Effect of Polymerization on the Subdomain 3/4 Loop of Yeast Actin*

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The Holmes F-actin model predicts a polymerization-dependent conformational change of a subdomain 3/4 loop with a hydrophobic tip (residues 266–269), allowing interaction with a hydrophobic surface on the opposing strand of the filament producing filament stabilization. We introduced cysteines in place of Val266, Leu267, and Leu269 in yeast actin to allow attachment of pyrene maleimide. Pyrene at each of these positions produced differing fluorescence spectra in G-actin. Polymerization decreased the fluorescence for the 266 and 267 probes and increased that for the 269 probe. The direction of the fluorescence change was mirrored with a smaller and less hydrophobic probe, acrylodan, when attached to 266 or 269. Following polymerization, increased acrylamide quenching was observed for pyrene at 266 or 267 but not 269. The 267 probe was the least accessible of the three in G- and F-actin. F-actin quenching was biphasic for the 265, 266, and 269 but not 267 probes, suggesting that in F-actin, the pyrene samples multiple environments. Finally, in F-actin the probe at 266 interacts with one at Cys374 on a monomer in the opposing strand, producing a pyrene excimer band. These results indicate a polymerization-dependent movement of the subdomain 3/4 loop partially consistent with Holmes’ model.

A number of additional modeling studies have addressed this aspect of the model with divergent results. This hypothesis was supported by a refinement of the F-actin model by Lorenz et al. (6). Using normal mode analysis, Tirion et al. (7) were unable to predict the occurrence of such a loop movement. Molecular dynamics simulations by Wrighers et al. (8) suggest that during the G to F transition, the closure of the nucleotide binding cleft destabilizes the binding contacts of the loop 262–272, leading to its detachment from the monomer surface, suggesting that the loop is capable of extending at least part way into the interstrand space. Schutt (4) has argued that a significant energetic barrier would have to be overcome to detach and restructure the loop as proposed by Holmes and considers such an event unlikely. He and co-workers have proposed an alternative model (4, 9) based on the profilin/β-actin ribbon structure, in which subdomains 1 and 2 lie near the filament axis whereas subdomains 3 and 4 are distal from the filament axis. The loop between subdomains 3 and 4 remains attached to the actin monomer surface, contributing to the stability of the monomer within the filament. However, they have yet to publish the coordinates of this filament model.

We had previously performed a series of experiments with mutant yeast actins designed to address Holmes’ hypothesis. Yeast actin is 87% identical to rabbit skeletal muscle actin, and it can interact with most of the actin-binding proteins of higher eukaryotes (10, 11). Yeast has a single copy actin gene, ACT1 (12), and it is thus possible to replace the wild-type actin gene with a mutant one in order to test the importance of specific residues or regions on actin function. Earlier results from in vitro studies in our laboratory are consistent with Holmes’ prediction of the “plug-pocket” interaction. The plug region in yeast actin consists of four residues, Val266-Leu267-Gly268-Leu269. Our results suggest that two of the three hydrophobic residues are required for filament formation. Insertion of a charged residue between two hydrophobic residues produced a cold-sensitive polymerization defect, whereas placement of a charged residue on either of the plug’s end positions had little effect. The cold sensitivity is what would be expected for a disruption of a hydrophobic interaction (13). Together, our results suggest that the plug-pocket interaction, if it exists, is flexible instead of the lock-and-key model proposed by Holmes. Simultaneous substitution of two hydrophobic residues, Val266 and Leu267, with glycine (GG-actin) prevented actin polymerization in vitro. However, phalloidin, BeFx, or equivalent amounts of wild-type actin restored the ability of GG-actin to polymerize, thereby suggesting the importance of the hydrophobicity of the plug for filament formation and stabilization.

To attempt to visualize the behavior of the loop (residues 262–272) during polymerization, two mutants, S265C and S265C/C374A, were previously created to enable the attachment of a fluorescent probe, N-(1-pyrenyl)maleimide (pyrene...
onaphthalene (acrylodan) to reveal polymerization-induced environmental changes around the loop residues. Using steady-state fluorescence measurements of these probes and the ability of the pyrene maleimide-labeled actins to be quenched by acrylamide, we have assessed the behavior of the labeled loop residues in the G- to F-actin transition.

EXPERIMENTAL PROCEDURES

Materials—The QuickChange site-directed mutagenesis kit was purchased from Stratagene Corp. Integrated DNA Technologies, Inc. synthesized oligodeoxynucleotides used for site-directed mutagenesis. Pyrene maleimide and ATP were purchased from Sigma. The Sequenase version 2.0 DNA sequencing kit was purchased from U.S. Biochemical Corp. Affi-Gel 10 active ester-agarose was purchased from Bio-Rad, and DNase I (grade D) was purchased from Worthington. Acrylodan, Alexa-phalloidin, and FM 4-64 were purchased from Molecular Probes, Inc. (Eugene, OR). Yeast cakes for preparation of wild-type (WT) actin were obtained locally. All other chemicals used were reagent grade quality. Myosin subfragment 1 (S1) was a generous gift from Dr. Larry Tobacman (University of Iowa).

Oligodeoxynucleotide-directed Mutagenesis—Site-directed mutagenesis was used to construct a mutant actin sequence carried in a centromeric plasmid pRS314 (15) marked with the TRP1 gene. The following oligodeoxynucleotides were used to generate the mutants we analyzed (in each case the mutant codon is underlined): V266C, 5'-CCATCCTTCAGTTGGGTTTTGGAAATC-3'; L267C, 5'-CATCCTTCTGGTGGTGGGAAATC-3'; L269C, 5'-CCATCTTGGTGGGTTTTGGGA-3'; C374A, 5'-GCTTACACCAAGGGTGCTTCTACTCTGC-3'. The DNA was sequenced in each case to verify the desired mutation with either the Sequenase kit or by the DNA Sequencing Facility at the University of Iowa.

Generation of Cells Producing Mutant Actins—pRS314 plasmids containing the mutant actin coding sequences were introduced into a trp1, ura3-52 Saccharomyces cerevisiae haploid cell in which the chromosomal ACT1 gene had been disrupted by replacement of the coding sequence with the LEU2 gene. Wild-type actin was expressed in these recipient cells from another centromeric plasmid containing the URA3 gene. Following transformation with the mutant plasmid and selection on tryptophan-deficient medium, surviving cells were subjected to plasmid shuffling to eliminate the plasmid carrying the WT actin gene. The mutant plasmid was rescued from surviving trp' ura' cells and sequenced to ensure that the mutation was still intact. Viable cells were readily obtained for all mutants.

Actin Purification—The Ca²⁺-forms of the actins were prepared by a combination of DNase I affinity chromatography and DEAE-cellulose chromatography according to Cook et al. (16) and stored in Ca²⁺-G-buffer (10 mM Tris-HCl, pH 7.5, containing 0.2 mM ATP, 0.2 mM CaCl₂, and 0.1 mM dithiothreitol) at 4 °C. Prior to labeling, dithiothreitol was removed from the actin by centrifuging through Sephacry G-50 columns equilibrated with dithiothreitol-free buffer (10 mM MOPS, pH 7.4, 0.2 mM CaCl₂, and 0.2 mM ATP). The WT actin was labeled on Cys²⁷⁴ with pyrene-maleimide; V266C, L267C, and L269C actins were labeled on both loop positions as well as Cys²⁷⁴ and C374A, and V266C/C374A, L267C/C374A, and L269C/C374A were labeled solely on the loop positions as described previously (14). Unreacted dye and denatured actin were removed by polymerization-depolymerization cycling. The extent of labeling was determined with the pyrene extinction coefficient, ε₂₉₅ = 22,000 M⁻¹ cm⁻¹.

The acrylodan labeling protocol was modified from Marriot et al. (17) as follows: V266C/C374A and L269C/C374A actins were incubated overnight at 4 °C in the G-form. The actin was then put through a cycle of polymerization and depolymerization to remove unbound dye and denatured actin. The extent of labeling was determined by using the acrylodan extinction coefficient, ε₂₉₅ = 15,500 M⁻¹ cm⁻¹, and the actin extraction coefficient, ε₂₉₅ = 26,000 M⁻¹ cm⁻¹. Corrections were made for the absorbance of acrylodan at 290 nm using the extinction coefficient ε₂₉₅ = 15,500 M⁻¹ cm⁻¹. The labeling efficiency was 100%.

Actin Polymerization Assays—In all cases, actin polymerization was initiated by the addition of KCl and MgCl₂ to final concentrations of 50 and 8 mM respectively. The final sample volume was 120 μl. For spectral comparisons of G- versus F-actin, polymerization was allowed to proceed for 20 min, a period sufficient for complete polymerization. For kinetic experiments (Fig. 7), actin polymerization was followed by the increase in light scattering as a function of time. The fluorimeter, either an SLM model 4860 fluorimeter or a Spex Fluorolog III, was set at 360 nm for both the excitation and emission wavelengths.

Fig. 1. Model of the rabbit skeletal muscle actin filament. In monomer 2, the loop is in the “parked” position, whereas the loops are extended as proposed by Holmes in monomers 1 and 3. Residues Phe²⁶⁶ in the subdomain 3/4 loop and Cys²³⁷ near the C terminus are presented as space-filled residues.

maleimide),¹ to monitor conformational changes that might occur (14). This position was chosen due to the similarities between the original Ser and the new residue, Cys. During polymerization, fluorescence of the loop at position 265 decreases, suggesting that the loop probe undergoes a conformational shift, placing it in a more hydrophilic or solvent-exposed environment. However, if Holmes’ model for loop unfolding is correct, then Ser²⁶⁵ would move the least (only about 0.5 Å) compared with residues in the plug, functioning, instead, as a pivot around which the remainder of the loop would unfold. Thus, Ser²⁶⁵ reports indirectly at best on loop movement.

Four major questions therefore remain unanswered regarding the loop model. First, during polymerization, does the plug detach from the surface of the actin monomer? Second, if detachment can occur, is it necessary for polymerization? Third, if the loop detaches, can it extend to interact with a hydrophobic pocket on the opposing strand of the filament? And fourth, if this is possible, is it required for filament stabilization? In this paper, to examine more thoroughly the contribution of this hydrophobic loop to F-actin structure and stability, we have mutated residues Val²⁶⁶, Leu²⁶⁷, and Leu²⁶⁹ at the tip of the loop individually to Cys. This would potentially allow stoichiometric attachment of thiol-specific fluorophors if the resulting sulfhydryl groups were accessible. The other reactive thiol group in actin, Cys²⁴⁷, was mutated to Ala in a duplicate set of the loop mutants to allow us to monitor the behavior of the loop fluorophor alone. A model of the actin filament showing the position of Cys²⁴⁷ and of phenylalanine at position 266 in the subdomain 3/4 loop of the muscle actin is shown in Fig. 1. The loop is depicted in both the extended and parked positions.

We have used these mutant actins labeled with the fluorescent probes pyrene maleimide and 6-acryloyl-2-dimethylaminonaphthalene; WT, wild-type; S1, subfragment 1; MOPS, 4-morpholinepropanesulfonic acid; NLLS, nonlinear least squares.

¹ The abbreviations and trivial names used are: pyrene maleimide, N-(1-pyrenyl)maleimide; acrylodan, 6-acryloyl-2-dimethylaminonaphthalene; WT, wild-type; S1, subfragment 1; MOPS, 4-morpholinepropanesulfonic acid; NLLS, nonlinear least squares.
Fluorescence Spectroscopy of Pyrene-labeled Wild-type and Mutant Actins—The change in pyrene fluorescence following actin polymerization was observed using a fluorimeter set at an excitation wavelength of 365 nm and emission wavelength of 386 nm for WT and V266C/C374A actins. The emission wavelength was 375 nm for L267C/C374A, and the emission wavelength for L268C/C374A was 386 nm. Emission spectra between 375 and 600 nm were obtained in a similar manner following excitation at 365 nm (18, 19). Acrylamide quenching studies were initiated to determine the degree of exposure of the loop residues to solvent in the monomeric and polymeric states of actin (20). Fluorescence quenching studies were performed by adding small volumes of 3 M acrylamide to achieve final concentrations of 25–500 mM in a cuvette containing 0.4 mg/ml actin in a volume of 120 µl. All of the experiments were repeated with at least two independent actin preparations.

The quenching data were initially analyzed using the Stern-Volmer equation,

\[ \frac{F_0}{F} = 1 + K_{SV}[Q] \]  

where \( F_0 \) and \( F \) represent the fluorescence intensities in the absence and presence of the quencher, \( K_{SV} \) is the Stern-Volmer quenching constant, and \([Q]\) is the total quencher concentration. Most of the actin samples show significant heterogeneity that cannot be analyzed in this fashion. Therefore, we correlated fluorescence intensity as a function of quencher concentration in a two-fluorophore system. We resolved two component spectra through nonlinear least squares (NLLS) fitting of the quenching data using the following formula (modified from Ref. 21),

\[ \frac{F_\lambda}{F_{\lambda0}} = \frac{f_1(\lambda)}{1 + K_{SV1}[Q]} + \frac{f_2(\lambda)}{1 + K_{SV2}[Q]} \]  

where \( F_\lambda \) and \( F_{\lambda0} \) are the fluorescence intensities in the absence and presence of the quencher at a particular wavelength \( \lambda \); \( f_1(\lambda) \) and \( f_2(\lambda) \) are the fractional contributions of the total fluorescence from fluorophores 1 and 2 in the absence of the quencher at wavelength \( \lambda \); and \( K_{SV1} \) and \( K_{SV2} \) are the Stern-Volmer quenching constants of the fluorophores. Assuming that the \( K_{SV} \) values are the same at all wavelengths for the two fluorophores, the fluorescence fractional contributions \( f_1 \) and \( f_2 \) of the two fluorophores could be derived at each wavelength through NLLS fitting. Fluorescence quenching data at all wavelengths were globally linked in the fitting. The component spectra were recovered through \( F_\lambda = f_1(\lambda)f_1(\lambda) \). The goodness of the fit was evaluated by the \( \chi^2 \) and the randomness of the residual distribution. The \( \chi^2 \) was calculated from Equation 3,

\[ \chi^2 = \sum \frac{[F_{\lambda m} - \left( \frac{F_{\lambda0}}{F_{\lambda0}} \right)]^2}{m} \]  

where the summation is over all of the quencher concentrations and all of the wavelengths (370–450 nm) in the measurement; the subscript \( m \) and \( t \) refer to measured and theoretical values from the two-component equation. For these determinations, the relative errors were 5% or less.

RESULTS

The focus of this work was to examine the behavior of the subdomain 3/4 plug during polymerization by labeling each of the positions with a fluorescent probe and assessing the effects of polymerization on probe fluorescence. Cells carrying each of the desired mutant actins were viable, and active actin was successfully obtained in each case. However, to properly interpret the results of our experiments, it was first necessary to gauge the effects of the mutations in vitro and in vivo in terms of actin function.

Effect of the Mutations in Vitro—We observed no adverse effects of the mutations in vitro in terms of growth rate, temperature dependence, or utilization of glucose and glycerol as carbon sources. Cellular morphological parameters such as vacuole inheritance, cytoskeletal arrangement, and cell morphology appeared normal as well.

Polymerization of Mutant Actins in Vitro—UV absorption results indicated a pyrene maleimide molar labeling ratio for WT, V266C/C374A, L267C/C374A, and L268C/C374A of 1:1, whereas the labeling ratio for the single mutants was approximately 2 mol of dye/mol of actin. The labeling efficiency ranged from 80 to 100%, suggesting that all of the newly introduced sulphydryl residues were accessible. We previously demonstrated that elimination of Cys374 from WT actin prevented labeling by pyrene maleimide (14). Therefore, attempts to label loop cysteines should not result in labeling at alternative sites.

We monitored the polymerization of the calcium form of the labeled actins at 25 °C by light scattering (data not shown). Light scattering showed no consistent differences between the wild-type and mutant actins, although small quantitative differences were observed from preparation to preparation. Thus, surprisingly in the context of the Holmes’ model, labeling did not significantly affect the polymerization of actin, although the probes were at or near the interstrand surface. Electron microscopy of negatively stained pyrene-labeled V266C/C374A, L267C/C374A, and L269C/C374A F-actins demonstrated the appearance of normal looking filaments (data not shown).

At 4 °C, the extent of polymerization of V266C/C374A, pyrene-labeled or not, was 30% less than that of WT actin, whereas neither labeled nor unlabeled L269C/C374A actin polymerized at 4 °C. V266C, L267C/C374A, and WT actins were not cold-sensitive. This cold sensitivity, regardless of whether or not the actin was pyrene-labeled, suggested that this behavior did not arise from the presence of free sulphydryls but from the absence of the original residues. The thermal stabilities of pyrene-labeled V266C/C374A, L267C/C374A, and L269C/C374A were determined using circular dichroism at 222 nm as a function of temperature (data not shown), and they were not significantly different from that of pyrene-labeled WT actin. This result suggested that despite the presence of an N-pyrenyl-succinimidyl group, the actin monomer structure was not grossly affected.

Fluorometric Behavior of the Loop Probes Labeled with Pyrene—We next examined the effects of polymerization on the fluorescence properties of the pyrene-labeled actins to determine whether polymerization caused a change in environment of the loop probe. The original hypothesis of Holmes involving movement of this loop predicted a change in environment for the three “plug” hydrophobic residues. We wished to determine whether this change occurred and, if so, to what extent the behavior of the probe would be uniform across the plug.

Position 266—The emission spectrum of pyrene at position 266 (Fig. 2B) behaves similarly to that of the probe at 265 (Fig. 2A), just outside the plug region. V266C/C374A G-actin shows the same two peaks as S265C/C374A at 385 and 405 nm and a shoulder at 429 nm when excited at 365 nm. Furthermore, like the case for 265 (Fig. 2A), the fluorescence intensities of the 266 peaks (Fig. 2B) decrease by 50% upon polymerization with no change in spectrum, suggesting that the probe is probably being pushed into a more polar or more solvent-exposed environment away from the “plug” so as not to disturb the hydrophobic “plug-pocket” interaction (14). Replacement of Ca2+ with Mg2+ had no effect on the fluorescence of the fluorophore at position 266 (data not shown).

To further assess the polymerization-related changes on the environment of the loop probes, we examined the effects of the small neutral collisional quencher acrylamide on pyrene fluorescence in both labeled G- and F-actin. Fluorescence quenching evaluates the degree of exposure of a fluorophore to the solvent and is characterized by the Stern-Volmer constant \( K_{SV} \). A change in the degree of protection against the quencher between the G- and F-actin states would indicate a change in exposure of the probe to solvent acrylamide (20). When pyrene is in a more protected environment, acrylamide will be prevented from colliding with the fluorophore, and \( K_{SV} \) will be lower. In previous studies, acrylamide produced minimal per-
turbation of muscle actin, demonstrating that changes in acrylamide quenching did not result from acrylamide-dependent opening of the actin (22).

Upon the addition of the quencher to V266C/C374A actin, the Stern-Volmer plot curves downward in both G- and F-actin states, indicating the presence of different conformational states (Fig. 3B). In G-actin, there are two populations; 70% is very buried, since the $K_{SV}$ of that population is 0, and the other 30% is very exposed, as evidenced by a high $K_{SV}$ of 12.8 (Table I). Upon polymerization, the buried population becomes much smaller and becomes more exposed, since it now has a $K_{SV}$ of 1.1. The exposed population increases (64%) with a high $K_{SV}$ of 10.8. Thus, the decrease in fluorescence upon polymerization is reflected in increased quenching constants, supporting the idea that the pyrene is in a more solvent-exposed environment upon polymerization.

The similarity in emission spectra of both V266C/C374A and S265C/C374A in both G- and F-actin states suggested that the probes at 265 and 266 might be in similar environments. Therefore, we also investigated the quenching behavior of the probe at position 267. The Stern-Volmer plots curve downward again for both G- and F-actins (Fig. 3A), suggesting the presence of the fluorophore in heterogeneous environments in both states. NLLS fitting showed that in G-actin the exposed component, although small (17%), has a high $K_{SV}$ of 9.8, whereas 83% of the fluorescence is buried and has a small $K_{SV}$ (Table I).

Position 267—In G-actin, the probe at 267 shows a 9-nm blue shift of the 385 nm to 376 nm as well as the resolution of the 385-nm shoulder into a distinct peak at 385 nm (Fig 2C). Polymerization results in a 30% decrease in fluorescence intensity when the probe is at position 267 (Fig. 2C), suggesting again that the probe at 267, like that at 266, is moving into a more polar or more exposed environment. L267C/C374A actin (Fig 3C) exhibits linear Stern-Volmer plots in both G- and F-actin states consistent with a dynamic quenching mechanism for acrylamide. The quenching curves were easily fitted by a Stern-Volmer equation, suggesting that at position 267, the fluorophor resides in a predominantly uniform, although not necessarily identical, environment in both G- and F-actin. The Stern-Volmer quenching constant is very small, and it doubled after polymerization (from 0.26 to 0.52 M$^{-1}$) (Table I). The increase in accessibility correlates well with the decrease in emission upon polymerization. The relatively small quenching constants in both G- and F-actin states for the 267 probe suggest that in both states the probe at this particular residue is largely buried and shielded from the quencher.

Position 269—Pyrene-labeled L269C/C374A G-actin (Fig. 2D) shows fluorescence peaks at 375, 385, 395, and 405 nm at 25 °C. In the polymerized state, the fluorescence intensity of the 375- and 395-nm peaks are greatly enhanced (76 and 51%, respectively) over that of background fluorescence in G-actin, and the peaks at 385 and 405 nm are enhanced by 20%. Upon the addition of the quencher, the 385- and 405-nm peaks are more quenched than the 375- and 395-nm peaks. The $K_{SV}$ values are larger at 385/405 than at 375/395 nm. The Stern-Volmer plot shows a downward curvature (Fig 3D).
This downward curvature can be explained in terms of heterogeneous populations of the fluorophor, which differ significantly in their individual exposure to the quencher. Assuming there are two major components and each of them has a unique emission spectrum and $K_{SV}$, NLLS fitting for the 269 probe reveals that both G- and F-actin states show a more exposed component and a more buried component. The component spectra are similar going from G-actin (Fig. 4A) to F-actin (Fig. 4C); however, the ratio of the two states changes during the transition. The more exposed component emits predominantly at 385 and 405, whereas the more buried component emits at 375 and 395 nm. The $K_{SV}$ for the buried component is 0.44 M$^{-1}$ (G-actin) and 0.75 M$^{-1}$ (F-actin), whereas the $K_{SV}$ for the more exposed component is 3.8 M$^{-1}$ (G-actin) and 5.1 M$^{-1}$ (F-actin). The population size of the two components changes from G- to F-actin. The exposed component drops from 34 to 16%, whereas...
the buried component increases from 66 to 84%. In summary, from G- to F-actin, a greater fraction of the fluorophor changes to a more buried state, which becomes resistant to quencher.

Kasha's rule (21) states that the emission spectrum should be the same irrespective of the excitation wavelength for a probe in a homogeneous environment. However, except for that at 267, the pyrene emission spectra of probes at all other positions in the loop that we examined are different at two different excitation wavelengths, 344 and 365 nm. An example of this behavior is seen when comparing probes at positions 266 (Fig. 5, A and B) and 267 (Fig. 5, C and D). In our case, this unusual behavior cannot be explained by covalent attachment of the label to multiple sites on the protein based on our observations with C374A actin discussed earlier (14). The uniform spectra observed at position 267 irrespective of the excitation wavelength indicate a single population of probe conformations in both G- and F-actin.

**Positional Dependence of Fluorescence Intensities**—When comparing the fluorescence spectra for the pyrene at each of the three positions, it is apparent that the fluorescence of the 266 probe is much greater than that at 267 or 269. To better quantify this difference, we integrated the fluorescence for each curve in G-actin (Fig. 2) between 370 and 500 nm with the following results expressed in arbitrary units: 266, 3.60 × 10^8; 269, 1.19 × 10^8. It was possible that this difference was due to differences in the degree of labeling, which were based on a single value for the molar extinction coefficient for the pyrene at 344 nm. We therefore assessed the absorption spectrum of the probe at positions 266 and 269. The results, shown in Fig. 6, indicate that between 280 and 340 nm, the spectra are qualitatively the same but quantitatively different. However, at 290 nm, the wavelength used for quantitation of the actin, and at 344 nm, the wavelength used to quantitate pyrene labeling, the absorption of the pyrene probes for 266 and 269 is identical. Thus, our determination that labeling occurs to the same extent for each of the probe positions is valid. However, for reasons we cannot explain, between 350 and 400 nm, the 266 spectrum showed a distinct bulge, which was not present for probes at positions 267, 269, and 374. This increased absorption for the range encompassing 365 nm, the wavelength used for our fluorescence studies, may have been the predominant reason for the enhanced fluorescence observed for the 266 probe in comparison with the other positions studied.

**Correlation between Fluorescence Change and Polymerization**—We next assessed the temporal relationship between polymerization and fluorescence change for each of the actins. The actins were excited at 365 nm, but different emission wavelengths were used depending on the actin, as their maximal emission wavelengths differed: 385 nm for V266C/C374A, 376 nm for L267C/C374A, and 397 nm for L269C/C374A. Fig. 7 shows that in each case, the net change in fluorescence is coincidental with the change in light scattering.

**Fluorometric Behavior of Acrylodan-labeled Actins**—To determine whether the changes seen in the emission spectra were probe-specific, we used another fluorescent molecule, acrylodan, to label the two loop positions. Pyrene maleimide, with a molecular weight of 297, is a bulky moiety of 9 Å in length and a hydrophobic equivalent to two leucine residues. Acrylodan (M_r 225), is a much smaller (3 Å) and less apolar probe. When attached to Cys^374_ of muscle actin, its fluorescence increases 40% following polymerization, and the emission maximum shifts from 492 to 465 nm (17). This result suggests that polymerization causes this probe to enter a more hydrophobic or less exposed environment consistent with data obtained using pyrenyl-actin.

When acrylodan is attached at position 266 in V266C/C374A actin, we observe a fluorescence spectrum with an emission maximum at 513 nm in both G- and F-actins. However, the fluorescence intensity drops by 11% upon polymerization (Fig. 8A), a result consistent with the pyrenyl-actin data. Acrylodan attached to position 269 also exhibits an emission maximum at 513 nm. However, contrary to the case for the label at 266, polymerization caused an 11% increase in fluorescence (Fig. 8B), again in agreement with our pyrene experiments. For most cases involving acrylodan, there is not only a change in the magnitude of the emission maximum but also in its position. Thus, the behavior of the probe on the loop of actin is unusual, although there has been at least one instance (23) in which a change in position did not accompany a change in peak magnitude.

**Excimer Fluorescence**—Previously, Feng et al. (14) observed an excimer peak between 450 and 550 nm with pyrene-labeled S265C actin, in which label was attached to both Cys^265_ and Cys^374_. This excimer formation indicated that the 374 probe on one monomer could interact physically with a 265 probe on another monomer in the opposing strand in the interstrand space within the actin filament to generate an interstrand stacking interaction. For these probes to physically stack in...
this manner, the sulfurs to which they are attached have to be within 18–20 Å of one another.

We observed a similar excimer peak with polymerized V266C actin (Fig. 9, compare A and B) or in a co-polymerized sample of labeled WT and V266C/C374A actin (data not shown), demonstrating the ability of a probe at 266 to form an interstrand interaction with one at Cys374 as well. The amplitude of the excimer peak with V266C actin is similar to that observed with S265C actin. The geometry of the filament is such that the excimer can only form if residue 266 is at or near the parked position and not in the fully extended position as suggested by the Holmes’ model. To further evaluate the significance of the excimer peaks obtained with probes at residues 265 and 266, we measured the excitation spectra of the pyrene-labeled S265C/C374A and V266C/C374A actins (Fig. 10). The amplitude of the excitation spectrum of V266C/C374A at 365 nm (line 2) is greater than that of S265C/C374A (line 1) but the excimer peak is smaller at this excitation wavelength. One might therefore speculate that there was less stacking interaction formed per monomer in V266C/C374A F-actin as compared with S265C/C374A F-actin.

![Figure 5](http://www.jbc.org/)

**FIG. 5.** Steady-state emission spectra of pyrenyl-actins with different excitation wavelengths. A, emission spectra of V266C/C374A using 344 nm as the excitation wavelength; B, emission spectra of V266C/C374A using 365 nm as the excitation wavelength; C, emission spectra of L267C/C374A using 344 nm as the excitation wavelength; D, emission spectra of L267C/C374A using 365 nm as the excitation wavelength. All actins were used at a concentration of 4.6 μM. Curve 1, G-actin; Curve 2, F-actin. The data are representative examples of results obtained with three independent actin preparations.

![Figure 6](http://www.jbc.org/)

**FIG. 6.** Comparison of absorption spectra of pyrenyl-actins. The experiments were done at 4.6 μM actin. Curve 1, V266C/C374A; curve 2, L269C/C374A. The arrows indicate the 290- and 344-nm positions, where the absorbance was identical. The absorbance at 344 nm was used for quantitating the extent of labeling.
with S265C/C374A F-actin. However, since we do not know what the excimer intensity would be for each position if 100% of the probe were involved, such a quantitative comparison is not possible. No excimer peak was observed with doubly labeled actins, "L267C (Fig. 9C) and L269C (Fig. 9D). The pyrene moieties on the neighboring monomers at 267 and 269 are thus not in a position to interact with one at position 374, consistent with the geometric predictions of the Holmes filament model whether or not the loop is extended.

**Effect of Myosin S1 Actin Interaction on Actin Pyrene Fluorescence**—Both spectroscopy and microscopy have revealed significant effects of myosin binding on the structure and dynamics of actin (18, 24, 25). The binding of S1 to the pyrene-labeled F-actins increased the fluorescence of all three loop pyrenes by
A similar increase in fluorescence was observed for the probe at 265 (14). This result is in contrast to the quenching of fluorescence of the C-terminal probe in labeled WT F-actin. Thus, myosin S1 binding to the exterior of the filament propagates a change in the interior of the filament that appears to cause all of the probes in the plug as well as the adjacent 265 probe to either move into a more hydrophobic environment or become less exposed to solvent (14).

**DISCUSSION**

A major part of the Holmes model for actin filament formation involves the repositioning of a subdomain 3/4 loop with a hydrophobic plug away from the surface of an actin in one strand so that it can interact with a hydrophobic surface on the opposing strand. Although, as detailed earlier, our previous work is consistent with the Holmes plug-pocket hypothesis, direct movement of the plug following polymerization has not been ascertained.

The experiments we describe here represent the first attempt to examine the effects of polymerization on the behavior of the actual plug residues. Except for the cold sensitivity of polymerization associated with two of the mutants, the cysteines introduced into the three plug positions appear to produce no adverse effects on actin polymerization at room temperature, in agreement with our previous studies.

The three loop cysteines in G-actin are as reactive toward pyrene maleimide as is the reactive cysteine at position 374. More interesting is the fact that the presence of the bulky probe at any of the three loop positions does not inhibit actin polymerization. This result is contrary to what might have been expected based on the original Holmes proposal of a tight association between an extended loop and a cross-strand hydrophobic pocket.

Our results clearly demonstrate that polymerization results in a change in environment of each of the three plug probes that might not be expected if the loop remained parked alongside the actin in the filament as it is in G-actin. At each of the positions, we observe a tight correlation between the change in

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**Fig. 9. Steady-state emission spectra of doubly labeled pyrenyl-actins.** Conditions are as described in the legend to Fig. 1. These actins (4.6 μM) have been labeled at both the loop position and Cys<sup>374</sup>. Curve 1, G-actin; curve 2, F-actin. A, S<sub>265</sub>C; B, V<sub>266</sub>C; C, L<sub>267</sub>C; D, L<sub>269</sub>C. The data are representative examples of results obtained with two independent actin preparations.

**Fig. 10. Steady-state excitation spectra of pyrenyl-actins.** Excitation spectra of the singly labeled actins were obtained with an emission wavelength of 385 nm. Curve 1, S<sub>265</sub>C/C<sub>374</sub>A; curve 2, V<sub>266</sub>C/C<sub>374</sub>A. 4.6 μM actin was used for these experiments. The data are representative examples of results obtained with two independent actin preparations.
fluorescence of the probe and increase in light scattering during actin polymerization. Coupled with our pyrene quenching and acrylodan data, these results suggest a close temporal relationship between alterations in the loop region of actin and its polymerization.

More striking is the position-dependent heterogeneity in fluorescence exhibited by these probes in both the G and F states. An apparent demarcation between 266 and 267 is observed following polymerization. The magnitude of the decrease in fluorescence of the 266 probe is much like that we had observed at 265, consistent with it being forced into the interstrand space by a polymerization-induced conformational change. On the other hand, there was a somewhat smaller decrease in fluorescence at 267 and an increase at 269, suggesting that these two probes ended up in a less hydrophilic or less solvent-exposed environment relative to their G-actin positions than do 265 and 266. The agreement of the direction of polymerization-induced change in acrylodan and pyrene fluorescence at positions 266 and 269 demonstrates that these changes reflect the general behavior of the loop and not specific behavior of the pyrene. The smaller magnitude of the acrylodan fluorescence change, in comparison with that of pyrene, may indicate that the smaller and more charged acrylodan requires less of a change in environment than pyrene to be accommodated in the interstrand space following polymerization.

The observation of a pyrene excimer from the interaction of pyrenes at 265 or 266 with a pyrene at 374 on an adjacent monomer in the opposing strand is very revealing. Based on the size of the probes and the requirement for an overlap of the two pyrenes to generate an excimer band, the sulfurs to which the probes were attached had to be about 20 Å apart with the two probes pointing toward each other through unobstructed space. We had previously demonstrated with pyrene-labeled S265C actin that the sulfurs to which the probes were attached were 25 Å apart based on the positioning of the C-terminal peptide, which was ill defined in the original crystal structure. The well documented ability of this peptide to move toward the center of the filament (26) must close the intersulfur distance sufficiently for the excimer to form. For V266C actin, in the context of the Holmes model, if the loop remained in the parked conformation, the two sulfurs to which the probes are attached would be separated by about 20 Å, and the probes would be in the proper orientation for excimer formation with ample room in the interstrand space to accommodate the stacked probes. The loop cannot be fully extended with the probe in the pocket defined by the Holmes model, because there is insufficient room to accommodate the bulky succinimidyl pyrene. However, rotation of the probe 120° away from but parallel to the plug tip would place the probe in a more open area that could possibly accommodate its size. In this case, though, the probe would be orthogonal to the 374 probe with mass from the actin between the two probes preventing excimer formation. Thus, the detection of an excimer at 266 in the F-actin precludes all of the subdomain 3/4 loops in the filament from being in a completely extended state and eliminates the requirement for complete loop extension for stable filament formation. However, it does not disallow such extension, especially with respect to the unmodified loop.

It is possible that in unmodified actin, the plug is equilibrating between a fully extended and parked conformation. A possible alternative explanation for an excimer involving 266 is that there is detachment and partial extension of the plug such
that the section involving the residue is still raised, permitting interaction of the 266 and 374 probes. These results also eliminate the need for an alternative cross-strand hydrophobic interaction for a loop probe outside of the “pocket” in order to allow filament formation to occur. Dynamic modeling studies (8) have also predicted this type of loop flexibility. Our previous mutational analyses suggest that involvement of only two of the three hydrophobic residues at any one time is required for filament stabilization, in agreement with this hypothesis (13). An explanation for the nonlinear Stern-Volmer plots for probes at 265, 266, and 269 is that polymerization causes the probe to detach, whereas in another the loop is detached and extended to some degree. Again, this behavior is consistent with the type of loop flexibility predicted by the modeling studies of Lorenz et al. (6) and Wriggers and Schulten (8). The degree of quenching is dependent on the accessibility of the fluorophor to the quencher. If the pyrenyl moiety is shielded by the surrounding protein matrix, the accessibility to the quencher is reduced. The easily accessible fluorophor population is easily quenched at the lower concentrations of acrylamide, and the fluorescence intensity is dominated by the less accessible fractions at the higher concentrations of acrylamide.

The quenching of fluorescence of the probe at residue 267 in F-actin is greater than in G-actin. However, the Ksv values for L267C/C374A actin in both G and F states are very low, suggesting that in both states, the probe at 267 is being shielded substantially from the quencher by the protein matrix itself. In summary, our loop fluorescence and quenching data together suggest that polymerization leads to detachment of the loop from the actin monomer body with the different residues of the plug behaving in a very heterogeneous manner, including the assumption of multiple conformations at the same residue. Such behavior was predicted on the basis of dynamic modeling studies and correlates with the results of our earlier mutagenesis studies of this region of the protein. The ability of myosin S1, which binds to the outside of the filament on the surface of subdomain 1 and 2, to affect the fluorescence of these loop probes in the interior of the filament is further evidence for this proposed loop flexibility and suggests that loop rearrangement may occur during actomyosin-dependent contraction. Finally, experiments carried out by Shvetsov and Reisler in conjunction with us2 show that polymerization is prevented by covalently tethering the loop to the surface of the actin monomer, thus supporting the need for loop separation from the actin body surface.

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Additions and Corrections

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Pro-survival function of Akt/protein kinase B in prostate cancer cells. Relationship with TRAIL resistance.

Hitesh Thakkar, Xufeng Chen, Frazier Tyan, Suzanna Gim, Heather Robinson, Calvin Lee, Sanjay K. Pandey, Chichi Nwokorie, Nneka Onwudiwe, and Rakesh K. Srivastava

The author has retracted this paper because of substantial overlap with Chen et al. (2001) Oncogene 20, 6073–6083.

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Effect of polymerization on the subdomain 3/4 loop of yeast actin.

Runa Musib, Gufeng Wang, Lei Geng, and Peter A. Rubenstein

Page 22704, Fig. 4: Line 3 is missing from panels A and C. The corrected figure is shown below.

FIG. 4. Spectral components of pyrene-labeled L269C/C374A actin obtained from NLLS fitting. A, emission spectra of 9.3 μM G-actin; B, residual plot of the NLLS fitting of the G-actin data; C, emission spectra of 9.3 μM F-actin; D, residual plot of the NLLS fitting of the F-actin data. Line 1, total fluorescence; line 2, fluorescence of the buried component; line 3, fluorescence of the exposed component.

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