Tropomyosin-Troponin Regulation of Actin Does Not Involve Subdomain 2 Motions*

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Dynamic properties of F-actin structure prompted suggestions (Squire, J. M., and Morris, E. P. (1998) FASEB J. 12, 761–771) that actin subdomain 2 movements play a role in thin-filament regulation. Using fluorescently labeled yeast actin mutants Q41C, Q41C/C374S, and D51C/C374S and azidonitrophenyl putrescine (ANP) Gln41-labeled α-actin, we monitored regulation-linked changes in subdomain 2. These actins had fully regulated acto-S1 ATPase activities, and emission spectra of regulated Q41CÆDANS/C374S and D51CÆDANS/C374S filaments did not reveal any calcium-dependent changes. Fluorescence energy transfer in these F-actins mostly occurred from Trp340 and Trp356 to 5-(2-(acetyl)amino)ethyl)amino-naphthalene-1-sulfonate (AEDANS)-labeled Cys41 or Cys374 of adjacent same strand protomers. Our results show that fluorescence energy transfer between these residues is similar in the mostly blocked (−Ca2+) and closed (+Ca2+) states. Ca2+ also had no effect on the excimer band in the pyrene-labeled Q41C-regulated actin, indicating virtually no change in the overlap of pyrenes on Cys41 and Cys374. ANP quenching of rhodamine phalloidin fluorescence showed that neither Ca2+ nor S1 binding to regulated α-actin affects the phalloidin-probe distance. Taken together, our results indicate that transitions between the blocked, closed, and open regulatory states involve no significant subdomain 2 movements, and, since the cross-linked α-actin remains fully regulated, that subdomain 2 motions are not essential for actin regulation.

Descriptions of the molecular mechanism of muscle function tend to focus on the dynamic properties of the myosin motor or the static shift of the tropomyosin/troponin (Tm/Tn) complex on actin. Actin appears to take the role of a static, rigid lattice upon which the other muscle proteins shift in order to produce and regulate muscle movement. However, with numerous studies showing the dynamic nature of filamentous actin (F-actin), we attached fluorescent probes (1,5-IAEDANS or pyrene maleimide) to three fully functional mutant yeast actins, Q41C/C374S, D51C/C374S, and Q41C at positions 41 or 51, and in the case of Q41C, also at position 374 (Fig. 1). The first two mutants have the reactive Cys374 replaced by Ser and new reactive cysteine residues replacing Gln41 and Asp51, respectively. The third mutant retains the reactive Cys374 while adding an additional reactive cysteine in place of Gln41. Using these labeled mutants, we monitored their fluorescence by means of direct excitation, energy transfer, and, in the case of the doubly labeled Q41C, excimer fluorescence during the various regulatory steps. We also used Gln41/ANP (azidonitrophenyl putrescine)-labeled α-skeletal actin (both cross-linked and regulatory states involve no

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The abbreviations used are: Tm, tropomyosin; Tn, troponin; S1, myosin subfragment-1; DTT, dithiothreitol; ANP, azidonitrophenyl putrescine; IAEDANS, 5-(2iodoacetamido)aminoethylamino-naphthalene-1-sulfonate; F-actin, filamentous-actin; FRET, fluorescence energy transfer; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]aminoethanesulfonic acid.

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TABLE I
Labeled actins used in this study and their regulation

| Actins | Description | ATPase activity ratio[^a] |
|--------|-------------|--------------------------|
| Yeast actins | | |
| Wt | Gln[^a] and Cys[^a] replaced by cysteine and serine, respectively, Cys[^a] labeled with AEDANS | +Ca[^a]/EGTA |
| Q41C[^a] | Cys[^a] replaced by cysteine and serine, respectively, Cys[^a] labeled with AEDANS | |
| D51C[^a] | Cys[^a] replaced by cysteine and serine, respectively, Cys[^a] labeled with AEDANS | |
| Skeletal α-actins | Uncross-linked skeletal α-actin with Gln[^a] labeled with ANP | |
| Q41[^d] | Cross-linked ANP F-actin | |
| Q41[^d] | | |

[^a] Regulation of acto-S1 ATPase was measured by determining the ratios of MgATPase activities in the presence of 0.2 mM CaCl$_2$ to those in the presence of 1.0 mM EGTA as described under “Materials and Methods.” Regulation of acto-S1 ATPase was measured on two separate preparations of each actin.

and uncross-linked) in conjunction with rhodamine phalloidin, and in this way, measured the quenching of rhodamine fluorescence by ANP during regulation. Also, using the cross-linked species, we were able to investigate the regulatory properties of F-actin with an immobilized subdomain 2.

Our results show that there are no significant changes in the probe environment, nor in the relative position of subdomain 2, in the course of Ca$^{2+}$ regulation by Tm/Tn. In addition, we found that despite intrstrand cross-linking, the ANP-labeled α-skeletal actin remained fully regulated, indicating that a mobile subdomain 2 is not a necessary component in actomyosin regulation.

MATERIALS AND METHODS

Reagents—ATP, ADP, dextrose, DTT, phalloidin, and phenethylsulfonfuryl fluoride were purchased from Sigma. 5-(6-Flosoacyetyl)aminoethylaminophthalene-1-sulfonate (1.5-AEADANS) and N-(1-pyrene)maleimide were purchased from Molecular Probes, Inc. (Eugene, OR). The QuikChange[^a] site-directed mutagenesis kit, DNA restriction enzymes, and plasmid purification kit were purchased from Stratagene (La Jolla, CA) to confirm the absence of random errors. The construction of the Q41C and Q41C/C374S mutants was reported previously (GeneMed, San Francisco, CA) to confirm the absence of random errors.

Weeds and Pope (33) and Kron (32), respectively. S1 and heavy meromyosin were prepared from myosin using the protocols of Weeds and Pope (33) and Kron et al. (34), respectively. The cardiac troponin and tropomyosin were generous gifts from Dr. L. Tobacman. Ca$^{2+}$-independent bacterial transglutaminase was a generous gift of Dr. K. Seguro, Ajimoto Co., Inc. Japan. Yeast actins were purified over a DNase I affinity column (35). To avoid possible contamination of yeast actin with cofolin, the DNase I column was washed with 1.0 mM NaCl in G-actin buffer (5 mM TES, 0.2 mM CaCl$_2$, 0.2 mM ATP, 1.0 mM DTG, pH 7.5) prior to the elution of yeast actin. After elution, the purified actins were stored on ice in G-actin buffer.

The labeling of both Q41C/C374S and D51C/C374S actin mutants with 1.5-AEADANS was done in the following manner. After elution from DNase I column, actin was exchanged into a DTT-free G-actin buffer using Sephadex G-50 spin columns. A 1.3-fold molar excess of 1.5-AEADANS dissolved in N,N-dimethylformamide was added to the actin and the airspace was then purged with N$_2$. The reaction, which was allowed to proceed overnight on ice, was stopped with an excess of DTT. The labeled actin was further purified via a polymerization-depolymerization cycle. An extinction coefficient of 5700 M$^{-1}$ cm$^{-1}$ was used to determine the degree of labeling, which was generally in excess of 90%. The labeling of Q41C, which has two reactive cystines, Cys[^a] and Cys[^a], with pyrene maleimide was performed according to a previously described protocol (37). The extent of labeling was ~1.4 pyrene maleimide/actin.

The ANP-labeled and uncross-linked skeletal actin was prepared using a procedure based on that of Hegyi et al. (18). 1.0 mg/ml G-actin in 5.0 mM Tris-Cl, 0.2 mM CaCl$_2$, and 0.4 mM ATP, pH 8.0, was incubated with 1.0 mM ANP and 0.4 unit/ml bacterial transglutaminase at room temperature, in the dark, for 2 h. This solution was placed under a stream of N$_2$ for 7 min to remove the dissolved O$_2$. To quench the attached ANP and prevent a cross-linking reaction, 2.0 mM DTT was added to actin prior to its UV irradiation with a Model C-62 UV Illuminator (Ultra-Violet Products Inc., San Gabriel, CA) under a constant N$_2$ stream (for 7 min). The labeled sample was polymerized by the addition of 2.0 mM MgCl$_2$. The resulting F-actin was pelleted by centrifugation at 40,000 rpm in a Beckman Ti 70 rotor, incubated on ice for 2 h, and homogenized in 5 mM Tris-Cl, pH 8.0, 2 mM MgCl$_2$, and 0.2 mM ATP. The degree of actin labeling was 98 ± 5%. The preparation of ANP-labeled and cross-linked F-actin proceeded similarly. G-actin was labeled by incubating with ANP and bacterial transglutaminase and was immediately polymerized by 2.0 mM MgCl$_2$. The labeled F-actin was pelleted and homogenized in 0.9 mM Tris-Cl, pH 8.0, 2 mM MgCl$_2$, and 0.2 mM ATP. After homogenization it was placed under N$_2$ stream and then irradiated by UV light as described above, except for the absence of DTT. The level of cross-linking achieved was ~85%.

Regulated Actin-activated ATPase—The rates of S1 Mg-ATPase activated by regulated actin ± Ca$^{2+}$ were obtained as described previously (38) using light scattering to monitor the clearing time of regulated F-acto-S1 solutions. Thin filaments were reconstituted using bovine cardiac troponin, and bovine cardiac tropomyosin and each of the following actins: wild type, Q41C/C374S, Q41C[^a] ARADANS/C374S, D51C/C374S, D51C[^a] ARADANS/C374S, Q41C, Q41[^d] pyrene, and skeletal α-actin ANP. The concentrations of actin, Tm, Tn, and S1 were 4.0, 2.0, 1.0, and 1.0 μM, respectively. Experiments were carried out at 23 °C using a Mg-ATP concentration of 0.1 mM and either 1.0 mM EGTA or 0.2 mM CaCl$_2$. The course of Mg-ATP hydrolysis was monitored by measuring the light scattering at 350 nm from the above solutions (38) in a Spex Fluorolog (Spex Industries Inc., Edison, NJ).

Fluorescence Measurements—Fluorescence emissions spectra for the
F-actin (37), the excitation wavelength was set at 344 nm. For measurements of rhodamine quenching in ANP-labeled actin, an excitation wavelength of 554 nm was used. Acrylamide quenching measurements of AEDANS-labeled actins were carried out using an excitation wavelength of 338 nm and an emission wavelength of 492 nm. Acrylamide was added in 10 mM increments and the data were fitted to the Stern-Volmer equation (39) in order to determine the quenching constant $K_{SV}$. The concentrations of actin, Tm, Tn, and S1 used in fluorescence experiments were 4.0, 2.0, 1.0, and 1.0 mM, respectively, unless stated otherwise. Yeast actin was stabilized by equimolar amounts of phalloidin.

In Vitro Motility Assays—The in vitro motility assays were performed according to a previously described protocol (29). Movement was initiated by applying an assay buffer (25 mM KCl, 1.0 mM EGTA, 5.0 mM MgCl$_2$, 10 mM DTT, 0.2% methylcellulose, 10 mM imidazole, pH 7.4) containing 1.0 mM ATP and an oxygen scavenging system (40). An ExpertVision System (Motion Analysis, Santa Rosa, CA) was used to quantify the sliding speeds of individual filaments. Individual filaments were judged to be moving smoothly and were used for statistical analysis if the standard deviation of their sliding speeds was less than one-third of their average velocity (41).

RESULTS

The Actins and Their Function—The flexibility of subdomain 2 in actin has been demonstrated by a number of studies (3, 9, 10) and there is evidence indicating that Tm has a sufficient influence on either this subunit or on the S1 bound to actin to abrogate their cross-linking (28). To test for possible changes in the conformational states of subdomain 2 due to regulatory proteins and the regulation of actin, we employed three fluorescently labeled actin mutants of subdomain 2 and Gln41- and Cys374-labeled skeletal α-actin (Table I, Fig. 1). All the polymerized actins used in this study, whether labeled or unlabeled, had fully regulated acto-S1 ATPase activities when complexed with Tm/Tn. Ca$^{2+}$-induced activation of acto-S1 ATPase, as measured by the ratio of Mg$^{2+}$ activities in the presence of 0.2 mM Ca$^{2+}$ and 1.0 mM EGTA, ranged between 10- and 16-fold for these actins (Table I), except for the double labeled Q41C actin, which showed 5-fold activation (Table I). The courses of Mg$^{2+}$-induced polymerization of the actins, both labeled and unlabeled, were similar, as were the speeds of the yeast actin filaments in the in vitro motility assays (3.0 ± 0.3 μm/s).

Previous work showed that ANP labeling of α-actin at Gln41 had no significant effect on its in vitro motility, while the intermolecular cross-linking by this reagent between residues Gln11 and Cys374 strongly impaired in vitro motility (42).

Emission Spectra of Unregulated Q41C_AEDANS/C374S and D51C_AEDANS/C374S—We monitored the fluorescence of the two AEDANS-labeled yeast actin mutants with an excitation wavelength of 338 nm (Figs. 2, A and B). Polymerization of Q41C_AEDANS/C374S Ca$^{2+}$-G-actin was accompanied by a marked increase in the fluorescence and a blue shift of ~9 nm (from a $\lambda_{max}$ of 495 nm for G- to a $\lambda_{max}$ of 486 nm for F-Q41C_AEDANS/C374S) (Fig. 2A), indicating the withdrawal of the probe from a more aqueous to a more hydrophobic environment. These changes are qualitatively similar to the changes observed for dansylethylenediamine attached to Gln41 in α-actin (43, 44).

The polymerization of D51C_AEDANS/C374S Ca$^{2+}$-G-actin decreased the AEDANS fluorescence significantly (solid and dashed line spectra in Fig. 2B) with no discernible shift in $\lambda_{max}$ (494 nm). This could indicate an extension of the probe into a more aqueous environment. To test the possibility of shifts to different environments for probes at Cys44 and Cys374, we performed fluorescence titration experiments using acrylamide as the quenching agent. The Stern-Volmer constants for the two Ca$^{2+}$-G-actins were very similar; 8.4 M$^{-1}$ for Q41C_AEDANS/C374S and 8.5 M$^{-1}$ for D51C_AEDANS/C374S, showing equal accessibility of the probes at Cys44 and Cys374 to acrylamide. For the actins the Stern-Volmer constants were different, 4.7 M$^{-1}$ for

![Diagram](Image)
Q41CAEDANS/C374S versus 5.9 \text{ M} \text{ Ca}^{2+} for D51CAEDANS/C374S. This suggests that at both locations AEDANS is buried upon actin polymerization, albeit to a smaller degree at Cys41 than at Cys51.

Emission Scans of Regulated Q41C AEDANS/C374S—To test for possible subdomain 2 changes that would account for the effect of Tm on S1 cross-linking to Lys 50 on actin (28), we examined the fluorescence of the probe at Cys 41 under regulatory conditions corresponding to the blocked and closed states of the McKillop and Geeves (46) three-state model. The sequential additions of Tm, Tm/Tn + Ca\textsuperscript{2+} (both resulting in the predominantly closed state (13, 46)), and Tm/Tn = Ca\textsuperscript{2+} (blocked state) had virtually no effect on the fluorescence spectra of F-actin alone and F-actin with Tm or Tm-Tn + Ca\textsuperscript{2+} were identical, with a \( \lambda_{\text{max}} \) of 486 nm, and are represented by a single line (dashed). B, emission spectra of D51CAEDANS/C374S Ca\textsuperscript{2+}-G-actin (solid line), F-actin alone and with Tm (dashed line), and F-actin with Tm-Tn + Ca\textsuperscript{2+} (dash-dotted line). The \( \lambda_{\text{max}} \) for all three plots is 494 nm.

Effects of Regulation on Subdomain 2

Based on the tryptophan substitution study of Doyle et al. (45) that revealed only small contributions of Trp\textsuperscript{70} (−1%) and Trp\textsuperscript{86} (−11%) to total actin fluorescence, and the relative proximity to the probe of Trp\textsuperscript{340} and Trp\textsuperscript{356} in same-strand actin protomers adjacent to (above) the labeled actins (Fig. 1), these last two tryptophan residues appear to be major energy donors to AEDANS on Cys41 (see “Discussion”). As shown in Fig. 3A, the presence of AEDANS on Cys\textsuperscript{41} led to a decrease in tryptophan fluorescence for both G-actin and F-actin. Since the decrease in tryptophan emission was much larger in F-actin than in G-actin, polymerization increases energy transfer from the tryptophans to the AEDANS probe. This increase in energy transfer can be accounted for by the contribution of Trp\textsuperscript{340} and Trp\textsuperscript{356} on the adjacent actin protomer in F-actin.

The addition of Tm had no effect on Q41CAEDANS/C374S F-actin emission (Fig. 3B). The formation of the F-actin/Tm-Tn complex increased tryptophan fluorescence (due to the tryptophan residues on Tn) but had no effect on AEDANS emission, both in the presence (closed state) and absence (blocked state) of Ca\textsuperscript{2+} (Fig. 3B). This evidence implies that not only does the creation of the closed state (through the addition of Tm) not
change the environment of the AEDANS probe, but the regulatory shift from closed to blocked states is also not accompanied by probe movements relative to the tryptophan residues in the adjacent actin.

Excimer Fluorescence Measurements in Unregulated and Regulated Q41CPYRENES—As a means of further investigating the possibility of dynamic transitions at the subdomain 2/1 interface in F-actin, we used the yeast actin mutant Q41C (that has two reactive cysteine residues at positions 41 and 374) labeled with pyrene (Fig. 1). Upon polymerization, the pyrene label at Cys41 forms an excimer with the label at Cys 374 of the next monomer in the strand (37). Movements of subdomain 2 relative to the C terminus of the following monomer would abolish or change the excimer and thus signal changes in subdomain 2 position in the closed versus blocked regulatory states. As expected (37), the spectrum of pyrene-labeled Q41C G-actin did not show an excimer band (Fig. 4). The F-actin spectrum, however, had a decreased pyrene peak and an excimer band at $\lambda_{\text{max}} = 476$ nm (Fig. 4). The addition of regulatory
proteins had no effect on the excimer peak, indicating no changes in the position of the stacked pyrene probes (Fig. 4). This is further evidence for the seemingly static nature of actin subdomain 2 in the transition from the blocked to closed regulatory states.

**Emission Scans of Regulated D51C\textsubscript{AEDANS}/C374S**—The second subdomain 2 mutant used in this work, D51C/C374S, gave us an additional point of reference for observing possible subdomain 2 movements during actin regulation. Furthermore, it allowed us to monitor such movements in the vicinity of a residue (Lys\textsuperscript{50}) involved in a cross-linking to S1 that is inhibited by Tm (28). The addition of Tm (i.e. creation of the closed state) had no effect on the AEDANS fluorescence of F-actin (Fig. 2B, dashed line) and cross-linked and cross-linked ANP-labeled F-actin alone, in the predominantly closed (+Tm, +Tm/Tn (+Ca\textsuperscript{2+})) and blocked (+Tm/Tm/ (−Ca\textsuperscript{2+})) regulatory states, and for the uncross-linked species in the open (+Tm/Tn and S1) state (Table II).

Our results indicate that there were no significant changes in the quenching of rhodamine fluorescence by ANP in any regulatory state, including that created in the presence of S1 (the open state). Thus, even the shift to the open state did not induce sufficient subdomain 2 movement to change the degree of rhodamine quenching by ANP.

Upon exposure to UV radiation the ANP moiety cross-links to Cys\textsuperscript{374} on the next actin protomer in the strand, immobilizing the DNase I loop (18). We found that the ANP cross-linked α-actin remained fully regulated. Hereafter, we have shown that regulation of actin is not accompanied by discernible subdomain 2 movements. The fact that the cross-linked actin re-
mains fully regulated shows that such movements, even if present, are neither a necessary nor an important part of actin regulation.

DISCUSSION

The purpose of this study was to test the hypothesis that subdomain 2 movements are an important component of actin regulation by the Tm-Tn complex. Squire and Morris (1) raised this possibility, suggesting that changes in x-ray diffraction patterns of regulated actin could, in part, be attributed to subdomain 2 movements and not solely to the shifting of the regulatory proteins. To explore this possibility, we used labeled skeletal α-actin and yeast actin mutants whose reactive cysteines on the DNase I loop (Cys41 or Cys374) were labeled with fluorescent probes. We anticipated that changes in the environment of these probes and in their distance from native actin tryptophan residues would be reflected in their fluorescence and in the fluorescence energy transferred to them. While such FRET measurements between tryptophans and the AEDANS probes in actin mutants were suitable for monitoring regulatory shifts between the blocked (+Tm-Tn; −Ca2+) and the closed (+Tm-Tn; +Ca2+) states, they could not be used to investigate the open state because of the strong tryptophan fluorescence of S1. By using ANP-labeled skeletal α-actin, we were able to look for changes in the quenching of rhodamine phalloidin fluorescence by ANP possibly stemming from shifts between the closed and open states. In its cross-linked state, ANP-actin also allowed us to investigate whether subdomain 2 movement is required for the regulation of actin by Tm-Tn.

Functional assays of labeled α-actin and yeast actin mutants confirmed their suitability for testing regulation-linked changes in actin structure. All actins polymerized well and were fully regulated in acto-S1 ATPase experiments (Table I). In vitro motilities were similar for wild type and both labeled and unlabeled mutant actins. A previous study showed that uncross-linked ANP-labeled skeletal α-actin has sliding speeds virtually the same as those of unlabeled α-actin, while the sliding of cross-linked ANP-actin is impaired (42).

Emission Spectra of Labeled Yeast Actin Mutants—As revealed by our results, adding regulatory proteins to Q41C/AEDANS/C374S produced no significant changes in probe fluorescence, indicating that whatever effect Tm/Tn have on this subdomain, it is not discernible in any environmental changes of the probe. In the case of D51C/AEDANS/C374S, addition of Tn to the F-actin/Tm complex increased the probe fluorescence by −10%. Yet, since Tn is added to actin-Tm in the presence of Ca2+, there should be no shift in Tm position, as both Tm alone and the Tm/Tn complex (+Ca2+) lie in the closed state (13, 25). As the presence or absence of Tn does not change the probe environment, the small effect of Tn on AEDANS fluorescence in this actin mutant appears unrelated to the state of regulated actin. This conclusion is confirmed by the lack of fluorescence differences between the blocked (−Ca2+) and closed (+Ca2+) regulatory states of this actin, indicating an absence of regulation-dependent changes in the probes environment. At this stage, the nature of the Tn-specific and Ca2+ insensitive perturbation at the Cys51 site on actin is unclear.

The availability of the actin mutants Q41C with its two reactive cysteines, Cys41 and Cys374, allowed us to examine the subdomain 2/1 interface in regulated F-actin. Upon polymerization this double pyrene-labeled mutant exhibits an excimer, indicating the stacking of pyrene labels attached to Cys41 and Cys374 on adjacent protomers within the same filament strand (37). As reported under “Results,” neither the addition of regulatory proteins nor the switch from the blocked to the closed state had any effect on the excimer fluorescence. This suggests that there is little, if any, change in the overlap of pyrene probes, and/or regulation-induced movement at the subdomain 2/1 interface in F-actin.

Fluorescence Energy Transfer Experiments—We took advantage of the inherent sensitivity of FRET measurements to distance changes (in the donor-acceptor pair), and of the now available information on the relative fluorescence levels of the four actin tryptophans, to monitor possible subdomain 2 movements due to actin regulation.

According to the Doyle et al. (45) analysis of tryptophan yeast actin mutants, the contributions of Trp79, Trp356, Trp340, and Trp356 to actin fluorescence are 1, 11, 37, and 51%, respectively, while the R0 value for the tryptophan-AEDANS donor-acceptor pair is 22 Å (47). These contributions permit the calculation of FRET efficiency in F-actin using the distances from the tryptophans to Gln41 and Asp51 derived from the Holmes et al. (48) (Fig. 1) and Lorenz et al. (49) models of F-actin. Obviously, such calculations of intra- and inter-protomer FRET in F-actin are model-dependent and their predictive value is further reduced by the unknown orientation of AEDEANS at the Cys31 and Cys51 sites. Despite these limitations and regardless of the exact partitioning of energy transfer between the different tryptophan residues, these calculations provide strong evidence for a major contribution to FRET by the inter-protomer (on adjacent actins within the same strand) transfer of energy from Trp356 and Trp340 to AEDANS on either Cys41 or Cys51. This conclusion is consistent with the much greater efficiency of FRET in F- than in G-actin (Figs. 3A and 5A). Thus, changes in AEDANS fluorescence during excitation at 295 nm (i.e. at the tryptophan excitation band) should monitor, to a large extent, distance changes between the probe and Trp79, Trp356 on the adjacent protomer.

Our main results show that the stimulated fluorescence of AEDANS attached to either Cys41 or Cys51 does not change upon the binding of Tm to F-actin or upon the switch of the actin-Tm complex from the blocked to the closed state (EGTA/Ca2+ switch). The fact that acceptor probes located at two different sites in subdomain 2 yield the same results regarding distance changes during Ca2+ regulation of these well regulated actins suggests that subdomain 2 remains static during this process. Our strategy of probing (by FRET) distance changes from tryptophan donors to two acceptor sites makes it unlikely that a movement of subdomain 2 escapes our detection due to compensating changes in energy transfer. Moreover, the absence of any perturbation of the pyrene excimer band in the course of regulatory transitions in Q41C yeast actin adds credence to the static picture of subdomain 2.

ANP-labeled Skeletal α-Actin—Our experiments involving the yeast actin mutants were limited to evaluating two states of the “three-state model” of actin regulation (46); the blocked and closed states. The transition to the open state requires the presence of S1, which would introduce additional energy donors to the environment of the AEDANS probes on actin. We were able to surmount this complication by using ANP-labeled (at Gln11) α-actin and monitoring the quenching of rhodamine phalloidin fluorescence by ANP. The ANP-labeled actin allowed us to look for subdomain 2 movements during the shift to the open state since the presence of S1 has no effect on rhodamine fluorescence. Not only did this particular experiment allow us to “fill-in the gap” left by our yeast actin mutant experiments, it provided yet another donor/acceptor configuration (with rhodamine phalloidin as a donor) for following actin regulatory transitions. Our results show no significant differences in energy transfer between rhodamine and ANP in any of the actin regulatory states, including the open state (Table II). These findings confirm our results obtained with AEDANS-labeled...
actin mutants, namely, that there seem to be no significant movements of subdomain 2 during actin regulation. The labeling of the skeletal actin with ANP had the additional advantage of allowing us to arrest subdomain 2 movement through the cross-linking of Gln41 to Cys374 on adjacent protomers (18). We found that despite this modification, which significantly impairs the motility of F-actin, the thin filaments remained regulated. This outcome alone does not preclude subdomain 2 movement during regulation; it simply demonstrates that such movement is not a requirement for regulation, and it serves to reinforce our results from the previous experiments that indicate no discernible regulation-based subdomain 2 movements.

Care must always be exercised in interpreting data generated through the use of probes. Probes may affect the characteristics of their host proteins and alter the dynamic properties of mobile sites. In the case of labeled actins, probes may stabilize or destabilize DNase I loop/C terminus interactions, or even produce a particular conformation that may not reflect normal function. However, the fact that the probes did not introduce marked changes in the in vitro motility of the actins, and especially in the regulation of their acto-S1 ATPase activities, indicates that they do not introduce noteworthy changes in the actins. Moreover, our multiple experiments, each yielding similar results, suggest that subdomain 2 movements seem neither to occur during, nor play an important role in, the regulation of actin by tropomyosin and troponin.

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