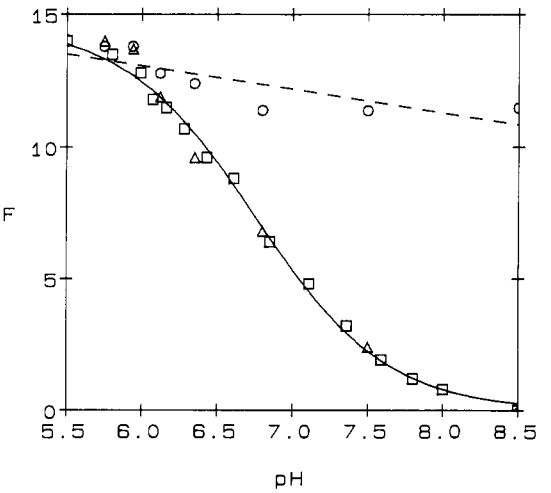


FIGURE 1: Effect of pH on AEDANS-actin fluorescence at 20 °C. The buffer contained 0.23 μM (10 mg/mL) IAEDANS-actin in 500 μM ATP, 2 mM MES, and 2 mM Tris-HCl in the presence of 10 μM (squares), 200 μM (triangles), and 2 mM (circles) $CaCl_2$. pH titrations were performed as described under Materials and Methods. The smooth line represents the best fit of the data to the equation $pH = pK_a + [A]/[AH]$ where $pK_a = 6.7$.

Effects of pH on AEDANS-actin Fluorescence in the Presence of Mg^{2+}. At pH 8, a biphasic 27–30% increase in fluorescence occurs upon addition of concentrations greater than 2 mM Mg^{2+} to 10 μg/mL AEDANS-actin in 200 μM ATP, 200 μM Ca^{2+}, and 2 mM Tris-HCl buffer (G-actin in G buffer). One phase is rapid and occurs within the dead time of the stop-flow (5 ms) while the other phase exhibits an apparent first-order process. This result is in agreement with previous reports at pH 8 (Frieden & Patane, 1985; Zimmerle et al., 1987; Carlier et al., 1986) and suggests the presence of two binding processes.

The AEDANS-actin fluorescence changes induced by Mg^{2+} are independent of whether the probe itself is directly excited or whether excitation occurs by energy transfer from actin tyrosine residues. Excitation at a wavelength of 295 nm instead of 340 nm yielded results identical with those just described for the addition of 2 mM Mg^{2+} in G buffer (data not shown). Since at this excitation wavelength nearly all the fluorescence observed at 470 nm is due to energy transfer between one or more actin tyrosine residues and the attached AEDANS probe, there would appear to be little change in the distance between the excited tyrosine residue(s) and the AEDANS moiety.

The magnitude of the rapid and slow phases depends upon the Mg^{2+} concentration added. With high concentrations of Mg^{2+} (>2 mM), the rapid and slow phases are about equal in size irrespective of the pH. With low Mg^{2+} concentrations, the rapid phase is small relative to the slow phase, indicating that, as with Ca^{2+}, the rapid phase results from binding to low-affinity sites. Since either Ca^{2+} or Mg^{2+} can induce this rapid phase, it can be implied that such sites are relatively nonspecific for either cation. As described in more detail elsewhere (Zimmerle et al., 1987), the slow phase reflects binding to moderate-affinity sites. Titration of the extent of the fluorescence of the slow phase as a function of pH (Figure 2) shows that the changes in the fluorescence extent follow a simple titration curve with a pK of about 6.8.

AEDANS-actin incubated with 2 mM Mg^{2+} (Figure 3), a condition where both the rapid and slow phases are induced, shows an instantaneous (<5 ms) fluorescence decrease upon reducing the pH from 8 to 6. This rapid decrease in fluorescence is also found for AEDANS-actin incubated with

Table II: Analysis of the Mg^{2+}-Induced Conformational Change at pH 7 and 20 °C by FITSIM[a]

mechanism[d]	k_{+Mg} (μM^{-1} s^{-1})	k_{-Mg} (s^{-1})	k_{+Ca} (μM^{-1} s^{-1})	k_{-Ca} (s^{-1})	k_{+iso} (μM^{-1} s^{-1})	k_{-iso} (s^{-1})	sum of squares[c]
$ACa \underset{k_{-Ca}}{\overset{k_{+Ca}}{\rightleftharpoons}} A' \underset{k_{-Mg}}{\overset{k_{+Mg}}{\rightleftharpoons}} A'Mg$	0.12	0.003	12	0.02			15
$ACa \underset{k_{-Ca}}{\overset{k_{+Ca}}{\rightleftharpoons}} A \underset{k_{-Mg}}{\overset{k_{+Mg}}{\rightleftharpoons}} A'Mg$	0.02	0.015	5	0.024			18
$ACa \underset{k_{-Ca}}{\overset{k_{+Ca}}{\rightleftharpoons}} A \underset{k_{-Mg}}{\overset{k_{+Mg}}{\rightleftharpoons}} AMg \underset{k_{-iso}}{\overset{k_{+iso}}{\rightleftharpoons}} A'Mg$	10[b]	16600	10[b]	390	0.018	0.005[b]	1

[a] FITSIM parameters: Marquardt algorithm, no weighting of data points, convergence at changes less than 1%. [b] Value fixed for this analysis. [c] Relative value, unitless value with the lowest sum of squares found set to 1. [d] Actin species represented by A′ are assumed to have a higher fluorescence yield than actin species represented by A.

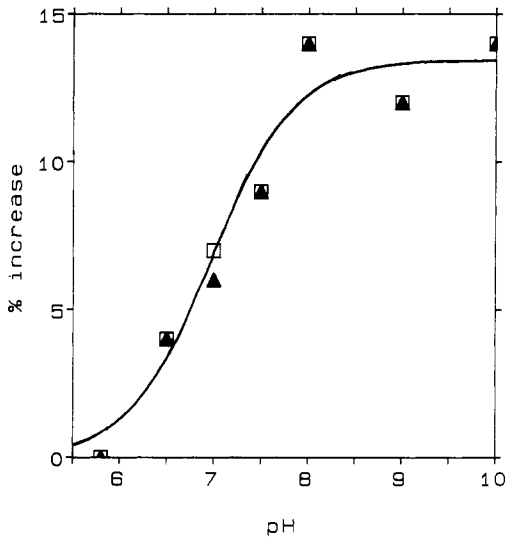


FIGURE 2: pH dependence of "fast" and "slow" phases at 20 °C. The "fast" (triangles) and "slow" (squares) phases of the AEDANS-actin fluorescence increases were induced by the addition of 10 mM Mg^{2+} to 0.23 μM (10 mg/mL) AEDANS-actin in the presence of 1 μM $CaCl_2$, 200 μM ATP, and either 2 mM Tris-HCl or 2 mM MES buffer at the pH shown. pH titrations were done as described under Materials and Methods. The smooth line through these points represents the best fit of the data to the equation $pH = pK_a + [A]/[AH]$ where $pK_a = 6.9$.

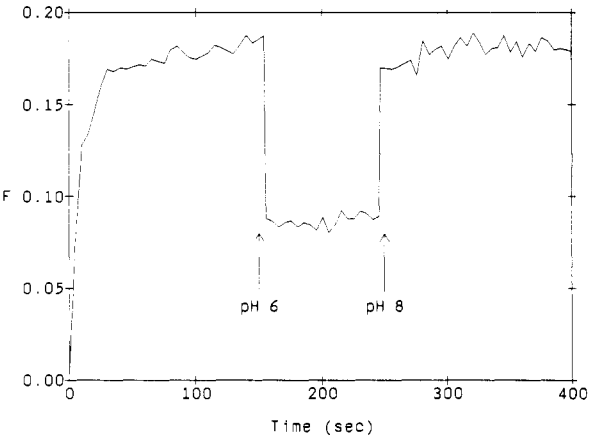


FIGURE 3: pH-induced AEDANS-actin fluorescence changes at 20 °C in the presence of Mg^{2+}. At time zero, 2 mM Mg^{2+} was added to 1.2 μM (mg/mL) AEDANS-actin at pH 8. At the arrow marked pH 6, the pH of the solution is dropped to pH 6 by the addition of a 0.1 M solution of MES. At the arrow marked pH 8, the pH of the solution is returned to pH 8 by the addition of a 1 M Tris buffer.

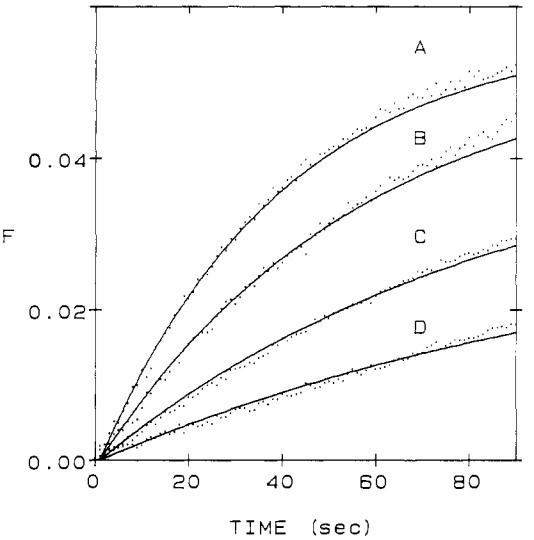


FIGURE 4: Time-dependent fluorescence change at 20 °C induced by Mg^{2+} addition to 1.2 μM AEDANS-actin (0.05 mg/mL) in the presence of 200 μM ATP and 2 mM MES buffer, pH 7.0. Curve A is the change recorded upon the addition of 25 mM Mg^{2+} in the presence of 6.25 μM Ca^{2+}, curve B for 6.25 mM Mg^{2+} and 6.25 μM Ca^{2+}, curve C for 6.25 mM Mg^{2+} and 200 μM Ca^{2+}, and curve D for 3.12 mM Mg^{2+} and 200 μM Ca^{2+}. The smooth line drawn through the experimental data points represents the best fit of the data obtained by FITSIM. The kinetic constants used are described in Table II.

only 100 μM Mg^{2+}, a concentration at which only the slow phase is induced (data not shown). Returning the pH to 8 results in a rapid reversal of the fluorescence changes just described.

The above experiments, taken together, suggest that pH changes do not signficantly alter Mg^{2+} affinity at the moderate-affinity sites. Thus, if Mg^{2+} had been released from the moderate-affinity sites when the pH was lowered from 8 to 6, we would have expected a slow fluorescence increase associated with rebinding of Mg^{2+} to the moderate-affinity sites when the pH was returned to 8. Indeed, this type of slow change does occur if the pH of a solution of actin containing Ca^{2+} at pH 6 is raised to pH 8 at the same time as Mg^{2+} is added. These experiments are described in more detail later.

Effect of pH on the Mg^{2+}-Induced Slow Conformational Change. Figure 4 shows stopped-flow experiments of the time-dependent fluorescence changes (solid lines) seen on Mg^{2+} addition to AEDANS-actin at pH 7. The fluorescence change induced by Mg^{2+} is smaller than that at pH 8, and considerably slower. As shown in Table II, we attempted to fit several different mechanisms to the data. As previously found using this procedure (Zimmerle et al., 1987), the data were found to fit well (solid lines) to the Mg^{2+}-induced conformational change mechanism (represented by the third mechanism of Table II or by row 2 of Scheme I) first described by Frieden (1982). The rate constant for the conformational change (k_{+iso}) is about 10-fold slower at pH 7 than at pH 8 (Table I). Unfortunately, only data at pH 7.0 could be analyzed in this way since at lower pH values, the time-dependent

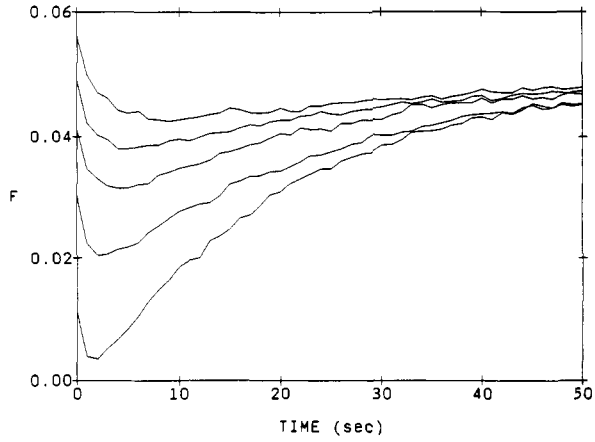


FIGURE 5: pH jumps (6 to 8.5) as a function of time after adding Mg^{2+}. At time zero, 550 μM Mg^{2+} was added to 1.2 μM (0.05 mg/mL) AEDANS-actin in 500 μM ATP and 1 mM MES buffer, pH 6 at 20 °C. After incubation times of 65, 160, 360, 480, and 640 s, going from the bottom to top curve respectively, the pH was increased to pH 8.5 and the change in AEDANS fluorescence induced by this pH change recorded over the next 40 s.

fluorescence changes induced by Mg^{2+} addition are too small for reliable fits to be obtained. The actin concentration could not be increased (thereby increasing signal) because under these conditions actin begins to polymerize.

Since the Mg^{2+}-induced conformational change at pH 6 cannot be measured directly by fluorescence, we chose a different experiment to test for this change. In this experiment, Mg^{2+} was added to Ca^{2+}–actin at pH 6 and the pH rapidly raised to 8. When Mg^{2+} addition and increase in pH are simultaneous, there is a slow fluorescence increase identical with that observed on Mg^{2+} addition to actin at pH 8 (Figure 5, lowest curve). However, the extent of this slow phase becomes smaller and eventually disappears with increasing time of incubation with Mg^{2+} prior to raising the pH. This result implies that the Mg^{2+}-induced conformational change is occurring at pH 6 but that the rate of this change is quite slow, as indicated by the time of incubation, since over 10 min is needed before the slow phase disappears. The slow rate is consistent with the data of Table I which show that the rate constant changes about 10-fold between pH 8 and 7.

Two observations were made which cannot be fully explained. First, close examination of the data of Figure 5 shows an initial slight decrease in fluorescence. The reason for this decrease is not clear but may be related to the fact that actin polymerizes somewhat at pH 6 with 550 μM Mg^{2+}, even at the low actin concentration used. Second, Mg^{2+} addition to Ca^{2+}–actin at pH 6 results in the induction of a slow 2–4% decrease in the IAEDANS-actin fluorescence (data not shown).

Discussion

In agreement with previous studies (Frieden et al., 1980; Carlier et al., 1986; Zimmerle et al., 1987), we find that a biphasic fluorescence increase in AEDANS-actin is induced by Mg^{2+} addition to actin in G buffer at pH 8. One fluorescence phase is a slow first-order process and is associated with Ca^{2+}–Mg^{2+} exchange at a site with a Mg^{2+} affinity of $\sim 10^{-5}$ M (i.e., the moderate-affinity site) (Frieden, 1982; Zimmerle et al., 1987). The other fluorescence phase is a rapid (occurring within 5 ms) salt-induced conformational change associated with Mg^{2+} binding at a site or sites with an affinity of $\sim 10^{-4}$ M (i.e., the low-affinity sites) (Carlier et al., 1986; Zimmerle et al., 1987). The rapid phase is referred to as salt induced since any general increase in ionic strength appears to induce it. We determined that the pH dependence for the maximum extent of both fluorescence phases induced by Mg^{2+} appears related to the protonation of a single class of sites on actin, with a pK similar to that of free histidine.

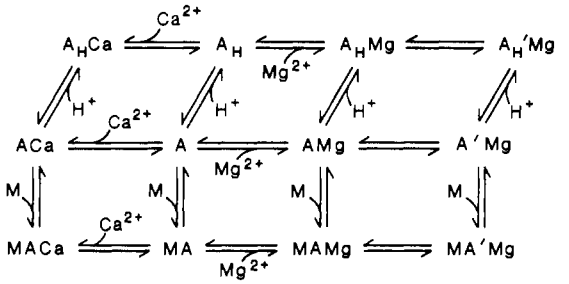
We found that changes in the fluorescence of AEDANS-actin as a function of pH depend not only on the cation species bound but also on the site to which it is bound. In the presence of free Mg^{2+} concentrations of less than 100 μM, AEDANS-actin undergoes a 6–10% decrease in fluorescence on lowering the pH 8 to 5.8; in the presence of Ca^{2+} concentrations of less than 100 μM, however, AEDANS-actin undergoes a 14–16% fluorescence increase over the same pH range. Under these conditions, little binding of metal at the low-affinity metal sites occurs. In the presence of high Mg^{2+} concentrations, a 14–16% decrease in fluorescence occurs on going from pH 8 to 5.8, while in the presence of high Ca^{2+} concentrations the fluorescence increases only slightly (2–3%) over this pH range. Under these conditions, metal is bound at the low-affinity sites.

Although fluorescent probes are sensitive indicators of protein conformation, knowledge of the exact step or steps responsible for inducing changes in the attached probe's local environment, thereby causing the fluorescence change, is nearly always unknown. Thus, conformational changes detected by alterations in the fluorescence of a covalently attached probe, such as AEDANS-actin, can be looked upon as black box. This makes it difficult to determine whether a fluorescence change upon ligand addition represents ligand binding directly or one or more conformational changes induced by ligand binding. Furthermore, since AEDANS-actin fluorescence is sensitive to two distinct processes, relationships between the fluorescence changes and cation binding on the actin molecule as a function of pH may not be straightforward. In the discussion to follow, we will attempt to sort through the various explanations for the pH dependence of metal-induced AEDANS-actin fluorescence changes, focusing first on the pH dependence of the slow fluorescence change associated with metal exchange.

In agreement with Frieden (1982), the maximum fluorescence change induced by Mg^{2+} becomes smaller at lower pH. This smaller change is due to both the instantaneous and time-dependent portions of the induced fluorescence change becoming smaller. The smaller change in the time-dependent response at lower pH could be the result of either weaker Mg^{2+} binding or a shift with pH in the isomerization equilibrium. Neither explanation appears valid, however, since the pH jump experiments from pH 6 to 8 in the presence of Mg^{2+} (Figure 5) suggest that a process related to the Mg^{2+}-induced isomerization does occur at pH 6. If protons prevented the Mg^{2+}-induced isomerization, actin incubated with Mg^{2+} at pH 6 would always demonstrate a time-dependent fluorescence increase upon a rapid pH jump from 6 to 8; that is, Mg^{2+} would induce the conformational change only after the pH is raised to 8. Instead, as Figure 5 shows, the proportion of the fluorescence increase which is time dependent is correlated with the incubation time of the actin with Mg^{2+}. This strongly suggests that protons alter by some manner the response of the AEDANS probe to conformational changes but that the actin conformer induced by Mg^{2+} binding at the moderate-affinity site still takes place at pH 6. Thus, the smaller Mg^{2+}-induced fluorescence change at lower pH is not related to a lower affinity of Mg^{2+}.

Fitting data at pH 7 was done in the same manner as reported previously for fitting at pH 8. The data could only be

Scheme I: Mechanism for AEDANS Fluorescence Changes



poorly fit to a mechanism relating Ca^{2+} release with an increase in fluorescence, as suggested by Estes et al. (1987), or to a mechanism where a fluorescence change is observed directly upon Mg^{2+} binding. In contrast, good fits to the data were obtained by using the scheme originally proposed by Frieden (1982) which related the fluorescence increase to a conformational change induced by Mg^{2+} binding. Although the maximum fluorescence extent induced by Mg^{2+} is less at pH 7 than at pH 8, this smaller change is assumed unrelated to a smaller extent of actin isomerization. However, neither the quality of the fits nor the rate constants obtained were dramatically altered if such an assumption was not made. Nonlinear regression fits of experimental data below pH 7 could not be obtained since the magnitude of the time-dependent AEDANS fluorescence change becomes too small.

Somewhat surprisingly, searches through the literature yielded no previous studies on the pH dependence of a proton conformational change monitored by an attached fluorescence probe. The results presented here indicate that studies of the variation of fluorescence yields with pH should be approached with extreme caution. With AEDANS-actin, the magnitude and even the sign of the fluorescence change observed upon metal exchange at the moderate-affinity site appear to vary with pH. Therefore, changes in the fluorescence magnitude upon metal exchange as a function of pH cannot be correlated with changes in affinity.

A mechanism consistent with the observed pH dependence of the time-dependent AEDANS-actin fluorescence change is shown in Scheme I. This mechanism is a modified version of the one first proposed by Frieden (1982). The top row of this scheme represents Ca^{2+}–Mg^{2+} exchange at the moderate-affinity site when actin is protonated; the middle row represents this exchange when actin is unprotonated (i.e., this row represents the original scheme proposed), while the bottom row represents this exchange on unprotonated actin with metal bound at the low-affinity sites. In Scheme I, A represents unprotonated monomeric actin while A_H represents actin protonated at a group of residues with a pK of 6.8. The symbols Ca or Mg to the right of A represent actin with bound Ca^{2+} or Mg^{2+} at the moderate-affinity sites, respectively, while the symbol M to the left of A represents actin with bound metal at the low-affinity sites. We believe that M can represent any monovalent or divalent cation. A'Mg or A'_HMg refers to the conformation induced by Mg^{2+} binding at high and low pH, respectively.

This scheme can describe all observed properties of AEDANS-actin related to the pH dependence of both the slow and instantaneous fluorescence increases if three assumptions are made. First, the fluorescence change associated with Mg^{2+} binding at the moderate-affinity site is altered upon protonation of the actin (A_H). This assumption explains why the magnitude of the slow fluorescence increase decreases as the pH is lowered from pH 8. Second, H^+ and M binding is rapid and results in an instantaneous fluorescence increase.

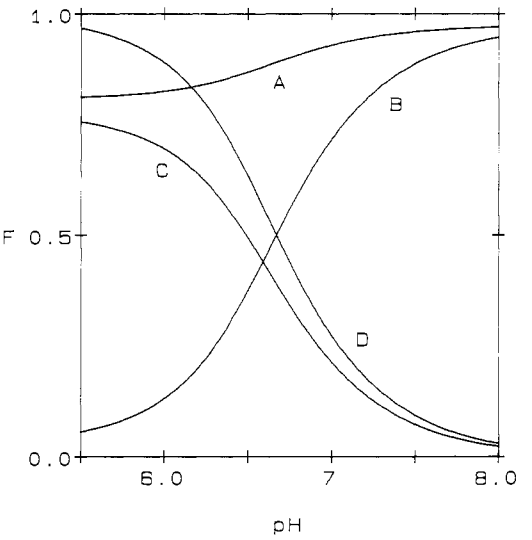


FIGURE 6: Simulation demonstrating the proposed pH variation of AEDANS-actin fluorescence changes upon Mg^{2+}–Ca^{2+} exchange at the moderate-affinity site. Curve A shows, as a function of pH, the fluorescence of AEDANS-actin with Mg^{2+} bound at the moderate-affinity site while curve D shows the fluorescence with Ca^{2+} bound at this site. These curves follow closely that of the actual titrations. The fluorescence of AEDANS-actin with Mg^{2+} bound is the result of the fluorescence contributions of the species A'Mg (curve B) and $A_H'Mg$ (curve C) in Scheme I. In all simulations, the pK_a for $A + H \rightleftharpoons A_H$ is 6.7; A_HCa and A'Mg have identical fluorescence yields (1.0) while the fluorescence yield of $A_H'Mg$ is 20% reduced (0.8).

Therefore, instantaneous fluorescence increases are found upon the binding of metal at the low-affinity sites (from row 2 to row 3) or upon protonation of the actin (from row 2 to row 1). Finally, we assume the species of metal bound at the high-affinity site does not affect AEDANS-actin fluorescence. This assumption appears valid on the basis of recent evidence we have presented (Zimmerle et al., 1987).

As no instantaneous fluorescence increase is observed upon metal cation addition at low pH, Scheme I does not allow metal binding at the low-affinity sites on protonated (A_H) actin. Thus, protons are assumed to compete with Mg^{2+} for binding at the low-affinity site, a conclusion consistent with that of Martonosi et al. (1964). Therefore, the percentage of actin in the salt-induced form is a function of both the pH and salt concentration. Interestingly, Martonosi et al. (1964) found that the pH dependence of Mg^{2+} binding to the low-affinity sites appeared similar to the titration curve of free histidine, a result consistent with the pK of about 6.8 determined for the proton-induced rapid fluorescence change found here.

Figure 6 shows a simulation using Scheme I with the pK_a and equilibrium values described in the figure legend. This figure attempts to summarize our observed results and demonstrates some of the changes which occur in the fluorescence of AEDANS-actin as a function of pH. In this simulation, the Mg^{2+} concentration is assumed low enough that metal binding at the low-affinity site does not occur (bottom row of Scheme I). The observed fluorescence change at a given pH upon addition of Mg^{2+} to actin containing Ca^{2+} at the moderate-affinity site will be the difference between the curve representing the fluorescence yield of Mg^{2+}–G-actin (curve A) and A_HCa–actin (curve D). Therefore, as the pH is decreased from 8 to 6.3, the fluorescence increase found upon the exchange of Mg^{2+} for Ca^{2+} at the moderate-affinity site will become smaller, and at pHs below 6.3, a slight fluorescence decrease will be recorded upon this exchange.

The variation of pH on actin properties found here suggests three points related to actin conformation and its ability to

polymerize. First, since the release of the tightly bound Ca^{2+} becomes slower with pH and polymerization becomes faster, the release of this Ca^{2+} is not a process which limits the polymerization reaction. Second, protonation of a class of residues, perhaps histidine, induces a conformer similar to cation binding at low-affinity sites. Third, despite a lack of AEDANS-actin sensitivity toward cation exchange at low pH, the conformational changes induced by Mg^{2+}–Ca^{2+} exchange still take place. In the following paper, we will attempt to validate these points by examining the polymerization properties of actin as a function of pH (Zimmerle & Frieden, 1988).

Changes in actin conformation have been suggested to be important in relation to the polymerization of actin (Miki et al., 1987; Keiser et al., 1986; Frieden, 1983). Indeed, the fact that interactions between metal binding sites may occur (Zimmerle et al., 1987) as well as interactions between metal binding sites and the nucleotide site (Frieden & Patane, 1988; Selden et al., 1987) suggests actin conformation may be modulated by a host of ligand-induced changes. We suspect actin may be able to undergo a relatively wide spectrum of individual conformational states between a state nearly unable to polymerize to a state able to self-associate rapidly. The population of actin molecules in a given conformational state will thus be relatively sensitive to a host of in vivo factors.

In conclusion, we have demonstrated that between pH 6 and 8 Ca^{2+}–Mg^{2+} exchange at the moderate-affinity site induces an actin conformational change, that a general salt-induced conformer is also induced by protonation of a class of sites of pK 6.8, and that this same protonation appears to alter the response of AEDANS-actin to cation exchange at the moderate-affinity site. Furthermore, we have demonstrated that the release of Ca^{2+} from the high-affinity site becomes slower with decreasing pH and appears unrelated to AEDANS fluorescence changes at pH 7 as was also the case at pH 8 (Zimmerle et al., 1987). All these results suggest the influence both the cation species and concentration, as well as the environmental pH, have on actin conformation.

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REFERENCES

Barany, M., Finkelman, F., & Therattil-Antony, T. (1962) *Arch. Biochem. Biophys. 98*, 28–45.

Barden, J. A., & dos Remedios, C. G. (1985) *Eur. J. Biochem. 146*, 5–8.

Barshop, B. A., Wrenn, R. F., & Frieden, C. (1983) *Anal. Biochem. 130*, 134–145.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Carlier, M., Pantaloni, D., & Korn, E. D. (1986) *J. Biol. Chem. 261*, 10778–10784.

Cooper, J. A., Buhle, E. L., Walker, S. B., Tsong, T. Y., & Pollard, T. D. (1983) *Biochemistry 22*, 2193–2202.

Estes, J. E., Selden, L. A., & Gershman, L. C. (1987) *J. Biol. Chem. 262*, 4952–4957.

Frieden, C. (1982) *J. Biol. Chem. 257*, 2882–2886.

Frieden, C., & Patane, K. (1985) *Biochemistry 24*, 4192–4196.

Frieden, C., & Patane, K. (1988) *Biochemistry 27*, 3812–3820.

Frieden, C., Lieberman, D., & Gilbert, H. R. (1980) *J. Biol. Chem. 255*, 8991–8993.

Gersham, L. C., Selden, L. A., & Estes, J. E. (1986) *Biochem. Biophys. Res. Commun. 135*, 607–614.

Houk, T. W., Jr., & Ue, K. (1974) *Anal. Biochem. 57*, 453–459.

Kasai, M., Asakura, S., & Oosawa, F. (1962) *Biochim. Biophys. Acta 57*, 13–21.

Keiser, T., Schiller, A., & Wegner, A. (1986) *Biochemistry 25*, 4899–4906.

Kouyama, T., & Mihashi, K. (1980) *Eur. J. Biochem. 114*, 33–38.

MacLean-Fletcher, S., & Pollard, T. D. (1980) *Biochem. Biophys. Res. Commun. 96*, 18–27.

Martonosi, A., Molino, C. M., & Gergely, J. (1964) *J. Biol. Chem. 239*, 1057–1064.

Mihashi, K., Nakabayashi, H., Yoshimura, H., & Ohnuma, H. (1979) *J. Biochem. (Tokyo) 85*, 359–366.

Miki, M., Barden, J. A., dos Remedios, C. G., Phillips, L., & Hambly, B. (1987) *Eur. J. Biochem. 165*, 125–130.

Oosawa, F., & Kasai, M. (1971) *Biol. Macromol. 5*, 261–322.

Rich, S. A., & Estes, J. E. (1976) *J. Mol. Biol. 104*, 777–792.

Selden, L. A., Gershman, L. C., Kinosian, H. J., & Estes, J. E. (1987) *FEBS Lett. 217*, 89–93.

Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866–4871.

Strzelecka-Golaszewska, H., Prochniewicz, E., & Drabikowski, W. (1978) *Eur. J. Biochem. 88*, 229–237.

Tawada, K., Wahl, P., & Auchet, J. C. (1978) *Eur. J. Biochem. 88*, 411–419.

Tellam, R. (1985) *Biochemistry 24*, 4455–4460.

Tsein, R. Y. (1980) *Biochemistry 19*, 2396–2404.

Tsein, R. Y., Pozzan, T., & Rink, T. J. (1982) *J. Cell Biol. 94*, 325–334.

Zimmerle, C. T., & Frieden, C. (1986) *Biochemistry 25*, 4899–4906.

Zimmerle, C. T., & Frieden, C. (1988) *Biochemistry* (following paper in this issue).

Zimmerle, C. T., Patane, K., & Frieden, C. (1987) *Biochemistry 26*, 6545–6552.