Residues 262–274 form a loop between subdomains 3 and 4 of actin. This loop may play an important role in actin filament formation and stabilization. To assess directly the behavior of this loop, we mutated Ser\(^{265}\) of yeast actin to cysteine (S265C) and created another mutant (S265C/C374A) by changing Cys\(^{374}\) of S265C actin to alanine. These changes allowed us to attach a pyrene maleimide stoichiometrically to either Cys\(^{374}\) or Cys\(^{265}\). These mutations had no detectable effects on the proteinase susceptibility, intrinsic ATPase activity, and thermal stability of labeled or unlabeled G-actin. The presence of the loop cysteine, either labeled or unlabeled, did not affect the actin-activated S1 ATPase activity or the in vitro motility of the actin. Both mutant actins, either labeled or unlabeled, nucleated filament formation considerably faster than wild-type (WT) actin, although the critical concentration was not affected. Whereas the fluorescence of the C-terminal (WT) probe increased during polymerization, that of the loop (S265C/C374A) probe decreased, and the fluorescence of the doubly labeled actin (S265C) was ~50% less than the sum of the fluorescence of the individual fluorophores. Quenching was also observed in copolymers of labeled WT and S265C/C374A actins. An excimer peak was present in the emission spectrum of labeled S265C F-actin and in the labeled S265C/C374A-WT actin copolymers. These results show that in the filaments, the C-terminal pyrene of a substantial fraction of monomers directly interacts with the loop pyrene of neighboring monomers, bringing the two cysteine sulfurs to within 18 Å of one another. Finally, when bound to labeled S265C/C374A F-actin, myosin S1, but not tropomyosin, caused an increase in fluorescence of the loop probe. Both proteins had no effect on excimer fluorescence. These results help establish the orientation of monomers in F-actin and show that the binding of S1 to actin subdomains 1 and 2 affects the environment of the loop between subdomains 3 and 4.

The crystal structure of the actin monomer has been elucidated as part of a 1:1 complex with three actin-binding proteins (1–3). However, the structure of the two-stranded actin filament at atomic resolution has not been determined due to the inability to date to crystallize F-actin. One F-actin model, proposed by Schutt \textit{et al.} (2, 4), is based on the profilin/β-actin ribbon structure in which actin monomers contact each other in a continuous fashion, with profilin molecules bridging between the actins on the outside of the structure. In this structure, actin subdomains 1 and 2 are near the center of the ribbon, whereas the subdomain 3/4 interface is near its exterior. Schutt \textit{et al.} have proposed that the ribbon can be transformed into a classical ADP-containing helical filament by a compression and a twist. However, coordinates of this filament model have not yet been published.

Holmes \textit{et al.} (5) have generated an alternative model based on fitting the coordinates of the monomer into a density map generated from low angle x-ray diffraction studies of oriented actin gels. This model has been further refined by Lorenz \textit{et al.} (6) and by Tirion \textit{et al.} (7). In this model, subdomains 1 and 2 are on the exterior of the filament, and the subdomain 3/4 interface is in its interior. In the G-actin structure, a loop consisting of residues 262–274, located between subdomains 3 and 4, is parked against the surface of subdomain 4. At the tip of this loop is a four-residue plug consisting of three hydrophobic amino acids and a glycine. Holmes \textit{et al.} (5) hypothesized that this loop rearranges during polymerization so that it points away from the surface of the protein in a direction perpendicular to the filament axis. The hydrophobic plug at the tip of the loop can now insert into a hydrophobic pocket formed by the interface of two monomers on the opposing strand of the helix, leading to an appreciable increase in the cross-strand stabilization available for the actin filament. Good agreement exists between this model and the results of low resolution structural studies (8–10), protein chemical studies (11), and antibody decoration studies (12–13). However, the model remains in large part unproven. At the existing level of resolution, the proposed interstrand contacts cannot be observed, and no direct experimental evidence exists for this conformational change involving the hydrophobic loop in actin polymerization.

A number of groups have previously studied the dynamics of actin polymerization by attaching fluorescent reporter groups to specific sites on Cys\(^{374}\) near the C terminus in subdomain 1 and on Gln\(^{11}\) near the top of subdomain 2 (14–16). However, no such studies have been performed with a reporter attached to subdomain 3 or 4 or to the interdomain loop due to the unavailability of a convenient site to attach the probe. Thus, the behavior of this part of the protein during polymerization is unknown.

The amino acid sequence of \textit{Saccharomyces cerevisiae} actin is 87% identical to that of skeletal muscle actin (17). Furthermore, it is easy to generate directed mutations in yeast actin,
making it an excellent model system for assessing experimentally the validity of the different actin filament models. In this paper, we have mutated Ser<sup>265</sup> adjacent to the hydrophobic plug, to cysteine (S265C). We then used this actin to generate a second mutant actin, which, in addition to the S265C alteration, contains a C374A mutation (S265C/C374A). Together with wild-type yeast actin, these mutant actins allow us to assess, separately or together, the behavior of the loop and C-terminal cysteines. We have used these actins labeled with pyrene maleimide to assess changes in the local environment of the loop cysteine during polymerization. We have further studied the interaction of this loop probe with the C-terminal domain of a neighboring actin monomer in the filament to gain additional insight into the spatial arrangement of actin monomers in F-actin. Finally, we have assessed the effects of the binding of myosin S1 and tropomyosin to F-actin on the environment of the pyrene attached to the loop.

EXPERIMENTAL PROCEDURES

Materials—The site-directed mutagenesis kit and [γ<sup>32</sup>P]ATP (6000 Ci/mmol) were purchased from Amer sham. Actin was obtained from Worthington. Pyrene maleimide, α-chymotrypsin, trypsin, and subtilisin were purchased from Sigma. The Sequenase Version 2.0 DNA sequencing kit was purchased from U. S. Biochemical Corp. Sphacryl S-200 resin was purchased from Pharmacia Biotech Inc. The BCA assay reagent was purchased from Pierce. The phagemid pRS314 was from Phil Hieter (The Johns Hopkins University, Baltimore, MD). The oligodeoxynucleotides used for site-directed mutagenesis were synthesized in the DNA Core Facility at the University of Iowa (Iowa City, IA). Rabbit actin and myosin were prepared from rabbit skeletal muscle according to the methods of Spudich and Watt (18) and Godfrey and Harrington (19). Myosin subfragment 1 (S1) was prepared according to Weeds and Pope (20). Tropomyosin was prepared from rabbit skeletal muscle according to Smillie (21). The SLM Model 4800 fluorescence spectrometer (SLM-AMINCO, Urbana, IL) used in this work is part of the Protein Structure Facility at the University of Iowa College of Medicine.

Oligonucleotide-directed Mutagenesis and Plasmid Construction—Plasmids were manipulated by procedures described previously. (22). The yeast actin coding sequence and the yeast actin promoter were inserted into the EcoRI and BamHI sites in the polylinker region of plasmid pRS314, which contains a TRP1 marker (23). Single-stranded DNA made from the phagemid with the actin coding sequence was used as a template for site-directed mutagenesis together with the oligodeoxynucleotide primer 5'GTTCCGATCCTGTCTGGTTTTCGAG-3' to generate the S265C mutant actin coding sequence (pRS-SC). The mutated rod was underlined. pRS-SC was used to generate diploid yeast cells carrying both the wild-type and mutant actin genes as well as haploid cells containing the mutant sequence as the only actin coding sequence in the cell (24). Sequencing of the plasmid rescued from the yeast verified that the transformed cells contained the desired mutation.

We next constructed a mutated actin plasmid with the codon for Cys<sup>374</sup> changed to that of Ala (pGEM-CA). The single-stranded DNA template was generated from phagemid pGEM-AC, a derivative of pGEM-3Z<sup>−</sup> (<sup>−</sup>), into which the actin coding sequence was inserted in the polylinker region between the BamHI and EcoRI sites, and the mutant oligodeoxynucleotide was 5'-ATCGTCCAGAGGTCTTATATCTCGT-3'. We next constructed a doubly mutated plasmid containing the S265C and C374A mutations. The 3'-end of the C374A mutant actin coding sequence was excised from plasmid pGEM-CA with EcoRI and KpnI. The excised fragment containing the C374A mutation was subcloned into pRS-SC, which had been pUC-18 digested. The resulting plasmid, pRS-SCCA, was used to generate haploid cells containing the mutant sequence as the only actin coding sequence.

Construction of Yeast Strains and Cell Growth Assay—S. cerevisiae strain TdyDD was a gift of Dr. D. Shortle (The Johns Hopkins University, Baltimore, MD). Rabbit actin and myosin were prepared from rabbit skeletal muscle according to Smillie (21). The SLM Model 4800 fluorescence spectrometer (SLM-AMINCO, Urbana, IL) used in this work is part of the Protein Structure Facility at the University of Iowa College of Medicine.

Electron Microscopy—Actin (5.0 μg) polymerized at 25 °C for 1 h was applied to carbon-coated Formvar grids and visualized following negative staining with 1.5% uranyl acetate and 28% using a Hitachi Model 7000 electron microscope (University of Iowa Central Electron Microscope Facility).

Determination of Critical Concentration—The critical concentration was determined using light scattering as described by Chen et al. (23). G-actin was polymerized at room temperature for 1 h as described above. F-actin was then diluted to different concentrations in Ca<sup>2+</sup>-free buffer (10 mM Tris-HCl, pH 7.5, 0.2 mM CaCl<sub>2</sub>, 0.2 mM ATP, and 0.5 mM dithiothreitol) and retention of the middle fractions of the actin peak. Mg<sup>2+</sup>-G-actin was obtained from the Ca<sup>2+</sup>-G-actin by the procedure of Pollard and Cooper (26). Mg<sup>2+</sup>-G-actin contained 10 mM Tris-HCl, pH 7.5, 0.05 mM CaCl<sub>2</sub>, 0.1 mM MgCl<sub>2</sub>, 0.2 mM EGTA, 0.2 mM ATP, and 0.5 mM dithiothreitol. We obtained only about one-third to one-half the amount of S265C/C374A actin as we did WT or S265C actin, although by Western analysis, the amount of actin/cell weight was about the same in all cases.

Actin Polymerization Assays—Light scattering was measured in an SLM Model 4800 fluorometer set at 380 nm for both the excitation and emission wavelengths. After isolation, actins were further purified by Sephacryl S-200 gel filtration column chromatography to remove pre-existing nuclei. After equilibration at the desired temperature with 15 min in a thermostatted cuvette chamber, actin polymerization was initiated by adding KCl and MgCl<sub>2</sub> to final concentrations of 50 and 2 mM, respectively (27). The change in light scattering was recorded as a function of time.

Fluorescence Spectroscopy of Pyrene-labeled Wild-type and Mutant Yeast Actins—The change in pyrene fluorescence following actin polymerization was observed using either an SLM Model 4800 fluorometer or a Spex fluorolog set at an excitation wavelength of 365 nm, an emission wavelength of 386 nm, and a slit width of 1 mm. Pyrene-labeled actin

1 The abbreviations used are: WT, wild-type; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.
and one on Cys374 near the C terminus. To study the loop site on the loop for a reporter group. This actin should have between 0.2 and 3.0 S265C actins was measured at 25 °C in a buffer containing 50 mM KCl, smoothly in the assay system. At least 150 filaments were analyzed for were used for statistical analysis (36) and were considered to move with standard deviations of less than one-third of the average velocity Analysis Inc., Santa Rosa, CA). The velocities of individual filaments and glucose oxidase as an oxygen-scavenging system. Quantification of phosphate was removed from actin stock solutions as described previously (34).

Effect on the Growth of Yeast Cells—To assess polymerization-dependent changes in the environment of the subdomain 3/4 conserved Ser265 was mutated to Cys to provide an attachment or Mg" form as measured as described by Chen et al. (23). Susceptibility of G-actin to digestion by α-chymotrypsin, trypsin, and subtilisin was determined according to Fievez and Carlier (32). Thermal denaturation of G-actin was monitored by following the change in circular dichroism at 222 nm as a function of temperature according to Chen et al. (23).

Actin-activated S1 ATPase Assays—Actin-activated S1 ATPase activity was measured at 25 °C in a buffer containing 5 mM KCl, 2 mM MgCl2, and 10 mM imidazole HCl, pH 7.4, using the malachite green assay (33). S1 concentration in these assays was 0.4 μM. Pyrene maleimide-labeled and unlabeled WT, S265C, and S265C/C374A actins were used at concentrations of 10 and 20 μM. Due to assay sensitivity, free phosphate was removed from actin stock solutions as described previously (34).

In Vitro Motility Assays—Assays of the ability of phallolidin-stabilized actin filaments to translocate over a heavy meromyosin-coated surface in the presence of ATP were performed as described previously (35) at 25 °C. The assay buffer contained 25 mM KCl, 1.0 mM EGTA, 4.0 mM MgCl2, 10 mM dithiothreitol, 10 mM imidazole, pH 7.4, 1.0 mM ATP, and glucose oxidase as an oxygen-scavenging system. Quantification of the sliding velocities was done with an Expertvision system (Motion Analysis Inc., Santa Rosa, CA). The velocities of individual filaments with standard deviations of less than one-third of the average velocity were used for statistical analysis (36) and were considered to move smoothly in the assay system. At least 150 filaments were analyzed for each sample.

Binding of S1 to Actin—The binding of S1 to pyrene-labeled WT and S265C actins was measured at 25 °C in a buffer containing 50 mM KCl, 2 mM MgCl2, and 5.0 mM Tris-HCl, pH 7.8. The labeled actin filaments (1.0 μM) were stabilized with phallolidin and then titrated with S1 (between 0.2 and 3.0 μM). The binding was monitored as described by Reeves and Jeffries (37) via quenching of pyrene fluorescence of the label attached to Cys747 in both WT and S265C actins.

RESULTS

The S265C and S265C/C374A Mutant Actins Have Little Effect on the Growth of Yeast Cells—To assess polymerization-dependent changes in the environment of the subdomain 3/4 loop, we generated two mutant actins. In S265C actin, the conserved Ser265 was mutated to Cys to provide an attachment site on the loop for a reporter group. This actin should have two accessible sulfhydryl residues, one on the loop and one on Cys747 near the C terminus. To study the loop cysteine alone, we mutated Cys747 of S265C actin to Ala and generated S265C/C374A actin. Haploid Td5SC and Td5SCCA cells, containing the mutant actins as the only actins in the cell, grew as well on YPD plates at 10, 30, and 37 °C as did the wild-type cells. No significant differences were found in the generation times of the S265C, S265C/C374A, and wild-type cells at the temperatures tested (data not shown). On solid hyperosmolar (0.9 mM NaCl) medium, the mutant cells grew slightly faster initially than WT cells. However, in liquid hyperosmolar medium, both mutant cells grew at rates similar to that of WT cells after a 1–1.5-h lag period (data not shown). The growth of mutant and WT cells was identical at 30 °C in medium containing 2% ethylene glycol as a sole carbon source (data not shown), indicating that the inheritance of mitochondria in both S265C and S265C/C374A cells is normal.

Purification and Characterization of Mutant Actins—To assess the effects of the S265C mutation on actin polymerization, we monitored by light scattering the polymerization kinetics of purified, gel-filtered S265C and S265C/C374A actins. Fig. 1 A shows that in the presence of 50 mM KCl and 2 mM MgCl2, mutant actins polymerized more rapidly than wild-type actin at 30 °C, but the extent of polymerization for all actins was the same. This result is consistent with the fact that the critical concentrations of mutant and WT actins were identical when determined by examining the extent of depolymerization at various concentrations of F-actin (data not shown). Although the main factor in the acceleration of polymerization of mutant actins appeared to be a shortened nucleation phase, the reason for this enhanced nucleation was not apparent. Electron microscopy of negatively stained S265C and S265C/C374A F-actins demonstrated the appearance of normal-looking filaments.

The increased rate of polymerization caused by the S265C mutation might arise from a localized conformational change in the loop or from a more general conformational change affecting multiple subdomains of the monomer. However, the intrinsic ATPase activity; the susceptibility to limited proteolysis by trypsin, subtilisin, and α-chymotrypsin; and the thermal stability of S265C and S265C/C374A G-actins were essentially the same as those of WT actin. Thus, the mutations appear to cause no gross conformational change in actin structure.

Pyrene Labeling of Mutant and WT Actins—To further study...
the role of the putative hydrophobic loop in actin polymerization (5), we labeled S265C and S265C/C374A actins with pyrene maleimide and studied their biochemical properties. UV absorption showed a molar labeling ratio of 2:1 for S265C actin and 1:1 for S265C/C374A and WT actins. C374A actin showed no labeling with coumarin maleimide and pyrene maleimide, indicating that only the sulfhydryls at positions 265 and 374, when present, were labeled (38). Therefore, WT actin was labeled at Cys$^{265}$ (tail-labeled), S265C actin was labeled at Cys$^{265}$ and Cys$^{374}$ (loop- and tail-labeled), and S265C/C374A actin was labeled only at Cys$^{265}$ (loop-labeled) with pyrene maleimide. Labeling produced little change relative to WT actin or the binding of DNase I to Ca$^{2+}$.

Effect of Polymerization on the Fluorescence of Labeled Ac-tins—Replacement of Ca$^{2+}$ with Mg$^{2+}$ in the nucleotide cleft of actin or the binding of DNase I to Ca$^{2+}$ G-actin had no effect on the fluorescence of labeled S265C/C374A and S265C G-actins. We next examined the fluorescence properties of the pyrene-labeled actins following their polymerization. Fig. 2 shows that as reported previously (14), the fluorescence of WT actin starts at a very low level and increases during polymerization. Surprisingly, the fluorescence of labeled S265C/C374A G-actin was greater than that of WT actin and decreased following polymerization, suggesting that polymerization caused the probe attached to the loop to move to a more hydrophilic environment. The fluorescence of labeled S265C G-actin was equal to the sum of the fluorescence values of equal amounts of WT and S265C/C374A G-actins. However, following polymerization, the final fluorescence of S265C F-actin was decidedly less than the sum of separate and equivalent amounts of labeled WT and S265C/C374A F-actins. This result indicated that in labeled S265C F-actin, the two probes were not acting independently of one another.

There are two possible explanations for the fluorescence quenching observed with labeled S265C F-actin. First, the presence of the probe on the loop could cause a change in the conformation of the actin monomer, resulting in a more hydrophilic environment for the C-terminal probe on the same monomer. Second, the presence of the loop probe on one monomer in the filament directly or indirectly affects the environment of the C-terminal probe on a neighboring monomer. We next performed experiments to distinguish between these possibilities. The pyrene fluorescence increased linearly with the percentage of labeled WT actin and decreased linearly with the percentage of labeled S265C/C374A actin in an otherwise unlabeled actin mixture (Fig. 3). Pyrene-labeled S265C actin behaved differently, however (Fig. 4A). At low percentages of labeled actin, the fluorescence change was equal to that of the sum of separate and equivalent amounts of labeled WT and S265C/C374A actins. However, as the percent of labeled S265C actin approached 50%, the fluorescence increase began to level off. This result suggested that the quenching we observed with labeled S265C F-actin, ~55% of the calculated fluorescence at 100% labeled actin (Fig. 4A), occurred as a result of intermonomer interactions of the pyrene moieties. To confirm this observation, we added increasing amounts of labeled S265C/C374A and WT actins in a 1:1 ratio to a 50:50 mixture of unlabeled S265C/C374A and WT actins (Fig. 4B). Again, at low percentages of labeled actins, the total change equaled the calculated sum of the two separate populations, whereas at higher percentages of labeled actins, we observed fluorescence quenching (up to 40% at full labeling).

Excimer Fluorescence—We next recorded the fluorescence spectra of the three labeled actins between 360 and 600 nm following excitation of the samples at 365 nm. If the quenching observed resulted from a direct interaction of two probes from neighboring monomers, a new excimer peak might be observed (39). Figs. 5 and 6 show that labeled S265C F-actin or a copolymerized mixture of labeled WT and S265C/C374A F-actins exhibited such an excimer peak between 450 and 550 nm, which was not observed when the spectra of WT and S265C/C374A F-actins were taken separately (data not shown). A similar excimer band was observed in copolymers of labeled S265C/C374A actin and rabbit skeletal actin. Formation of this excimer requires that the sulfurs to which the probes are attached in the filament are within 18 Å of one another. We observed no excimer peak in a solution containing a mixture of preformed WT and S265C/C374A F-actins, indicating that the interactions were between monomers in the same filament, not between filaments (data not shown).

No excimer peak was observed with labeled S265C G-actin immediately following chromatography on Sephadex S-200 to remove actin oligomers. However, small amounts of excimer...
appeared in solutions of S265C G-actin 1 or 2 days following gel filtration, and the size of this band increased somewhat over time during the week-long period in which we used the actin following purification (Fig. 5). In labeled samples that were 3 days old, small amounts of this excimer were also seen with pyrene maleimide-labeled S265C/C374A actin alone following polymerization. Thus, labeling of the loop caused a time-dependent conformational change in the actin allowing monomer-monomer interaction in some alternative manner that led to probe stacking. Thus, experiments were performed within 2 days following actin labeling.

Excitation spectra of the pyrene monomer in labeled S265C actin were recorded with the emission monochromator set at $\lambda_{\text{max}}$ for the two monomer bands (386 and 405 nm). At these two settings, the excitation spectra were identical, except for a small difference in fluorescence intensity (Fig. 7). However, when the excitation spectrum of the same actin sample was obtained for the excimer band (the emission monochromator set at 490 nm), the spectrum (solid trace) was clearly different from the above two spectra (Fig. 7). This is consistent with the presence of two ground-state populations of labeled S265C actin, with unstacked and stacked pyrenes, yielding the monomer and excimer emission spectra, respectively (39–40).

Copper Bound to the WT Actin C Terminus Affects the Fluorescence of Pyrene-labeled S265C/C374A Actin in an S265C/C374A-WT Copolymer—Our results showed that the C-terminal probe of one monomer can directly interact with the loop
ATPase as well as WT actin did. A small decrease (between 15% and 20%) in the ATPase activities was observed with actins containing the pyrenyl probe attached to Cys374. This is consistent with the previous report on the effect of pyrene labeling of Cys374 in rabbit actin on the functional properties of skeletal α-actin (41).

The in vitro motilities of the different actins mirrored their behavior in acto-S1 ATPase measurements. The mean sliding speed of actin filaments (3.6 ± 0.3 μm) was reduced by up to 25% only for the actins labeled at Cys374. A similar decrease in sliding speeds was observed before for pyrene-labeled rabbit actin (41). The sliding of other actins, including labeled S265C/C374A and CA actins, was the same as that of WT actin. Thus, mutation of Ser265 to cysteine and the attachment of the pyrene probe to this site appear to have no effect on actomyosin interactions. Labeled S265C actin was more resistant to fragmentation in the motility assays than the other actins. Table I shows the ATPase activation and the sliding data for the various actins examined.

Binding of S1 and Tropomyosin to Actin and Their Effect on Pyrene Fluorescence—Fig. 9A shows the emission spectrum of pyrene-labeled S265C/C374A actin (2.0 μM) in the presence and absence of S1 (8.0 μM). The binding of S1 to actin enhanced the fluorescence of pyrene attached to Cys374 by ~25%. This is in contrast to the well documented quenching of pyrene fluorescence of the probe located at Cys874 in actin. Such a quenching of pyrene fluorescence can be easily seen in Fig. 9B, which shows the spectra of pyrene-labeled S265C actin with and without bound S1. This mutant actin has two pyrene probes attached to it, at Cys874 and Cys265. The quenching of the Cys874 pyrene fluorescence by S1 dominates the overall monomer emission of the two probes on S265C actin. Consequently, despite the enhanced fluorescence from the Cys265 pyrene due to S1 binding (Fig. 9A), the fluorescence intensity of S265C actin decreases by ~60% at 405 nm. Strikingly, the quenching by S1 of monomer pyrene emission in labeled S265C actin is not accompanied by any changes in the excimer spectrum. With our evidence for two labeled S265C actin populations, this result suggests that in S265C actin, stacking of pyrene probes stabilizes the Cys265 region against S1-induced conformational changes.

To verify that the population of pyrene-stacked actin (i.e. the excimer population) binds S1 well, as indicated by the results of our functional assays, we determined the binding constants of
S1 for pyrene-labeled WT and S265C actins. Fig. 10 shows, based on the quenching of pyrene fluorescence at 405 nm by S1, that the binding results can be fitted with curves corresponding to binding constants ($K_b$) of $(2.5 \pm 0.5)$ and $(7.5 \pm 0.9) \times 10^6$ M$^{-1}$ for labeled WT and S265C actins, respectively, and to a stoichiometric binding of S1 to both actins. Thus, S1 has an increased affinity for labeled S265C actin relative to that for pyrene-labeled WT actin.

The addition of tropomyosin (1.0 μM) to pyrene-labeled S265C and S265C/C374A actins (3.0 μM) had no effect on either the monomer or excimer emission spectra of these actins (data not shown). The spectra were recorded under the same conditions as those described in the legend to Fig. 9 except for the addition of 3.0 mM MgCl$_2$. To verify that tropomyosin binds to both actins, the samples were pelleted after the fluorescence measurements, and the resolubilized and denatured pelleted proteins were examined by SDS-polyacrylamide gel electrophoresis. Similar binding of tropomyosin to WT and pyrene-labeled S265C/C374A and S265C actins was observed on these gels.

**DISCUSSION**

To directly assess the behavior of the subdomain 3/4 loop during and following polymerization, we generated two mutant actins containing a cysteine residue in place of the serine at position 265, just adjacent to the hydrophobic plug hypothesized by Holmes et al. (5) to be important for stabilization of the actin helix. This modification allowed the attachment of a fluorescent pyrene moiety and provided the first opportunity to investigate environmental changes involving this part of the protein during polymerization.

*In vitro*, the mutation had little if any observable effect in yeast. It also had little if any effect on a number of properties of G-actin. Although the critical concentrations for polymerization of the wild-type and two mutant actins were essentially the same, the presence of the mutation in the loop resulted in a greatly accelerated nucleation phase during the polymerization process regardless of whether the cysteine was derivatized or not. This is a rare instance in which a mutation, which to our knowledge is not present in another actin, enhances the speed of actin polymerization. The reason for this effect is not clear yet.

The normal appearance of filaments generated with pyrene-labeled S265C and S265C/C374A actins suggests strongly that residue 265 in the loop is not involved directly in the formation of a cross-strand interaction stabilizing the F-actin filament. The presence of a bulky pyrene group would have otherwise blocked such an interaction, preventing filament formation. The lack of disruption by this modification is further demonstrated by the ability to decorate these filaments with myosin S1 and the normal or near-normal ability of these filaments to bind S1, to activate myosin ATPase activity, and to move in the *in vitro* motility assay. The loop pyrene is in a more hydrophobic environment that that on the C terminus of G-actin as demonstrated by the much stronger fluorescence of labeled S265C/C374A G-actin compared with labeled WT G-actin. This fact is consistent with the atomic structure of the actin monomer, in which the loop, and probably the attached probe, is folded against the surface of the protein, while the C terminus is more flexible and solvent-exposed. Replacement of G-actin's Ca$^{2+}$ with Mg$^{2+}$ affects the conformation of several sites on actin (42), but has no apparent effect on the environment of the Cys$^{265}$ pyrene.

According to the model of Holmes et al. (5) for F-actin, there is unoccupied space between the two strands of the helix in F-actin. During polymerization, the loop undergoes a conformational change from its “parked” position against the actin surface and extends away from the protein such that the hydrophobic plug (residues 266–269) at its tip inserts into a...
Hydrophobic pocket formed by two subunits on the opposing strand. In this model, residue 265 serves as a pivot point for the rotation of the plug and does not itself move to any great extent. If such a model is correct, the probe attached to Cys\(^{265}\), which is outside the hydrophobic plug, may reside in a more hydrophilic space in the middle of the F-actin helix (compared with its position in G-actin), while the hydrophobic plug residues are buried in the hydrophobic pocket on the opposing strand. We show that the pyrene fluorescence of labeled S265C/C374A actin decreases after polymerization, suggesting that the loop probe does move into a more hydrophilic environment.

The quenching of pyrene fluorescence observed during the polymerization of labeled S265C actin indicates that the two probes in labeled S265C F-actin are not acting independently. Experiments on mixtures of labeled and unlabeled S265C actins as well as on a 50:50 mixture of labeled S265C/C374A and WT actins suggested that the probes affect one another in an inter- rather than intra-monomer fashion. This conclusion was further confirmed by the appearance of an excimer peak upon the polymerization of either labeled S265C actin or a 50:50 mixture of labeled S265C/C374A and WT actins, even though an excimer was not observed for labeled S265C/C374A or WT actin alone. According to excitation spectra, the formation of this excimer peak is most likely due to a direct interaction of the two fluorophores in the ground state (39). Although a quantitative estimate of the excimer population cannot be made on the basis of our data, the large quenching of pyrene monomer fluorescence of S265C F-actin (and of S265C/C374A and WT actin copolymers) indicates the stacking of at least 50% of the pyrene probes. The stacking of pyrenes suggests that in the filament, the two sulphydryls to which the probes are attached must be able to occupy positions within 18 Å of one another, with no steric hindrance by intervening structures. Furthermore, this proximity is not limited to yeast actin because an excimer band was formed also in copolymers of yeast and rabbit actins. In the model of Holmes et al. (5), the C-terminal cysteine sulphydryl of monomer N is -24 Å from the loop sulphydryl of monomer N+1, in good agreement with our data. A stereo diagram depicting the relationship of these two cysteines in neighboring monomers in the filament model of Holmes et al. is shown in Fig. 11. In the ribbon structure of Schutt et al. (2, 4), a similar interaction would be allowed between the C terminus of monomer N and the loop of monomer N+2 with an inter-sulphydryl distance of 40 Å, which should decrease somewhat following the rotation and compaction necessary to generate a filament, although the exact distance cannot be ascertained since no published coordinates exist for this filament model.

Our study indicates that there is likely to be substantial communication between the C-terminal Cys\(^{274}\) and residue 265 in the loop, even when the C-terminal cysteine is not derivatized by a 9-Å-long adduct. The binding of copper to the C-terminal cysteine on WT actin decreased the fluorescence of the probe on the loop of S265C/C374A actin in a copolymer of WT and labeled S265C/C374A actins. This result suggests that the binding of copper, instead of a large fluorescent probe, to subdomain 1 of one monomer affects the environment of the loop on a neighboring monomer in one of two ways. First, this change could occur if the binding of the copper traps the C-terminal peptide at a position farther away from the loop nearer the outside of the filament, thereby reducing the amount of hydrophobic mass in the vicinity of the loop. Alternatively, the hydrophilicity of the loop environment may have been increased by the placement of the C-terminal bound cop-

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**Fig. 10.** Binding of S1 to pyrene-labeled WT and S265C F-actins. The binding of S1 (between 0.2 and 3.0 \(\mu\)M) to labeled WT (○) and labeled S265C (●) F-actins (1.0 \(\mu\)M) stabilized by phalloidin was monitored via quenching of pyrene fluorescence at 405 nm. The excitation wavelength was 365 nm. The binding was measured in the presence of 50 mM KCl, 2.0 mM MgCl\(_2\), and 5.0 mM Tris-HCl, pH 7.8. The calculated curves describe equimolar binding of S1 to labeled WT (\(K_a = 2.5 \times 10^6\) M\(^{-1}\)) and labeled S265C (\(K_a = 7.5 \times 10^6\) M\(^{-1}\)) F-actins.

**Fig. 11.** Stereo diagram, based on the F-actin model of Holmes et al. (5), depicting the relationship between the Cys\(^{274}\) sulphydryl of one actin monomer and the loop sulphydryl on Cys\(^{265}\) of another actin monomer in the filament that allows excimer formation to occur when both residues have been modified with pyrene maleimide. Numbers refer to the positions of selected residues, and the letters following the numbers refer to the identity of the actin monomer in the repeating triplet of monomers that make up the filament. The loop cysteine is labeled as S265 for the residue that occupies that position in wild-type actin. The dashed line shows that the two atoms to which the probes are attached are separated by 23.6 Å, with no intervening mass between them. The diagram was constructed using the coordinates of Lorenz et al. (6).
per near the loop. Although we cannot distinguish between these possibilities, it has been suggested in a related case that the labeling of the C-terminal cysteine may move loop 38–52 in subdomain 2 to a greater radius from the helix axis (38).

The binding of myosin S1 to labeled S265C/C374A F-actin caused an increase in fluorescence of the loop pyrene. In terms of the model of Holmes et al. (5), this result shows for the first time that myosin binding to the outside of the filament results in an alteration of the environment in the filament interior. From the discussion above, this change might result from the effect of S1 on the position of the C-terminal arm in the neighboring monomer. This result is consistent with the hypothesis that actomyosin-dependent contraction may, in part, be dependent on conformational changes in the thin filament, as proposed previously (9–10). The inhibition of pyrene monomer fluorescence by the binding of S1 to labeled S265C F-actin and the lack of any S1 effect on the excimer band can be rationalized by the presence of two populations of actin, as inferred from excitation spectra of pyrene monomer and excimer fluorescence. The stacking of pyrenes in the “excimer population” may stabilize the Cys265 environment and inhibit S1-induced changes at that site. Such a stabilization of S265C F-actin is implied by its decreased fragmentation in the in vitro motility assays. In contrast to S1, tropomyosin affected neither the monomer nor excimer fluorescence of the labeled mutant actins, even though tropomyosin is thought to lie near the groove between the two strands of the actin filament.

The work we describe here establishes the S265C mutation as a valuable system for examining the behavior of the subdomain 3/4 interface. Neither the mutation nor the attachment of pyrene to Cys265 has any significant effect on the main functions of actin, the activation of myosin ATPase, and the in vitro motility of actin. The successful use of pyrene maleimide as a label opens up the possibility of labeling the loop cysteine with smaller spin probes, which will allow us to assess the behavior of this loop under different conditions over a wide range of time scales. Finally, combining the S265C mutation with other plug mutations that we have generated (23, 43, 44) should enable us to directly assess the effect of these mutations on the conformation of the hydrophobic plug residues in both G- and F-actins.

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