Binding of Troponin I and the Troponin I−Troponin C Complex to Actin−Tropomyosin. Dissociation by Myosin Subfragment 1†

X. Zhou,‡ E. P. Morris,§ and S. S. Lehrer*

Muscle Research Group, Boston Biomedical Research Institute, 20 Staniford Street, Boston, Massachusetts 02114, and Department of Neurology, Harvard Medical School, Boston, Massachusetts 02115

Received October 6, 1999; Revised Manuscript Received November 18, 1999

ABSTRACT: Troponin I (TnI) is the component of the troponin complex, TnI, TnC, TnT, that is responsible for inhibition of actomyosin ATPase activity. Using the fluorescence of pyrene-labeled tropomyosin (Tm), we probed the interaction of TnI and TnIC with Tm on the reconstituted muscle thin filament. The results indicate that TnI and TnIC($-Ca^{2+}$) bind specifically and strongly to actin−Tm with a stoichiometry of 1 TnI or 1 TnIC/1 Tm/7 actin, in agreement with previous results. The binding of myosin heads (S1) to actin−Tm at low levels of saturation caused TnI and TnIC to dissociate from actin−Tm. These results are interpreted in terms of the S1-binding state allosteric-cooperative model of the actin−Tm thin filament, closed/open. Thus, TnI and TnIC($-Ca^{2+}$) bind to the closed state of actin−Tm and their binding is greatly weakened in the S1-induced open state, indicating that they act as allosteric inhibitors. The fluorescence change and the stoichiometry indicate that the TnI-binding site is composed of regions from both actin and Tm probably in the vicinity of Cys 190.

In vertebrate skeletal and cardiac muscle, the troponin−tropomyosin (Tn−Tm) complex interacts with actin to confer Ca^{2+} sensitivity to muscle contraction and to actin−myosin ATPase, see reviews (*1*−*3*). Tm is an α-helical coiled coil molecule which interacts with seven actin subunits and with itself in a head to tail fashion to form a continuous filament along actin. Tn contains three subunits, TnC, TnT, and TnI. TnT interacts with Tm from a region near Cys 190 (*4*) to the head−tail overlap and is the component which anchors the troponin I−troponin C (TnIC) complex to actin−Tm. In the absence of Ca^{2+}, TnI interacts with actin, which results in the inhibition of contraction (*5*). Binding of Ca^{2+} to TnC causes local dissociation of TnI from actin (*6*), relieving the inhibition.

Early studies have indicated that the binding to actin of TnI and the TnIC complex in the absence of Ca^{2+} is enhanced by Tm (*7*−*9*). In this study, we used the fluorescence of pyrene-labeled Tm (*10*) to probe the interaction of TnI and TnIC with Tm in the actin−Tm complex.

We found that TnI and TnIC ($-Ca^{2+}$) bind strongly to actin−Tm with a stoichiometry of 1 TnI/1Tm/7actin but do not bind to Tm. In view of the stoichiometry and the large fluorescence change (40%), the TnI-binding site probably jointly involves Tm in the region of Cys 190 as well as actin. We also show that the binding of S1 caused dissociation of TnI and TnIC from actin−Tm at S1/actin ratios less than 0.5. These results can be interpreted with the allosteric-cooperative model of the thin filament (closed/open) (*11*, *12*), whereby TnI and TnIC binding is inhibited when the actin−Tm filament is shifted into the open state (*12*). Preliminary reports of this work and effects on actin−Tm activated myosin ATPase have been presented (*13*, *14*).

EXPERIMENTAL PROCEDURES

Rabbit skeletal actin was prepared from an acetone powder of rabbit muscle and purified by cycles of polymerization/depolymerization (*15*, *16*). Rabbit skeletal Tm was purified by isoelectric precipitation and ammonium sulfate fractionation (*17*, *18*). For the fluorescence experiments, Tm was labeled with pyrene iodoacetamide as described earlier (*10*). RSTm, which consists predominantly as a 60/40 mixture of αα/αβ isoforms (*19*), was used without further fractionation. Whereas the α isoform contains a Cys at 190, the β isoform contains an additional Cys at 36. Since the labeling was done in the unfolded state, both sites should label with the same efficiency. Thus, 83% of the pyrene fluorescence should originate from the pyrenes at Cys 190. We therefore interpreted fluorescence changes as originating from the Cys 190 region. The concentration of the labeled Tm was determined by BCA-protein assay (Pierce) using unlabeled Tm as a standard, and the concentration of pyrene bound to Tm was determined with $\epsilon_{344nm} = 2.2 \times 10^4\ M^{-1}$. The labeling ratio, pyrene/Tm, was 1.6. Singly labeled Tm in which pyrenes occupied only one of the two Cys 190 was prepared by mixing 20% highly labeled Tm with 80% unlabeled Tm in 4 M GdmCl to unfold the chains and dialyzing out the denaturant to refold Tm. TnC and TnI were prepared by standard methods (*20*). S1 was prepared by chymotryptic digestion of myosin (*21*). Concentrations were determined spectrophotometrically using values $(mg/mL)^{-1}$

† Supported by NIH Grants HL 22461 and AR 41637.

* To whom correspondence should be addressed, Boston Biomedical Research Institute. Phone: (617) 912-0381. Fax: (617) 912-0308. E-mail: Lehrer@bbri.org.

‡ Present address: Department of Biochemistry, Boston University School of Medicine, 80 East Concord St., Boston MA 02118.

§ Present address: Biochemistry Department, Imperial College, London SW7 2BZ, U.K.

10.1021/bi992327m CCC: $19.00
Published on Web 01/12/2000

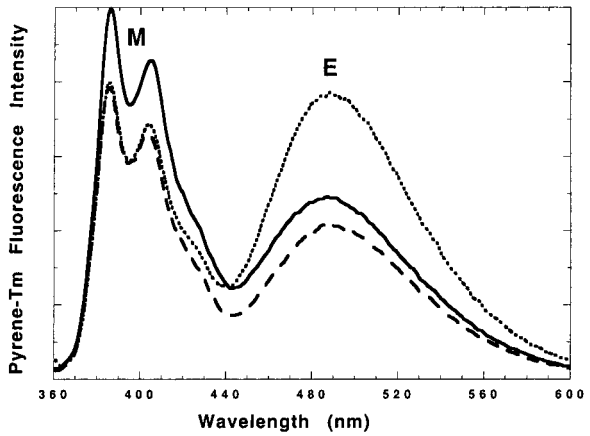

FIGURE 1: Pyrene monomer (M) and excimer (E) spectral changes on binding TnIC to doubly labeled actin−Tm* and reversal of monomer change by S1. Actin−Tm* (- - -); +TnIC (—); +S1 (···). [actin] = 2 μM, [Tm] = 0.3 μM; [TnIC] = 2 μM; [S1] = 2 μM in 10 mM Hepes buffer, pH 7.5, 5 mM $MgCl_2$, 50 mM NaCl, and 2 mM EGTA, 25 °C.

at the indicated wavelengths: 0.63 (290 nm, G-actin); 0.74 (280 nm, S1); 0.40 (280 nm, TnI); 0.18 (280 nm, TnC).

Steady-state fluorescence data were obtained with a SPEX Fluorolog 2/2/2 photon-counting fluorometer (Edison, NJ) in the ratio mode with a 2.5 nm band-pass for both excitation and emission on samples incubated in the 25 °C thermostated housing. Titrations were carried out with excitation at 340 nm and emission at 405 nm to monitor pyrene monomer fluorescence (*10*).

The titration data were fit to a quadratic binding equation (*4*) to obtain the binding constant and stoichiometry using the Kaleidegraph nonweighted least squares program (Synergy).

RESULTS

Binding Using Pyrene−Tm Fluorescence. The fluorescence of rabbit skeletal Tm, highly labeled with pyrene iodoacetamide (Tm*) consists of a structured monomer band in the 400 nm region and a broad excimer band in the 480 nm region (*10*). The excimer band arises from an interaction between pyrenes on adjacent chains and is conformationally sensitive (*22*), whereas the monomer band, arising from noninteracting pyrenes, is sensitive to environment polarity. Singly labeled Tm which is mainly labeled at one of the two equivalent Cys of each chain only exhibits monomer fluorescence. A 30−40% increase in monomer fluorescence and a smaller change in excimer fluorescence was caused by the binding of TnIC to the actin−Tm* complex (Figure 1). Addition of S1 reversed the monomer fluorescence change and produced a large increase in the excimer band. The S1-induced excimer fluorescence change is due to a change in conformation of Tm* on actin associated with a change in thin filament state (*10*). The S1-induced monomer fluorescence change is due to the displacement of TnIC from actin−Tm* when S1 binds to actin. A similar magnitude of monomer fluorescence change occurs on binding TnIC to singly labeled actin−Tm* (Figure 2B), indicating that there was little contribution to the monomer fluorescence change associated with the S1-induced actin−Tm* conformational change.

The change in monomer fluorescence was used in titrations to obtain binding constants and stoichiometry (Figure 2A).

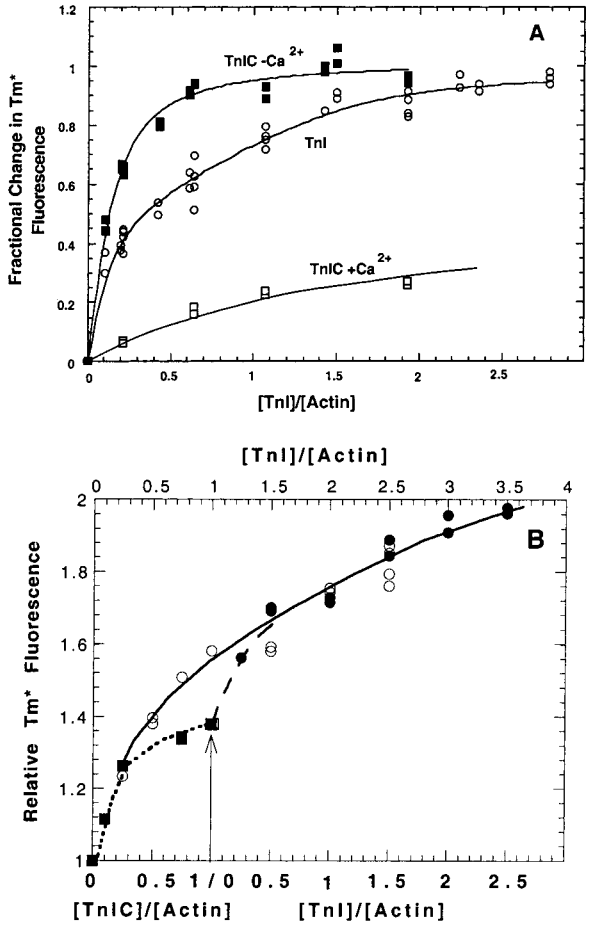

FIGURE 2: Binding of TnI and TnIC to actin−Tm* monitored by pyrene monomer fluorescence. (A) Doubly labeled Tm*. Conditions as for Figure 1 except [NaCl] = 100 mM and [Ca^{2+}] = 0.3 mM for TnIC(+Ca^{2+}). The curves are the best fits to the data. For TnIC, K = 6×10^6 M^{-1}, n = 0.14 TnIC/actin subunit. For TnI 2 classes of binding sites were used by fixing $K_1 = 6 \times 10^6$ M^{-1}, n_1 and n_2 = 0.14 and 0.86 TnI/actin subunit, respectively and obtaining $K_2 = 3 \times 10^5$ M^{-1}. The binding of TnIC + Ca^{2+} was weak and not analyzed. (B) Singly labeled Tm*. Conditions as for Figure 1. Two titrations were done: (1) TnI titration (○, top abcissa); (2) TnIC (■) added to [TnIC]/[actin] = 1, then TnI was titrated (●, bottom abscissa, 0−2.5 TnI/actin).

The results indicated similar binding constants and a stoichiometry of 1 TnI or TnIC/1 Tm/7 actin. Additional weaker binding of TnI but not TnIC which influenced Tm fluorescence was also observed. To further explore the weaker additional binding of TnI and competition with TnIC, the fluorescence of singly labeled Tm* bound to actin was monitored during binding of TnI before and after binding TnIC (Figure 2B). First TnIC was added to actin−Tm* in the absence of Ca^{2+} to ensure saturation at the TnIC specific site (dotted curve) before adding TnI, then TnI was titrated into the TnIC bound actin−Tm system. TnI produced a further increase in fluorescence (dashed curve), similar to that produced by binding TnI in the absence of TnIC (solid curve). These data suggest that TnI displaced TnIC in the specific site and also bound to additional sites on actin to increase the fluorescence. Thus, it appears that TnI and TnIC in the absence of Ca^{2+} bind strongly to a specific site on actin−Tm* near the label on Tm at Cys 190. TnI binds additionally to sites of lower affinity on actin, affecting the environment of the label via a long-range effect.

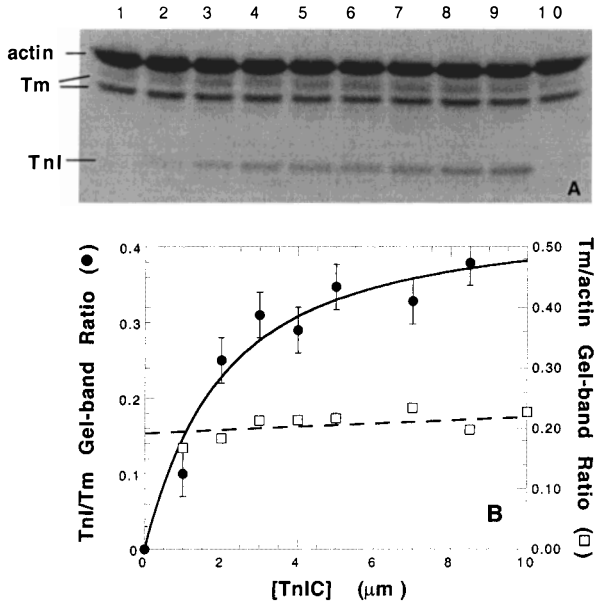

FIGURE 3: Binding of TnIC to actin−Tm monitored by actin sedimentation and gel electrophoresis (16% polyacrylamide−SDS). (A) Gel bands of proteins bound to actin in pellet. Bands 1−9; Increasing concentrations of TnIC from 0 to 10 μM were mixed with actin−Tm in the absence of Ca^{2+} (2 mM EGTA). Band 10; 3 μM TnIC mixed with actin−Tm in the presence of Ca^{2+} (1 mM $CaCl_2$). [actin] = 5 μM, [Tm] = 1 μM, 100 mM NaCl, 10 mM sodium phosphate buffer, pH 7.0, 2 mM $MgCl_2$, and 1 mM DTT, 20 °C. (B) Densitometry of gel bands.

Binding by Actin Sedimentation. Direct evidence for the binding properties of TnIC to actin−Tm was obtained by actin sedimentation and gel electrophoresis. Increasing amounts of TnIC were mixed with actin−Tm and sedimented at high speed. The proteins in the actin-containing pellets were visualized on gels (Figure 3A). In the presence of Ca^{2+}, no TnIC was observed in the pellet (lane 10). Densitometry indicated that TnIC was bound to actin−Tm close to the Tm−actin stoichiometry of 1/7 and a binding constant of 6×10^5 M^{-1} (Figure 3B). The 10 times lower binding constant for TnIC binding to unlabeled actin−Tm compared to pyrene-labeled actin−Tm* (Figure 2) could be due to the influence of the label since an increased binding constant was observed on binding Tn to pyrene labeled Tm* (*10*).

Displacement of TnI and TnIC from Actin−Tm by S1. As indicated in Figure 1, the binding of S1 to actin−Tm* reverses the increase in monomer fluorescence produced by TnI and TnIC. To determine the mechanism of the S1-induced displacement, the monomer fluorescence of actin−Tm*−TnI and actin−Tm*−TnIC mixtures was monitored during titrations with S1. To determine the S1 binding stoichiometry, the light scattering associated with the binding of S1 was also monitored (Figure 4**A**). The data indicate that the binding of S1 to actin−Tm* causes the dissociation of TnI and TnIC from the specific sites at low levels of saturation of S1/actin, i.e., half of the TnI and TnIC is dissociated from actin−Tm when only about 1/5 of the actin sites are occupied with S1.

Direct sedimentation and gel electrophoresis data verified the dissociation of TnIC from actin−Tm at low ratios of binding of S1 (Figure 4B). In the presence of Ca^{2+} (lanes a−f), very little binding of TnIC was observed compared to the absence of Ca^{2+} (lanes g−i). Increasing [S1] decreased the level of TnI and TnC in the actin pellets after sedimentation in the absence and presence of Ca^{2+}. Densitometry verified that about half of TnI and (TnC) were displaced when S1/actin $\approx$ 0.2, much before full saturation of the actin by S1 (Figure 4C).

DISCUSSION

Our work extends the early studies of the binding of TnI and TnIC to actin−Tm (*7*−*9*) with the use of a fluorescence probe primarily at Cys190 of Tm (Tm*). Titrations showed that both TnI and TnIC bind to actin−Tm* with a stoichiometry of 1 TnI or TnIC/7 actin, the same stoichiometry as Tm binding to actin. The 30−40% fluorescence increase is probably due to a local environmental change due to shielding of the pyrene group from solvent. A similar fluorescence increase was observed on binding Tn and TnT to pyrene-labeled Tm (*10*). Thus, the fluorescence change is most probably due to binding in the region of Cys 190, the main site of labeling. However, a long-range effect cannot be entirely ruled out. In view of the stoichiometry and the magnitude of the fluorescence change, it appears that the TnIC−TnI specific binding site combines a region of Tm and a region of 1 of the 7 actin subunits that interact with the Tm. In addition to the specific binding site involving Tm, TnI, but not TnIC, binds to other sites on actin−Tm, probably to actin, in agreement with earlier evidence of binding to actin in the absence of Tm (*7*).

The dissociation of TnI and TnIC from actin−Tm by S1 binding can be explained in two ways; via S1 directly competing with TnI-binding sites on actin or via S1 changing the state of Tm on actin, which causes the loss of the TnI-binding site. Although there is evidence that S1 and TnI bind in the same region of actin, a direct effect would require that the loss of TnI and TnIC binding would occur in parallel to the binding of S1. The above data which show that S1 binding at low S1/actin ratios to actin−Tm is sufficient to dissociate TnI and TnIC argues for the indirect effect. The actin−Tm thin filament equilibrates between open and closed S1-binding states or on and off activity states (*12, 23*). The inhibition of actin-S1 ATPase on binding TnI is due to allosteric shifting of the actin−Tm equilibrium to the off state. However, S1 and S1-ADP, which make strong complexes with actin, trap the actin−Tm filament in the on or open state. Only a few bound S1s per Tm are required in view of the large cooperative unit size (*12*). By shifting the thin filament to the on or open state, the TnI and TnIC thin filament interaction is weakened, causing dissociation. Thus, it appears that TnI and TnIC do not bind well to actin−Tm in the on or open state as required for allosteric inhibitors (*12*). Evidence for a different position of Tm on actin in the on state has been obtained from structural studies (*24*). The loss of a TnI-binding site made up of parts of both Tm and actin due to binding of S1 would be consistent with a movement of Tm which would eliminate that common site.

In the complete thin filament (actin−TmTn), Ca^{2+} causes local dissociation of TnI from actin−Tm (*6*), but interaction of TnIC with the C-terminal region of the TnT component keeps the TnIC attached to the thin filament. As shown previously and verified in this work, in the absence of TnT, complete dissociation of TnIC is facilitated by Ca^{2+} (*9, 25, 26*). Low binding ratios of rigor bonds of S1, such that occur

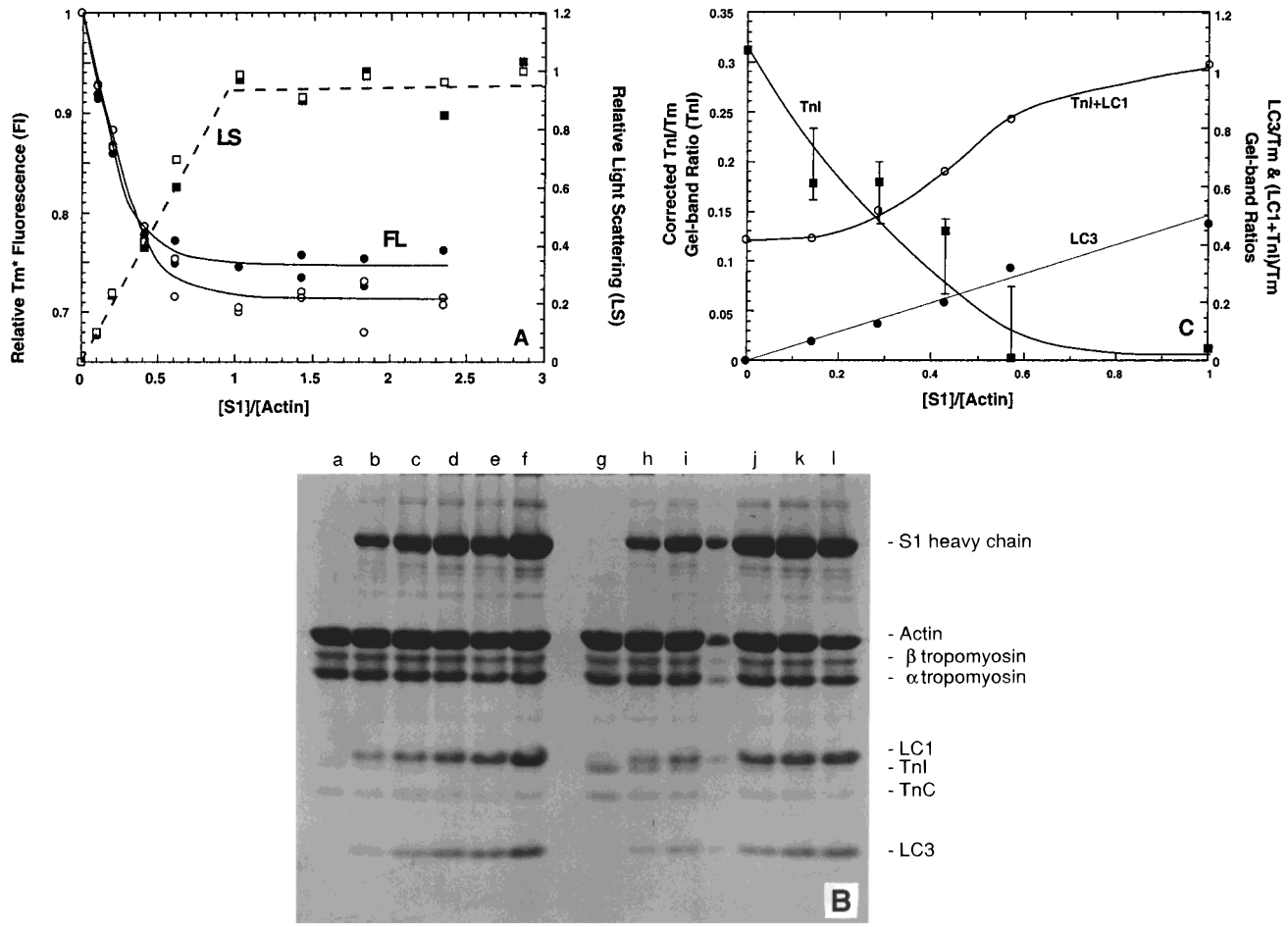

FIGURE 4: Displacement of TnI and TnIC from actin−Tm* by S1. (A) Pyrene monomer fluorescence, (○, ●). S1 binding to actin (□, ■). TnIC, (●, ■); TnI, (○, □). [actin] = 1.4 μM, [Tm*] = 0.2 μM, [TnIC] = 0.7 μM in 50 mM NaCl, 10 mM Hepes buffer, pH 7.5, 5 mM $MgCl_2$, 2 mM EGTA, and 1 mM DTT. [TnI] = 1.4 μM in the same solution except [NaCl] = 100 mM. (B) Intensity of gel bands of proteins bound to actin in pellet after sedimentation and 9 to 18% gradient gel electrophoresis. Increasing concentrations of S1 were added to solutions containing [actin] = 16 μM, [Tm] = 4 μM, [TnI] = 6.7 μM, [TnC] = 12 μM, in 100 mM NaCl, 2 mM $MgCl_2$, 0.4 mM CaCl2 (lanes a−f), or 0.8 mM EGTA (lanes g−i). Sodium phosphate buffer, 10 mM, pH 7.0, 25 °C. Polyacrylamide gradient gel, 9 to 18% . LC1 and LC3 are S1 light chains, which increase in proportion to the bound S1. (C) Densitometry of gel bands in panel B, as a ratio to the constant amount of Tm present. Because the TnI band was not sufficiently resolved from the LC1 band, they were integrated together, and the corrected value of LC1 bound was obtained by subtracting the density of the LC1 + TnI bands at high Ca^{2+} where little TnI was bound. The linear increase in LC3 with S1 added indicates the validity of the correction procedure.

at low [ATP] (*27*) and *N*-ethyl maleimide treated S1, which binds to actin strongly in the presence of ATP, have been shown to activate the actin−TmTn thin filament even in the absence of Ca^{2+} (*28*). Clearly, the activation most probably involves a shift to the on state, releasing TnI. In this case, the TnI is locally dissociated from the specific site but is held on the thin filament by the presence of TnT.

In the absence of Tm, TnI binds weakly to actin to cause inhibition of ATPase (*7*). Since actin does not equilibrate between off and on states, the inhibition is probably due to a direct competition between TnI and S1 for a site on actin. This is supported by the requirement of each actin subunit to bind TnI to give full inhibition. Preliminary S1-binding kinetic studies indicate that TnI binding to actin at high saturation inhibits the S1-binding rate (*14*). However, when TnI binds to actin−Tm with the stoichiometry of 1 TnI/1 Tm/7 actin, the S1-binding rate is inhibited to the same extent as the removal of Ca^{2+} from actin−TmTn, indicating that the significant occupation of the blocked thin filament state is a major cause of the ATPase inhibition. If TnI interacts with the same site on an actin subunit in the absence of Tm and Tn as for actin−Tm and actin−TmTn, TnI could interfere with the binding of S1 to 1 of the seven actin sites, resulting in a contribution to the ATPase inhibition. It thus appears that, in the actin−TmTn thin filament, TnI binds to actin in the absence of Ca^{2+} to inhibit ATPase in two ways: (1) by causing a block of the initial or weak binding of S1 and (2) by stabilizing Tm in the off state which inhibits the isomerization of S1.

ACKNOWLEDGMENT

The excellent technical assistance of Ms. Minhua Chai is appreciated. We thank Dr. Michael Geeves for critical reading of the manuscript.

REFERENCES

1. Leavis, P. C., and Gergely, J. (1984) *CRC Crit. Rev. Biochem. 16*, 235−305.
2. Farah, C. S., and Reinach, F. C. (1995) *FASEB J. 9*, 755−767.
3. Zot, A. S., and Potter, J. D. (1987) *Annu. Rev. Biophys. Biophys. Chem. 16*, 535−559.
4. Morris, E. P., and Lehrer, S. S. (1984) *Biochemistry 23*, 2214−2220.

5. Perry, S. V. (1999) *Mol. Cell. Biochem. 190*, 9−32.
6. Tao, T., Gong, B.-J., and Leavis, P. C. (1990) *Science 247*, 1339−1341.
7. Perry, S. V., Cole, H. A., Head, J. F., and Wilson, F. J. (1972) *Cold Spring Harbor Symp. Quantum Biol. 37*, 251−262.
8. Potter, J. D., and Gergely, J. (1974) *Biochemistry 13*, 2697.
9. Hitchcock, S. E. (1975) *Eur. J. Biochem. 52*, 255.
10. Ishii, Y., and Lehrer, S. S. (1990) *Biochemistry 29*, 1160.
11. McKillop, D. F. A., and Geeves, M. A. (1993) *Biophys. J. 65*, 693−701.
12. Lehrer, S. S., and Geeves, M. A. (1998) *J. Mol. Biol. 277*, 1081−1089.
13. Zhou, X., Morris, E. P., and Lehrer, S. S. (1995) *Bophys. J. 68*, A167.
14. Lehrer, S. S., Chai, M., and Geeves, M. A. (1997) *Biophys. J. 72*, A60.
15. Spudich, J., and Watt, S. (1971) *J. Biol. Chem. 246*, 4866−4871.
16. Lehrer, S. S., and Kerwar, G. (1972) *Biochemistry 11*, 1211−1217.
17. Lehrer, S. S., and Morris, E. P. (1982) *J. Biol. Chem. 257*, 8073−8080.
18. Lehrer, S. S., and Morris, E. P. (1984) *J. Biol. Chem. 259*, 2070−2072.
19. Lehrer, S. S. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3377−3381.
20. Van Eerd, J., and Kawasaki, Y. (1973) *Biochemistry 12*, 4972−4980.
21. Weeds, A. G., and Pope, B. (1977) *J. Mol. Biol. 111*, 129−157.
22. Lehrer, S. S. (1997) *Methods Enzymol. 278*, 286−295.
23. Lehrer, S. S. (1994) *J. Muscle Res. Cell Motil. 15*, 232−236.
24. Vibert, P., Craig, R., and Lehman, W. (1997) *J. Mol. Biol. 266*, 8−14.
25. Hitchcock, S. E., Huxley, H. E., and Szent-Gyorgi, A. G. (1973) *J. Mol. Biol. 80*, 825−836.
26. Potter, J. D., and Gergely, J. (1975) *J. Biol. Chem. 250*, 4628−4633.
27. Bremel, R. D., Murray, J. M., and Weber, A. (1972) *Cold Spring Harbor Symp. Quantum Biol. 37*, 267−275.
28. Nagashima, H., and Asakura, S. (1982) *J. Mol. Biol. 155*, 409−428.

BI992327M