The Regulation of Subtilisin-cleaved Actin by Tropomyosin/Troponin*

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Vertebrate striated muscle contraction is regulated in a Ca\(^{2+}\)-dependent fashion by tropomyosin (Tm) and troponin (Tn). This regulation involves shifts in the position of Tm and Tn on actin filaments and may include conformational changes in actin that are then communicated to myosin subfragment 1 (S1). To determine whether subdomain 2 of actin plays a role in this regulation, the DNase-I loop 38–52 of this subdomain was cleaved by subtilisin between residues Met\(^{47}\) and Gly\(^{48}\). Despite impaired unregulated function, the potentiation of regulated function in the in vitro motility assay was not significantly different from that of uncleaved actin. Stopped-flow measurements of ADP release from regulated and unregulated cleaved acto-S1 showed a marked increase in ADP release from acto-S1 in the presence of the regulatory complex. The enhancement of the actin affinity for S1 in the presence of regulatory proteins was greater for uncleaved than for cleaved F-actin. Finally, both cleaved and uncleaved actins protect myosin loop 1 from papain cleavage equally well. Our results suggest that the potentiation of actin function in the in vitro motility assay by regulatory proteins stems from changes in cross-bridge cycle kinetics. In addition, the unimpaired calcium-sensitive regulation of cleaved actin indicates that subdomain 2 conformation does not play an essential role in the regulation process.

The contraction of vertebrate striated muscle is controlled by the thin filament-associated proteins, tropomyosin (Tm) and troponin (Tn), which regulate the interaction of actin and myosin in a Ca\(^{2+}\)-dependent fashion (1). In the absence of Ca\(^{2+}\), Tm appears to bind to subdomain 1 of actin and bridge over subdomain 2 to the next protomer, thereby blocking myosin access to the strong binding sites on actin (2, 4–6). In the presence of Ca\(^{2+}\), Tm moves toward subdomain 3 and 4 (4, 6, 7), and this motion is completed upon binding of S1 (8, 9).

Based on structural models of regulated F-actin (2–5, 8, 10, 11), subdomain 2 does not seem to have a marked role in interactions with regulatory proteins. However, Squire and Morris (12) raised the possibility that changes seen in the structural studies of regulated thin filaments may be due not only to shifts of the Tm/Tn complex, but also to subdomain 2 movements within each actin protomer. The observations that selected subdomain 2 alterations impair regulation by tropo-
nin-troponin (13, 14) and that the presence of Tm and Tn/Tn interferes with cross-linking between subdomain 2 and S1 (15) are consistent with this hypothesis. Moreover, the recent paper of Luo et al. (16), reporting the cross-linking of Tn-I to Met\(^{47}\) on subdomain 2 of actin, suggests that TnI interacts with this region of actin. A similar conclusion was reached in an electron microscopic study of thin filaments containing mutant troponymosin, in which troponin density was found near Met\(^{47}\) (although troponin more strongly contacted subdomain 1 than subdomain 2) (7). Although previous work from our laboratory (17) did not detect any subdomain 2 movements during regulation, all of our experiments used probes attached to Gln\(^{41}\) (in α-skeletal actin) or Cys\(^{41}\) and Cys\(^{51}\) (in mutant yeast actins). Because such probes may have altered the dynamics, if not the conformation, of an intrinsically dynamic subdomain 2, and because TnI cross-links to Met\(^{47}\) on actin, we tested for Tm/Tn interactions with this region using subtilisin-cleaved actin (3, 18).

Subtilisin cleaves between Met\(^{47}\) and Gly\(^{48}\) in loop 38–52 of actin’s subdomain 2 (3). The resultant protein exhibits modified interpomter interactions in F-actin and impaired function with myosin (i.e. myosin binding, acto-S1 ATPase, and in vitro motility) (3, 18). Since these changes occur subsequent to subdomain 2 cleavage, the question addressed in this work is whether, and to what extent, the subtilisin-cleaved actin is still regulated by Tm/Tn. Alterations in regulation would indicate that subdomain 2 indeed has a direct or indirect role in regulation.

Previous studies have shown that the presence of the Tm/Tn regulatory complex not only introduces Ca\(^{2+}\)-based regulation to F-actin filaments but also potentiates their function, i.e., the in vitro motility of actin filaments and the force generated by actomyosin are enhanced (19–21). Depending on the assay conditions and the types of Tm and Tn used, thin filament sliding speed increases of up to 100% have been reported (19–22). Although several explanations for such actin speed increases can be considered, the most likely possibility is that of accelerated actomyosin detachment through faster ADP release and/or ATP binding. Because the stopped flow technique limits measurements of the kinetic rates of these processes to 500–700 s\(^{-1}\), the effect of Tm/Tn on these rates is not easily

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The abbreviations used are: Tm, tropomyosin; Tn, troponin; DTT, dithiothreitol; HMM, heavy meromyosin; PMSF, phenylmethylsulfonyl fluoride; S1, myosin subfragment 1; MOPS, 4-morpholinepropanesulfonic acid.
measured. Such measurements, however, are feasible using the cleaved actin, and they were carried out in this study.

In the experiments described below, we investigated the effect of regulatory proteins on the in vitro motility of subtilisin-cleaved actin, the binding of S1 to such actin, and the rate of ADP release from S1 bound to actin. We compared the degree of protection of myosin loop 1, which is part of the myosin nucleotide binding cleft, afforded by cleaved and uncleaved actin. Our findings indicate a modulation of cross-bridge cycle kinetics by the Tm/Tn regulatory complex, while also supporting the conclusion that subdomain 2 conformation does not play an essential role in Tm/Tn-based regulation of actomyosin interactions.

MATERIALS AND METHODS

Reagents—ATP, ADP, DTT, phalloidin, and PMSF were purchased from Sigma. N-(1-pyrene)-maleimide was purchased from Molecular Probes, Inc.

Proteins—Skeletal myosin and actin were prepared from rabbit back muscle according to Godfrey and Harrington (23) and Spudich and Watt (24), respectively. S1 and heavy meromyosin (HMM) were prepared from myosin using the protocols of Woods and Pope (25) and Kron et al. (26), respectively. The cardiac troponin and tropomyosin were produced from myosin using the protocols of Weeds and Pope (25) and Kron et al. (26). Subtilisin was purchased from Sigma.

The labeling of skeletal actin with pyrene maleimide was performed according to a previously described protocol (28). The extent of labeling was ~100%.

Actin was cleaved by adding subtilisin to skeletal G-actin in a 1:1000 weight ratio. The cleavage was carried out at room temperature for 15 min at 20 °C. The reaction was terminated by the addition of PMSF to 1.0 mM. The cleaved actin was then polymerized by raising the MgCl2 and KCl concentrations to 2.0 mM and 50 mM, respectively. The cleaved G-actin was run on a 12.5% SDS-polyacrylamide gel to verify the complete cleavage and was used in experiments within 24 h after the cleavage.

Acto-HMM ATPase Measurements—The rates of HMM Mg-ATPase were determined at 25 °C under steady-state conditions, by monitoring the inorganic phosphate release after ATP hydrolysis as described before (18). Thin filaments were reconstituted using bovine cardiac troponin, rabbit skeletal tropomyosin, and the subtilisin-cleaved or intact skeletal actin. The assay solutions contained 10 mM imidazole-HCl, pH 7.0, 10 mM NaCl, 2.0 mM K-EGTA, 3.0 mM MgCl2–ATP. Protein concentrations were 0.3–0.5 μM for HMM (0–16 μM S1 heads) and 0–40 μM actin (intact and cleaved).

Competition Binding Experiments—The binding of S1 to ADP-actin, subtilisin-cleaved and pyrene-labeled actins was monitored by measuring the quenching of pyrene fluorescence by S1 in samples containing 1.0 μM pyrene-labeled actin and 5.0 μM subtilisin-cleaved actin with and without regulatory proteins (14). When present, the molar ratios of Tm and Tn to actin were 0.35:1.0. S1 was added to actin solutions in 0.25-μM increments to a total concentration of 3.0 μM. To ensure that all binding was strong (in the absence of ATP), 5 units of hexokinase and 2.0 mM ADP, phalloidin in equimolar concentrations with the actins, 4.0 mM MgCl2, 150 mM NaCl, and 2.0 mM DTT. All measurements were recorded at 23 °C in Spex Fluorolog using excitation and emission wavelengths of 365 nm and 405 nm, respectively. Dissociation constants were determined using the program Nfit 1.0 (University of Texas, Galveston, TX) and fitting the data with the equation

\[
S_0 = (K_{d,S}(F_0 - F)/(F - F_0)) + (A_{o,p}(F_0 - F)/(K_d,A_0(p)(F_0 - F) + K_{d,pp}(F_0 - F))) \tag{Eq. 1}
\]

where \(S_0\) represents total S1 concentration, \(K_{d,S}\) = dissociation constant for pyrene-labeled actin; \(F =\) fluorescence; \(F_0 =\) maximum fluorescence, i.e. the fluorescence of pyrene-labeled actin before the addition of S1; \(A_{o,p} =\) concentration of pyrene-labeled actin; \(F_0 =\) minimum fluorescence, i.e. the fluorescence with actin alone; \(S_1 =\) concentration of subtilisin-cleaved actin; \(K_{d,pp} =\) dissociation constant for subtilisin-cleaved actin.

The above equation, connecting the measured fluorescence with the concentrations of the bound and free actins, was derived by algebraic manipulation of the following expressions,

\[
S = S_0 + A_{o,p} + A_{o,p} \tag{Eq. 2}
\]

where \(S_0\) represents total S1 concentration, \(S =\) unbound S1, and \(A_{o,p}\) and \(A_{o,p}\) represent S1 complexes with pyrene-labeled and subtilisin-cleaved actin, respectively. Each of these species is expressed in terms of a dissociation constant (\(K_{d}\)), using the mass action law relationships

\[
K_{d,S} = A_{o,p}/S \tag{Eq. 3}
\]

and

\[
K_{d,pp} = A_{o,p}/S \tag{Eq. 4}
\]

and is related to the observed fluorescence (\(F\)) via the following equation.

\[
F = F_0/(S_0 + A_{o,p} + A_{o,p}) \tag{Eq. 5}
\]

In Vitro Motility Assays—The in vitro motility assays were performed according to a previously described protocol (29). Tm and Tn, when present, were added to the assay system at the concentration of 0.1 μM; Tm alone was present at 0.4 μM in these assays. Movement was initiated by adding an assay buffer (2.0 mM K-EGTA/CaCl2–K-EGTA (with the ratio varying depending on the pCa desired), 20 mM KCl, 2.0 mM MgCl2, 10 mM DTT, 25 mM MOPS, pH 7.3; total ionic strength, 50 mM) containing 1.0 mM MgATP and an oxygen scavenging system (30) at 25 °C. An ExpertVision System (Motion Analysis, Santa Rosa, CA) was used to quantify the sliding speeds of individual filaments. Actin speeds (mean ± S.D.) were determined from measurements of motion of 150–250 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-half of their average speed (31). Filament speeds at different pCa values were fitted to a Hill equation in the form

\[
V = V_0/(1 + (\text{pCa}/V_C)^n) \tag{Eq. 6}
\]

where \(V\) is the measured mean speed of actin filaments, \(V_0\) is the mean filament speed at pCa = 5.0 (maximal speed), \(\text{pCa}\) is the calcium concentration at which \(V = 0.5 V_0\), and \(n\) is the Hill coefficient. Significance was determined using Student's t-test, and the confidence level was set at \(p < 0.05\).

Stopped-flow Experiments—The stopped-flow measurements were carried out at 20 °C in a buffer containing 100 mM KCl, 25 mM MOPS, pH 7.3, 2.0 mM MgCl2, 2.0 mM CaCl2–K-EGTA, 2.0 mM DTT, and 1.0 mM PMSF. The final concentration of S1, F-actin (whether cleaved or uncleaved), and ADP (when present) were 2.0, 3.0, and 100 μM, respectively. Phallolidin was added at an equimolar concentration to actin for filament stabilization. When applicable, rabbit skeletal Tn and bovine cardiac Tn were each present at a concentration of 0.75 μM. The final concentration of MgATP, after mixing, was 5.0 mM. The rate-limiting dissociation of ADP from acto-S1 was followed via a decrease in light scattered at 90° to the exciting light that accompanied the subsequent ATP-induced dissociation of the acto-S1 complex. Excitation and emission wavelengths were both 345 nm.

Papain-digestion Assays—Papain was activated by dissolving it in a solution containing 50 μM DTT, 1.5 mM EDTA, and 5.5 mM cysteine, pH 6.0, and incubating for 30 min on ice before use. The digestions were performed at room temperature, at S1 concentration of 8.7 μM and, where applicable, F-actin concentration of 17.4 μM. Papain was added to each sample to a final concentration of 50 μg/ml, and the digestions were terminated by adding 10 mM iodoacetate acid. The extent of S1 digestions lasting 0, 5, 10, 15, 25, and 40 min was estimated from scans of SDS-PAGE gels.

RESULTS

Actin Sliding in the in Vitro Motility Assays—As documented earlier (3, 18), our limited digestion of actin by subtilisin yielded homogenous preparations with virtually no uncleaved or further degraded actin. As also reported before (18), the in vitro motility of subtilisin-cleaved actin was strikingly impaired. The results of these measurements are listed in Table I. The in vitro motility speeds for the cleaved and uncleaved actins without regulatory proteins were 1.9 ± 1.1 and 4.1 ± 0.7 μm/sec, respectively. We found that in the presence of CaCl2–, at pCa = 5.0, the regulatory complex increased the sliding speed of the subtilisin-cleaved and uncleaved actin filaments by...
about 2-fold, to 3.57 ± 1.2 and 7.45 ± 1.1 μm/s, respectively (Table I). The presence of the regulatory proteins also increased the percentage of smoothly moving cleaved actin filaments ~2.5-fold, from 34.4% to 88.5%. Tm alone had a much smaller effect than Tm/Tn on the motility of the cleaved and intact actin (Table I). In the presence of Tm the speeds of actin increased by up to 20% (± 10%).

Measurements of the sliding speeds of regulated actin versus pCa revealed similar dependence on Ca^{2+} for the cleaved and intact actin (Fig. 1, inset). This is easily seen after normalizing the actin filament speeds to 100% at pCa = 5.0 (Fig. 1). Thus, the regulatory mechanism seems little affected by the cleavage; both the cleaved and uncleaved actins have similar pCa_{50} and Hill coefficients (Fig. 1).

S1 Binding—The affinity of S1 to pyrene-labeled skeletal actin and subtilisin-cleaved actin in both the presence and absence of Tm/Tn was measured in binding competition assays. The goal of these measurements was to determine the effect of the regulatory proteins on the affinity of cleaved actin for S1 and to assess the possibility that Tm/Tn might “restore” their binding to that of intact actin and S1. The presence of the regulatory complex increases the affinity of the S1 for the pyrene-labeled actin by about 5-fold, with the K_{d} decreasing from 0.18 ± 0.003 to 0.037 ± 0.003 μM (mean ± S.E.) in the absence and presence of Tm/Tn, respectively (Fig. 2). A similar effect of Tm/Tn on the affinity of S1 to pyrene-labeled actin was reported by Korman et al. (14). The cleaved and unlabeled actin bound S1 less well than the intact labeled actin, with a K_{d} of 0.39 ± 0.006 μM. Taking into account the ~5-fold increase in the K_{d} value of S1 and actin due to pyrene labeling of actin (14), our measurements show about a 10-fold lower binding constant of S1 to cleaved than to intact actin. This result is consistent with prior measurements of the effect of subtilisin cleavage of actin on S1 binding (3). In the presence of regulatory proteins, the affinity of S1 to cleaved actin increased by 2-fold, with a K_{d} of 0.22 ± 0.004 μM. This improvement in binding is less than the 5-fold effect of troponin-tropomyosin measured using the uncleaved labeled actin. Thus, even in the presence of Tm/Tn the uncleaved actin binds S1 less strongly than intact actin in the absence of regulatory proteins.

Besides decreasing the affinity of S1 binding, subtilisin cleavage of actin also decreases weak acto-S1 interactions as evidenced by its increase of the K_{m} (from 33 to 200 μM) value of the actomyosin ATPase reaction (3, 18). We verified in acto-HMM ATPase measurements that the difference between the K_{m} values of cleaved and intact actin in such assays is retained in the presence of Tm/Tn (at pCa = 5.0; data not shown). Taken together, the small effect of Tm alone on the motility of cleaved actin, and the S1 binding and acto-HMM ATPase results indicate that the regulatory proteins do not offset the cleavage damage to the interaction with myosin.

Stopped-flow Measurements of ADP Release from Regulated and Unregulated Acto(cleaved)-S1—Stopped-flow measurements of the ATP-dependent rate of dissociation of rigor acto-S1 complex at 20 °C did not reveal any major slowing of this process when using cleaved actin. The rates of dissociation of S1 from both cleaved and uncleaved actin were linear with the [MgATP] and were >800 s⁻¹ at 5 mM ATP (the highest rate we could reliably measure). Such fast rates preclude the measurement of any possible effect of regulatory proteins on acto-S1 dissociation. Although lowering the temperature of the solution would slow acto-S1 dissociation, this strategy is not useful because regulatory proteins lose their ability to potentiate thin filament sliding speeds at low temperatures (<12 °C).2

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TABLE I

| Actin                  | Speeds in μm/sec |
|-----------------------|-----------------|
|                       | No regulatory proteins | Tm added | Tm/Tn added |
| Intact                | 4.1 ± 0.7       | 4.9 ± 0.7 | 7.45 ± 1.1 |
| Cleaved               | 1.9 ± 1.1       | 2.2 ± 1.0 | 3.57 ± 1.2 |

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2 E. Homsher, personal communication.
Stopped-flow measurements of ADP release from S1 at 20 °C in the presence of both regulated and unregulated subtilisin-cleaved actin filaments were fitted using a single exponential equation. The results reveal an almost 8-fold increase in the rate of ADP release by the regulated compared with the unregulated cleaved actin, with release rates of $>900$ s$^{-1}$ and $125 \pm 12$ s$^{-1}$, respectively (Fig. 3). We were unable to reliably measure the release rates of ADP from uncleaved acto-S1 as these rates were beyond instrument resolution, even without regulatory proteins ($>850$ s$^{-1}$). The nucleotide release rates can be decreased considerably by using the ADP analog eADP in lieu of ADP as a substrate in such experiments. The results of such measurements showed that the rates of eADP release from S1 are about the same in the presence of unregulated and regulated actin (32, 33). Our in vitro motility experiments using eATP instead of ATP also did not reveal any effect of Tm/Tn on actin speeds. In the presence of eATP unregulated and regulated intact actin moved at $1.37 \pm 0.45$ and $1.29 \pm 0.41$ μm/s speeds (mean ± S.D.), respectively.

Protection of Loop 1 in S1 from Proteolysis: Cleaved versus Uncleaved Actin—Modification of loop 1 of S1 has been shown to affect the rate of nucleotide release from acto-S1 (34–36). In addition, despite the fact that actin does not directly contact this area of S1, it protects S1 loop 1 from digestion by papain (37). By comparing the degree of protection afforded by cleaved versus uncleaved actin, we sought to determine whether the actin cleavage by subtilisin affected the ability of actin to modulate this site. However, the degree of protection from papain proteolysis afforded S1 by the cleaved actin was virtually the same as that by uncleaved actin (Fig. 4). This shows that actin cleavage alters ADP release from acto-S1 by weakening the impact of actin on structural elements of S1 other than loop 1.

**DISCUSSION**

Recently, Luo et al. (16) reported on the photocross-linking of residues 104 and 133 on mutant troponin I (TnI) to Met 47 on actin in reconstituted thin filaments in the absence of Ca$^{2+}$. On this basis, Luo et al. (16) suggested that TnI binds to actin in the vicinity of Met 47, probably to the adjacent segment 50–58. Binding of this actin region to TnI may form a Tm interaction site, which can stabilize the latter protein at the outer domain of actin i.e. in the blocked state. In the presence of Ca$^{2+}$, the release of TnI from its binding region on actin would allow the movement of Tm to its thermodynamically preferred position between the outer and inner domains of actin, i.e. to the closed state position (16). This regulation model does not evoke any companion dynamic changes on actin and, therefore, is not in conflict with the apparent absence of subdomain 2 motions during actin regulation (17).

The proposed location of the TnI binding site on actin (segment 50–58) is consistent with the easy availability of actin specifically cleaved by subtilisin between Met 47 and Gly 48 (3, 18) suggested that such material could be used to examine the dependence of regulation on subdomain 2 interactions, the conformational changes in this region, and the role of subdomain 2 in transmission of regulatory allosteric changes to other parts of actin.

Several aspects of actin structure and function are impaired by its subtilisin cleavage. These include the previously described (3, 18) and confirmed above degradation of interactions with myosin, increased critical concentration for polymerization (3), and decreased inter-strand interactions (38). The cleaved actin filaments are also more susceptible to tryptic proteolysis (at Lys 61 and Lys 89), indicating a more open conformation of actin (39) and/or greater mobility of its subdomain 2 within the polymer. It is possible that the decreased interactions of cleaved actin with myosin are due to either a change in the binding site for myosin (if it includes residues 38–52) or a decreased communication pathway in actin by the cleavage between Met 47 and Gly 48.

Despite changed interprotomer interactions in F-actin and the functional impact of DNase-I binding loop cleavage, the regulatory mechanism was not impaired in the cleaved actin. When compared with normal thin filaments, the degree of Tm/Tn-induced potentiation remained the same, with no significant shift in pCa dependence of the regulation of cleaved actin (Fig. 1). This indicates that the propagation of allosteric regulatory changes in actin occurs despite impaired interprotomer interactions and that these effects may be propagated.
through the regulatory proteins themselves. Thus, subdomain 2 does not seem to play an active role in the regulation of actomyosin interactions. If TnI binds to segment 50–58 on actin (16), it may act as a mechanical latch for Tm rather than allosteric modulator of subdomain 2 and its dynamics. This conclusion is in line with the results of our previous study in which we used probes attached to actin’s subdomain 2 to monitor its conformational transitions during the regulatory process.

The steric-blocking model of actomyosin regulation does not provide a simple explanation for the Tm/Tn-induced potentiation of actin speed and force production in the in vitro motility assay (19). Variations in filament speed and force generation are generally attributed to changes in the kinetics of ADP release and recruitment of myosin heads by the thin filament. Since previous studies (19–22) have indicated increases in both force and speed in the presence of Tm/Tn, it appears that the presence of regulatory proteins affects both myosin head recruitment and nucleotide release through direct interactions between the regulatory proteins and myosin, interactions mediated by actin, or both. An increase in myosin recruitment, which would increase force generation because of a greater number of heads attached at a given time, is suggested by the increased binding of S1 to actin in the presence of Tm/Tn (27, 40–42). However, under conditions of HMM saturation in the in vitro motility assay, increases in unloaded filament sliding speed can not be produced by increases in myosin head recruitment (43). Instead, filament sliding speed increases are more likely produced by increased cross-bridge cycle turnover rate or faster ADP release. This scenario is supported by a recent study of Homsher et al. (19), showing that the enhancement of actin speed by Tm/Tn is progressively lost by increasing the [Mg-ADP].

We showed in our binding competition assays that the presence of regulatory proteins increases the affinity of S1 to the thin filament reconstituted with intact actin ~5-fold (Fig. 2). For thin filaments containing cleaved actin, the increase in affinity is less than 2-fold. These results indicate that the difference in the binding of myosin heads by the two types of actin is not decreased in the reconstituted thin filaments. Despite this difference, both types of regulated thin filaments display 2-fold increases in the in vitro motility speeds. This confirms the contention that increases in speed are not tied to increases in recruitment, but are most likely due to increases in the rate of ADP release. Consistent with this, when such acceleration in ADP release does not occur, as in the case of eADP (32, 33), we have measured similar actin speeds with and without Tm/Tn.

Due to the extremely rapid ADP release rate from acto-S1 at room temperature, we were unable to determine the effect of Tm/Tn on the ADP off rates from uncleaved actin/S1. Using lower temperatures to slow the kinetics of this reaction was not an option because as low as 12 °C Tm/Tn cause a decrease in the in vitro motility performance instead of the potentiation that is seen at room temperature. However, given the performance of the regulated subtilisin-cleaved actin filaments in the in vitro motility assay, this system provided a convenient model for the study of ADP release from the acto-S1 complex. As reported above, S1 in the presence of unregulated cleaved actin filaments released ADP at a much slower rate than in the presence of their uncleaved counterparts, but this rate was increased severalfold upon the addition of Tm/Tn. Thus, our finding provides an experimental link between the in vitro motility speed increases in the presence of the regulatory complex and the increase in the rate of ADP release from myosin. The improved binding of S1 to cleaved actin in the presence of Tm/Tn, which increases the S1 affinity to actin by less than 2-fold, cannot account fully for the acceleration of ADP release. It appears that while the cleaved actin fails to open the nucleotide cleft on S1 to the same extent as the intact actin does, Tm/Tn restores that ability to the cleaved protein. At present, it is unclear whether this effect is due to direct Tm/S1 interactions or allosteric changes on actin (induced by Tm/Tn) that potentiate its effect on the nucleotide cleft on S1. To test one possible structure-function relationship behind the effect of actin on ADP release from S1, we compared the ability of intact and cleaved actins to protect loop 1, which spans the 25/50-kDa junction of S1, from papain cleavage. Despite the fact that actin does not directly contact this site, the binding of actin to S1 causes a conformational change in loop 1, which inhibits its cleavage by papain. Loop 1 is located over the nucleotide binding cleft, and various studies have shown that modifying it affects nucleotide binding. Rovner et al. (36) have shown a marked increase in the in vitro motility of actin and suggested a concomitant increase in ADP release when this loop is altered through mutations. In line with this, Sweeney and Holzbaur (34) also demonstrated the postulated rate increases in ADP release. Additional studies by the same laboratory showed that the rate of ADP release is correlated to the length and flexibility of loop 1 (35). Thus, given the reported effect of loop 1 on ADP release and our own results, which showed changes in motility consistent with shifts in ADP release, we investigated the possibility that subtilisin-cleaved actin had a different effect on loop 1 than the intact actin. Our results indicate no changes in the degree of protection provided by the cleaved actin. Thus, the mechanism involved in the shift in ADP release brought about by the cleaved actin does not involve changes in actin impact on loop 1 of S1.

In summary, our work shows that the presence of regulatory proteins increases the speed of cleaved actin filaments in the in vitro motility assay, and this effect is correlated with the increase in the rate of ADP release from myosin. Despite the functional differences between the modified and unmodified actins, actin regulation and the effect of the regulatory complex on speed is unchanged; it is unaffected by the impairment of interprotomer actin interactions. While the cleavage in actin subdomain 2 does affect interactions between actin and S1, these changes do not appear to include the influence of actin on loop 1 in S1. Therefore, the mechanism that alters the rate of ADP release must be due to other changes in acto-S1 interactions.

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