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Preparation and Characterization of a New Molecular Cytochemical Probe:

5-Iodoacetamidofluorescein-labeled Actin[1]

YU-LI WANG and D. LANSING TAYLOR

Cell and Developmental Biology, The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

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The new technique of molecular cytochemistry (Taylor DL, Wang YL (1978): Proc Natl Acad Sci USA 75:857) requires the use of functional fluorescent analogs of cellular components with optimal fluorescence characteristics. An analog of actin suitable for this technique is prepared by reacting purified rabbit striated muscle actin with 5-iodoacetamidofluorescein (5-IAF). The conjugate is purified by DEAE-cellulose ion exchange chromatography and cycles of polymerization–depolymerization, yielding a relatively homogeneous product with the fluorescein group covalently attached to cysteine 373. The fluorescently labeled actin maintains normal polymerizability and activates heavy meromyosin Mg^{2+} adenosine triphosphatase to the same extent as unlabeled actin. Furthermore, fluorescent paracrystals are readily detectable in fluorescence microscope upon adding excess Mg^{2+} or Ni^{2+} ions. Spectrofluorimetric studies of the bound fluorescein indicate that the peak excitation and emission wavelengths, the shapes of the spectra, and the peak fluorescence intensities are somewhat sensitive to polymerization and heavy meromyosin binding. Possible causes of these spectral changes are analyzed and future applications of this fluorescently labeled actin in vitro as well as in vivo are discussed.

KEY WORDS: Molecular cytochemistry; Fluorescently labeled actin; 5-Iodoacetamidofluorescein; Fluorescence spectroscopy.

Introduction

The self-assembly of actin and the interactions between actin and other muscle proteins have been studied intensively during the past two decades (for a review, see ref. 22). Recent investigations on nonmuscle cells have revealed important new roles of actin in many nonmuscle processes such as ameboid movement, cytokinesis, and membrane receptor redistribution (for reviews, see refs. 13, 24, and 30). Many proteins have been shown to interact with actin, including various gelatin factors (for reviews, see refs. 13 and 30), profilin (1), nonmuscle actinins (9), spectrin (18), DNase I (14), and even some glycolytic enzymes (13), making actin one of the most versatile proteins in eukaryotic cells.

Fluorescence spectroscopy has been a powerful tool for studying protein–protein interactions. Fluorescent probes such as *N*-(3-pyrene) maleimide (11), *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)-ethylenediamine (10, 25, 29), *N*-(*p*-(2-benzimidazolyl)-phenyl) maleimide (26), dansyl cystine (2), and dansyl aziridine (15, 25), have been coupled to actin. Using various fluorescence spectroscopic techniques, much information has been obtained regarding the biochemical activities of actin in vitro. In an attempt to extend the applicability of fluorescence techniques to living cells, we have recently introduced the technique of in vivo molecular cytochemistry, using functional fluorescent analogs of cellular components (31). The procedure involves purifying the cellular component under study, labeling with appropriate fluorophores, testing the conjugates for functional activities in vitro, and incorporating the conjugates into living cells using microinjection techniques. Subsequent recording of fluorescence images from injected cells allows direct determinations of the distribution of the conjugate and opens the possibility of extending spectroscopic methods to living cells.

To be suitable for applications in living cells, the fluorescent analog must meet at least four criteria: 1) The fluorophore should be excited by a relatively long wavelength of light, so that radiation damage to living cells and interference from autofluorescence can be minimized; 2) The fluorophore should emit intense fluorescence (by having a high extinction coefficient and/or a high quantum yield), in order to maximize the signal level from single cells; 3) The bond between the fluorophore and the labeled component must be covalent and

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stable, so that the two will remain associated inside living cells; and 4) The labeling reaction should be mild enough not to abolish the functional activities of the cellular component. Optimally, it is preferable to have a homogeneous preparation of conjugates with the fluorophore attached to well-defined sites, in order to avoid ambiguities involved in the evaluation and interpretation of biochemical as well as spectroscopic data.

Attempts have been made to prepare a fluorescently labeled actin that meets the above criteria. It is known that a sulfhydryl group, located at cys-373, can be blocked without significantly affecting known biochemical activities of actin (17). Therefore, a long-wavelength, highly fluorescent reagent that reacts specifically and irreversibly with sulfhydryl groups should be appropriate for the present purpose. 5-Iodoacetamidofluorescein (5-IAF) has been chosen based on these considerations. In this article, we describe the detailed procedure for preparing 5-IAF-labeled actin (hereafter referred to as 5-AF-actin). The reactive site is characterized by testing the effect of *N*-ethylmaleimide (NEM) pretreatment. The functional activities are assessed based on polymerizability, activation of heavy meromyosin (HMM) Mg^{2+} adenosine triphosphatase (ATPase), formation of paracrystals, and incorporation into a cell-free contractile system. Furthermore, the fluorescence properties are characterized and the application to molecular cytochemistry in vivo is discussed. The incorporation of 5-AF-actin into living ameba and sea urchin eggs has already been reported elsewhere (31–33).

Materials and Methods

Reagents. 5-IAF and lissamine rhodamine B sulfonyl chloride were purchased from Molecular Probes (Plano, TX). NEM, fluorescein isothiocyanate (FITC), dithiothreitol (DTT), and Sephadex G-25 were obtained from Sigma Chemical Co. (St. Louis, MO). ATP was purchased from Boehringer-Mannheim and DEAE-cellulose (DE52) was obtained from Whatman.

Protein preparation and assay. Rabbit striated muscle actin was prepared according to the method of Spudich and Watt (27). Rabbit striated muscle myosin was prepared according to Kieley and Harrington (12). HMM was prepared according to Lowey and Cohen (16). Ovalbumin (Worthington, 2 times crystallized) was labeled using FITC or lissamine rhodamine B sulfonyl chloride as described previously (32, 33). The motile cell-free extract, S3, from *Dictyostelium discoideum* was prepared according to Condeelis and Taylor (3).

Concentrations of labeled proteins were determined by the Lowry procedure. The concentration of unlabeled G-actin and HMM were measured by absorbance at 280 nm, using extinction coefficients of 1.08 and 0.65 $cm^{-1}(mg/ml)^{-1}$, respectively. The molecular weight of actin was assumed to be 42,000 daltons and that of HMM 350,000 daltons.

Extinction coefficient of bound fluorescein. The concentrations of fluorescein bound to actin were determined by absorbance measurements at 495 nm in a buffer (Buffer E) containing 10 mM Tris-HCl, pH 8.0, 0.2 mM $CaCl_2$, 0.5 mM ATP. The extinction coefficient was obtained by the method of Hartig et al. (8). The extinction coefficient of 5-IAF at 495 nm in 7.5 M urea, 10 mM Tris-HCl, pH 8.0, was 6.9×10^4 $cm^{-1}M^{-1}$. The absorbance of 5-AF-actin at 495 nm in Buffer E was 87% that in 7.5 M urea, 10 mM Tris-HCl, pH 8.0. Assuming that 5-IAF and 5-AF-actin had the same extinction coefficient in 7.5 M urea, 10 mM Tris-HCl, the extinction coefficient of 5-AF-actin in Buffer E should be 87% of 6.9×10^4 $cm^{-1}M^{-1}$, or 6.0×10^4 $cm^{-1}M^{-1}$.

NEM treatment of actin. Aliquots of 20 mM NEM stock solution in 3 mM imidazole, pH 7.5, 0.2 mM $CaCl_2$, 0.4 mM ATP, 0.2 mM ascorbic acid were added to 4.0 mg/ml solutions of G-actin in the same buffer. The final concentration of NEM was 0.75 mM. The mixture was put under N_2 atmosphere and stirred at room temperature for 1 hr. The reaction was terminated by adding 2-mercaptoethanol to 1 mM. Quenched NEM was subsequently removed by dialysis.

Fluorescence measurements. Fluorescence intensities and spectra were measured using a Fluorolog spectrofluorometer (Spex Industries, Metuchen, NJ) equipped with an XBO 150 xenon lamp. The intensity fluctuations of the xenon lamp and the variations in the efficiency of excitation monochromator were corrected using a rhodamine B quantum counter in the reference detector. To record excitation spectra, the emission monochromator was fixed at 545 nm with 10 nm bandpass and the excitation monochromator scanned from 400 to 535 nm with 1 nm bandpass. To record emission spectra, the excitation monochromator was fixed at 465 nm with 10 nm bandpass and the emission monochromator scanned from 480 to 600 nm with 1 nm bandpass. To measure peak fluorescence intensities, the excitation and emission monochromators were fixed at 500 and 522 nm, respectively, with 8 nm bandpass for both monochromators. A quartz square microcuvette of 4 mm pathlength was used for all measurements.

The concentration of 5-AF-actin in all samples were 0.01 mg/ml, which was below the critical concentration. A buffer containing 2 mM piperazine-*N.N′*-bis (2-ethane sulfonic acid) (PIPES), 2 mM $MgCl_2$ and 0.5 mM DTT (Buffer M) was used for all samples. Samples of G-actin were prepared by direct dilution of monomeric 5-AF-actin into Buffer M immediately before measurements. To prepare polymerized 5-AF-actin, 0.25 mg/ml monomeric 5-AF-actin was first copolymerized with 2 mg/ml unlabeled actin. The copolymers were subsequently diluted 25-fold into 0.5 mg/ml solutions of unlabeled actin in Buffer M. For acto-HMM, F-actin copolymers as described above were diluted 25-fold into solutions of 1.7 mg/ml HMM in Buffer M. These procedures ensured that 1) the inner filter effect was negligible, 2) the G-state was stabilized by subcritical concentrations, 3) the F-state was stabilized by copolymerization with unlabeled actin and dilution with excess unlabeled F-actin, so that the total actin concentrations were well above the critical concentration, and 4) all samples were under identical ionic conditions, so that effects of ionic environment could be ruled out.

Preparation of paracrystals. Mg^{2+} paracrystals were prepared by adding $MgCl_2$ to monomeric 5-AF-actin to a final concentration of 25 mM. Ni^{2+} paracrystals were prepared by adding $NiCl_2$ to monomeric 5-AF-actin to a final concentration of 4 mM. DTT was removed from the solution, since it reacted with Ni^{2+} ions. Ni^{2+} paracrystals from the same test tube were used for both fluorescence microscopic and electron microscopic observations.

Observation and recording of fluorescent images. Fluorescent images were observed with a Zeiss Photomicroscope III equipped with an epi-illumination condensor, using either 25× Neofluar objectives (N.A. 0.60) or 100× oil immersion objectives (N.A. 1.25). A 60 W quartz-halogen lamp was used as the light source. The images were recorded with an RCA TC 1030/H TV image intensifier coupled to an NEC video tape recorder and an RCA TV monitor.

Selected images on the video tapes were photographed from the

TV screen with Kodak Tri-X film ASA 400 at ⅛ sec exposures and developed in D-76 developer for 10 min at 22°C.

Miscellaneous procedures. For viscosity measurements, G-actin was polymerized by adding KCl to 100 mM and $MgCl_2$ to 1.5 mM. After an 8 hr incubation at 25°C, the viscosity was measured using a Cannon-Manning Semi-microviscometer Size 150. The buffer flow time was 29 sec at 25°C. The relationship between specific viscosity and actin concentration is analyzed by linear regression. The reduced viscosity is calculated from the slope of the line; the critical concentration is determined from the *x*-axis intercept.

Actin-activated HMM Mg^{2+} ATPase was measured in a buffer of 15 mM Tris-HCl, pH 7.5, at 24°C, 2 mM $MgCl_2$,0.4 mM DTT, 2 mM ATP. After incubation for 15 min at 24°C, the concentration of Pi was determined by the method of Pollard and Korn (23). Less than 50% ATP was hydrolyzed during the assay period. The ATPase activity due to HMM alone was substracted from all values.

The Ni^{2+} paracrystals were negatively stained with 2% uranyl acetate and observed with a Philips 301 electron microscope operated at 80 kV.

Results

Preparation of 5-AF-actin

All steps involving fluorescein were carried out under minimum illumination. Specifics of the procedure were as follows.

Step 1: Reaction. A fine slurry of 10 mg 5-IAF in 300 μl acetone was added dropwise to 3 ml of a buffer containing 100 mM boric acid, 100 mM KCl, 4 mM $MgCl_2$, pH 8.5. The dissolved dye solution was mixed with 3 ml of 5 mM Tris-HCl, pH 8.0, 0.2 mM $CaCl_2$, 100 mM KCl, 1 mM ATP, 0.4 mM ascorbic acid, containing 5 mg/ml F-actin. The molar ratio of dye to protein during reaction was 55 to 1. The reaction was carried out for 2 hr at room temperature under N_2 atmosphere, and terminated by adding excess 2-mercaptoethanol. F-actin was then pelleted by centrifugation at 100,000 × *g* for 2 hr.

Step 2: Depolymerization and desalting. The F-actin pellet was resuspended in 2 ml of 5 mM Tris-HCl, pH 8.0, 0.2 mM $CaCl_2$, 1 mM ATP, 1 mM DTT, and depolymerized by adding dropwise, 1 ml of 1.8 M KI, 3 mM $CaCl_2$, 15 mM ATP, pH 7. After stirring for 30 min at 0°C, the depolymerized actin was clarified and loaded onto a 0.5 cm × 50 cm Sephadex G-25 gel filtration column equilibrated with 10 mM Tris-HCl, pH 7.5, 0.1 mM $CaCl_2$, 0.5 mM ATP, 0.5 mM DTT (Buffer B) at 4°C. Fluorescent fractions in the void volume were collected. Following desalting, the molar ratio of bound fluorescein to actin was 0.4–0.5.

Step 3: DEAE-cellulose ion exchange chromatography and second cycle of polymerization–depolymerization. Since the fluorescein group carries net negative charges at neutral pH (20), labeled actin could be separated from unlabeled actin by ion exchange chromatography. The DEAE-cellulose ion exchange chromatography was carried out essentially as described by Gordon et al. (6). A 0.5 cm × 12 cm DE-52 column was saturated with ATP and preequilibrated with Buffer B containing 0.1 M KCl at 4°C. Two to three milliliters of Buffer B were added to the column immediately before and after the desalted fractions were loaded, in order to minimize actin polymerization during loading. Elution was accomplished with a linear gradient of KCl from 0.1 to 0.4 M in Buffer B. The presence of protein and fluorescein in eluted fractions was monitored by their absorbance at 290 and 495 nm, respectively (Figure 1). Two major absorption peaks at 290 nm were detected, corresponding to 0.20 and 0.27 M KCl, respectively. Fractions of both peaks showed increases in viscosity upon warming, but only the second peak absorbed light at 495 nm. Therefore, the first peak was identified as unlabeled actin and the second peak as 5-IAF-labeled actin.

Fractions 31–40 were pooled and warmed to room temperature after the addition of 5 mM $MgCl_2$. Polymerized actin was subsequently collected by centrifugation at 100,000 × *g* for 2 hr. The pellet was resuspended in 0.5–1.0 ml Buffer A at

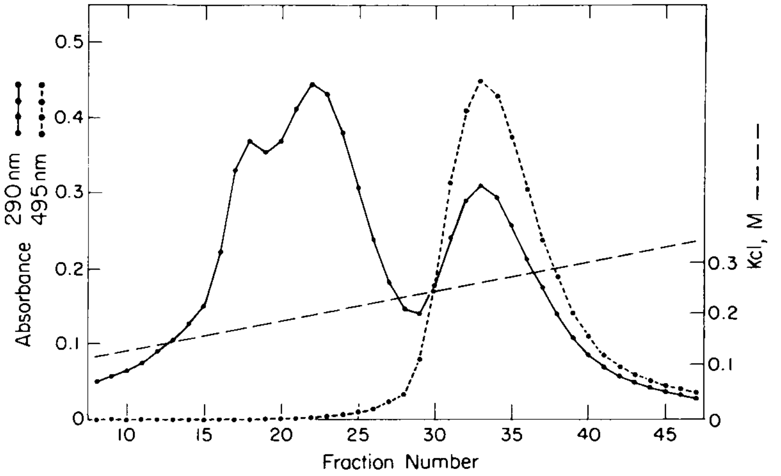


Figure 1. DEAE-cellulose ion exchange chromatography of actin reacted with 5-IAF. The column is eluted with a gradient of KCl (– – –). Absorbances at 290 nm (——) and 495 nm (- · - ·) are measured to follow the elution of protein and fluorescein, respectively. The volume of each fraction is 1 ml.

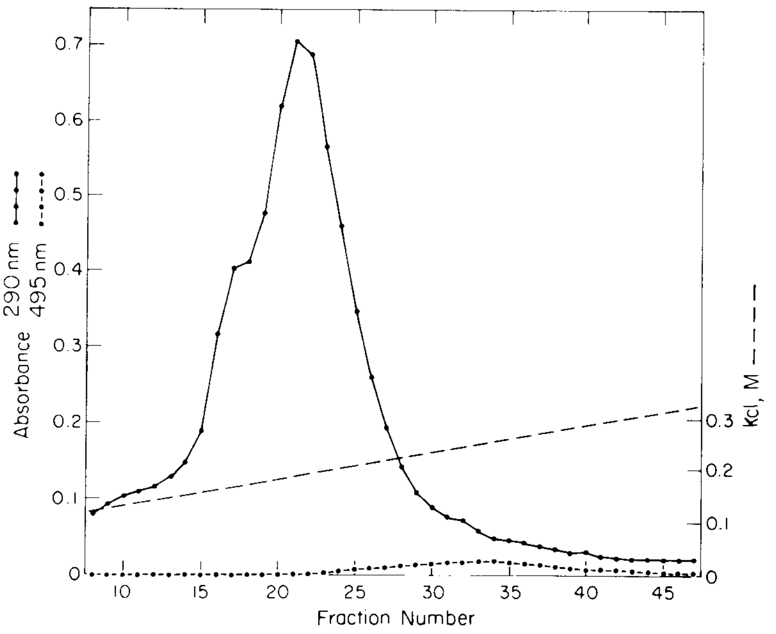


Figure 2. DEAE-cellulose ion exchange chromatography of actin treated with NEM prior to 5-IAF labeling. The total amount of actin is the same as that in Figure 1. Therefore, the peak heights can be directly compared.

4°C (2 mM Tris-HCl, pH 8.0, 0.2 mM CaCl$_2$, 0.2 mM ATP, 0.5 mM DTT, 0.02% NaN$_3$) and dialyzed for 3 days to depolymerize the 5-AF-actin. The yield of 5-AF-actin was about 20–25% with a fluorescein/actin molar ratio of 0.7–0.9.

Bond Stability and Site of Reaction

To test for the presence of unbound fluorescein and the stability of the association between fluorescein and actin, a solution of 5-AF-actin was made in 1% sodium dodecyl sulfate (SDS) and 1 mM DTT. Following heating to 100°C for 3 min the solution was passed through a Sephadex G-25 column preequilibrated with 10 mM Tris-HCl, pH 8.0, 1 mM DTT, 1% SDS. A single fluorescent peak was detected in the void volume. This result suggested that 1) no unbound fluorescein was present, and 2) the association between fluorescein and actin was covalent and irreversible.

To locate the site of reaction, actin was pretreated with NEM before labeling. A comparison of the elution profile of ion exchange chromatography (Figures 1, 2) indicated that after NEM treatment, the peak of 5-AF-actin decreased by more than 95%, while the peak of unlabeled actin increased correspondingly. This result suggested that 5-IAF reacted predominantly with the same site as NEM, previously determined to be cys-373 (5).

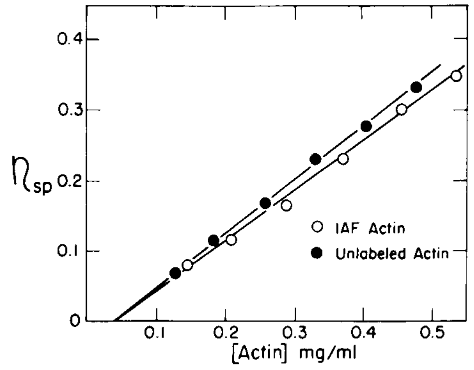
Functional Properties of 5-AF-Actin

The effect of 5-IAF labeling on the biochemical properties of actin was assessed by functional assays in vitro. The reduced viscosity of polymerized 5-AF-actin was 7.2 dl/g under the specified condition, which was 94% that of unlabeled actin (7.7 dl/g). The critical concentration of 5-AF-actin was indistinguishable from that of unlabeled actin (about 0.04 mg/ml under the specified condition, Figure 3). These values fell within the range of values reported previously (7), and suggested that 5-AF-actin maintained normal polymerizability.

5-AF-actin activated the HMM Mg^{2+} ATPase to the same extent as does unlabeled actin (Figure 4). Up to 200-fold activation was observed under the assay condition. The specific activities for both 5-AF-actin and unlabeled actin were $\sim 3\ \mu$M Pi/μM HMM · μM actin · sec.

Native actin aggregates into paracrystals in the presence of 25–50 mM Mg^{2+} ions (28). The ability of 5-AF-actin to form paracrystals was tested using fluorescence microscopy. Large fluorescent paracrystals (up to 40 μm in length) against a very low background were readily detectable upon adding 25 mM MgCl$_2$ (Figure 5), indicating that essentially all of the 5-AF-actin was incorporated into the paracrystalline structure. The much smaller Ni^{2+} paracrystals (about 3–5 μm in length)

Figure 3. Viscosity assays of 5-AF-actin (○) and unlabeled actin (●). Both are polymerized for 8 hr (25°C) before measuring viscosities in a Cannon-Manning 150 Viscometer. Critical concentrations are indicated by the intercepts on the x-axis.



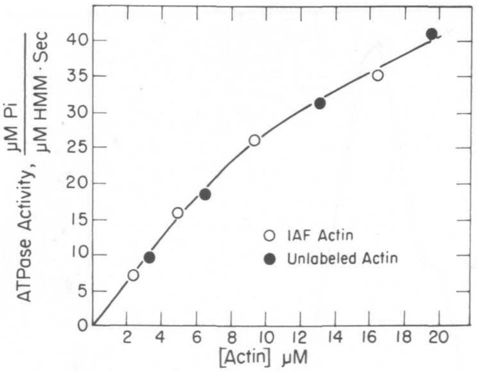
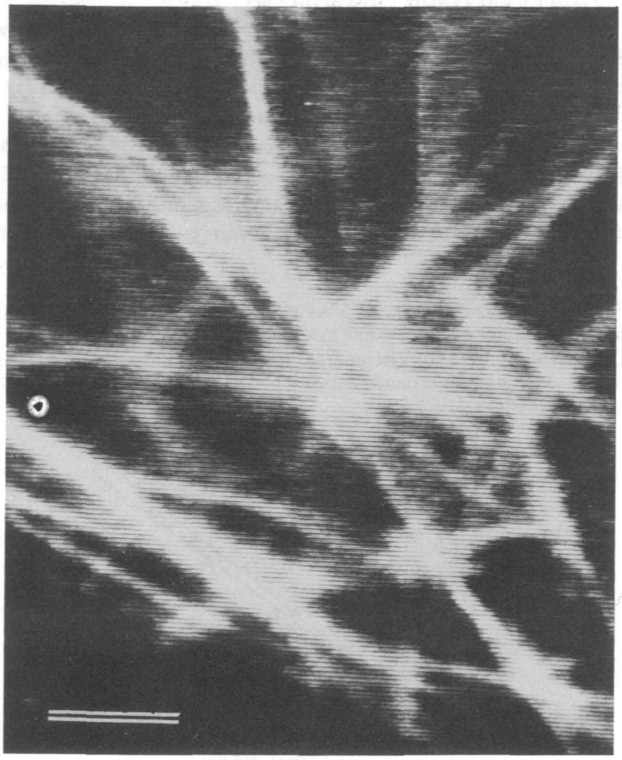


Figure 4. Activation of HMM Mg^{2+} ATPase as a function of 5-AF-actin concentration (○) or unlabeled actin concentration (●). The concentration of HMM varies from 8 to 40 μg/ml, in order to attain about the same rate of ATP hydrolysis (~ 0.06 mM Pi/min). The ATPase activity due to HMM alone (~ 0.2 μM Pi/μM · sec) has been substracted from all values. The conditions for the assay are described under Materials and Methods.

Figure 5. Mg^{2+} paracrystals of 5-AF-actin as detected by a fluorescence microscope coupled to a TV image intensifier. Bar = 5 μm.



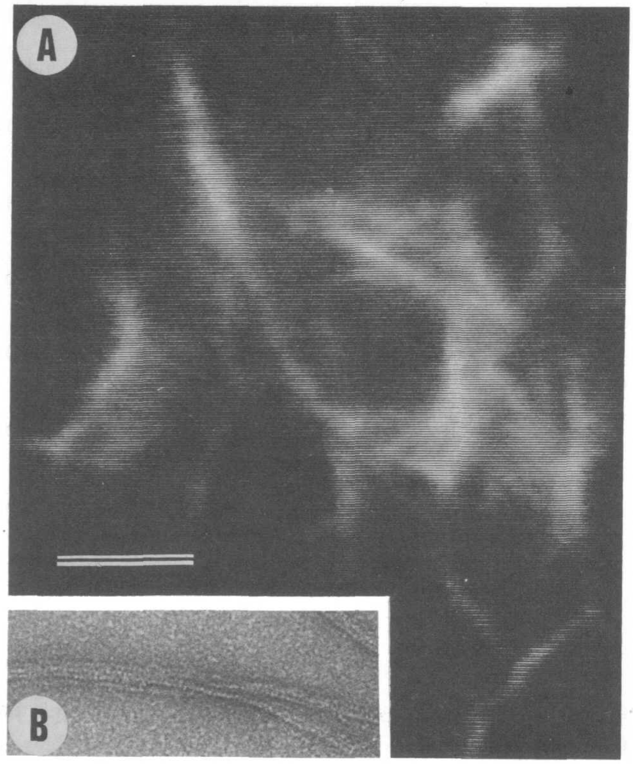


Figure 6. Ni^{2+} paracrystals of 5-AF-actin. (A) As detected by a fluorescence microscope coupled to a TV image intensifier. Bar = 5 μm. (B) Electron microscopy of negatively stained Ni^{2+} paracrystals.

could also be detected (Figure 6A). Electron microscopy of negatively stained Ni^{2+} paracrystals indicated that each paracrystal contained up to 20 actin filaments (Figure 6B), similar to what has been reported for unlabeled actin (28). Samples of F-actin had the appearance of a barely detectable dense meshwork in the fluorescence microscope. However, the signal is too weak to be recorded by the present methods.

5-AF-actin was further tested to see if it could be incorporated into a cell-free extract from *D. discoideum,* which exhibited Ca^{2+} and pH sensitive contraction with concomitant formation of highly birefringent actin fibrils (3). A mixture of 5-AF-actin and lissamine rhodamine B ovalbumin was added to the extract in order to follow the distribution of the two fluorescent components simultanelusly. The fluorescence of both 5-AF-actin and lissamine rhodamine B ovalbumin remained uniform during the sol-gel transition. However, when solation–contraction of the gel was induced by raising the pH or free Ca^{2+} concentration, fluorescent fibrils containing 5-AF-actin were observed (Figure 7A). In contrast, the lissamine rhodamine B fluorescence remained uniform (Figure 7B). The final contracted pellet consisted of a dense meshwork of small fibrils (Figure 7C). These observations were independent of 5-AF-actin concentrations in the range of 0.15 mg/ml to 0.05 mg/ml (which is close to the critical concentration), suggesting that the incorporation of 5-AF-actin into fibrils was not due to the entrapment of preexisting 5-AF-actin filaments by the contracting gel.

Fluorescence Properties of 5-AF-actin

The fluorescence spectra and peak fluorescence intensities of 5-AF-actin were studied in the monomeric, polymeric, and