Inhibition of Actin−Myosin Subfragment 1 ATPase Activity by Troponin I and IC: Relationship to the Thin Filament States of Muscle†

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ABSTRACT: Troponin I (TnI) is the component of the troponin complex that inhibits actomyosin ATPase activity, and Ca^{2+} binding to the troponin C (TnC) component reverses the inhibition. Effects of the binding of TnI and the TnI−TnC (TnIC) complex to actin−tropomyosin (actinTm) on ATPase and on the binding kinetics of myosin subfragment 1 (S1) were studied to clarify the mechanism of the inhibition. TnI and TnIC in the absence of Ca^{2+} bind to actinTm and inhibit ATPase to similar levels with a stoichiometry of one TnI or one TnIC per one Tm and seven actin subunits. TnI also binds to actinTmTn in the presence of Ca^{2+} with a stoichiometry and inhibition constant similar to those for the binding to actinTm of TnI and Tn in the absence of Ca^{2+}. Thus, in the presence of Ca^{2+}, the intrinsic TnI which is released from its binding site on actinTm does not interfere with the binding of an extra molecule of TnI to actinTmTn. The rate of S1 binding to actinTmTnI and to actinTmTnTnI in the presence of Ca^{2+} was inhibited to the same extent as upon removal of Ca^{2+} from actinTmTn. These studies show that TnI inhibits ATPase by the same mechanism as Tn in the absence of Ca^{2+}, by shifting the thin filament equilibria from the open state to the closed and blocked states.

In vertebrate skeletal and cardiac muscle, the troponin−tropomyosin (TnTm)[1] complex interacts with actin to confer Ca^{2+} sensitivity to muscle contraction and to actin−myosin ATPase (see reviews in refs *1*−*5*). Tm is an α-helical coiled coil molecule which interacts with seven actin subunits (0.14 Tm per actin subunit) and with itself in a head to tail fashion to form a continuous filament along actin (*6*). Tn, which interacts with each Tm, contains three subunits, TnC, TnT, and TnI. An extended region of TnT interacts with a region of Tm that includes Cys 190 to the C-terminus at the head−tail overlap (*7*, *8*). The C-terminal region of TnT also interacts with the TnIC complex, anchoring it to actinTm. In the absence of Ca^{2+}, TnI binds to one or two actin subunits (*9*, *10*), which results in the inhibition of contraction. Binding of Ca^{2+} to TnC causes local dissociation of TnI from actin, relieving the inhibition (*11*).

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 (*12*−*14*). The bound TnI was sufficient to almost completely inhibit actinTm−S1 ATPase activity, but the activity was not similarly inhibited by the binding of TnIC in the absence of Ca^{2+} (*15*−*17*). Recent studies showed that TnI and TnIC in the absence of Ca^{2+} bind specifically to actinTm to a site that probably jointly involves actin and Tm near position 190 of Tm, and the binding of myosin heads (S1) at low degrees of saturation (S1/actin < 0.5) causes dissociation of TnI and TnIC from actinTm (*18*).

Here we show that the binding of both TnI and TnIC in the absence of Ca^{2+} inhibits actinTm−S1 ATPase activity like the binding of Tn in the absence of Ca^{2+}. The stoichiometry of the inhibition is one TnI or one TnIC per one Tm and seven actins, similar to that of whole Tn in the absence of Ca^{2+}. We also show that an additional molecule of TnI is bound to actinTmTn in the presence of Ca^{2+} to inhibit ATPase to the same level and with the same stoichiometry and binding constant as TnI binding to actinTm, indicating that Ca^{2+} causes considerable movement of the intrinsic TnI away from its site on actin and Tm. Kinetic data indicated that the rate of binding of S1 to all three systems, actinTmTn in the absence of Ca^{2+}, actinTm-TnI, and actinTmTnTnI in the presence of Ca^{2+}, was inhibited to the same extent.

These results indicate that TnI and TnIC in the absence of Ca^{2+} inhibit actinTm−S1 ATPase by shifting the equilibrium from the open (on-activity state) to the closed and blocked states (both off-activity states) of the three S1-binding states of the thin filament (blocked, closed, and open) (*19*). A preliminary report has been presented (*20*).

EXPERIMENTAL PROCEDURES

Rabbit skeletal actin was prepared from an acetone powder of rabbit muscle and purified by cycles of polymerization and depolymerization (*21*, *22*). Rabbit skeletal Tm was purified by isoelectric precipitation and ammonium sulfate fractionation (*22*, *23*). TnC and TnI were prepared by

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[1] Abbreviations: S1, myosin subfragment 1; Tm, tropomyosin; Tn, troponin; TnC, troponin C; TnI, troponin I; TnT, troponin T.

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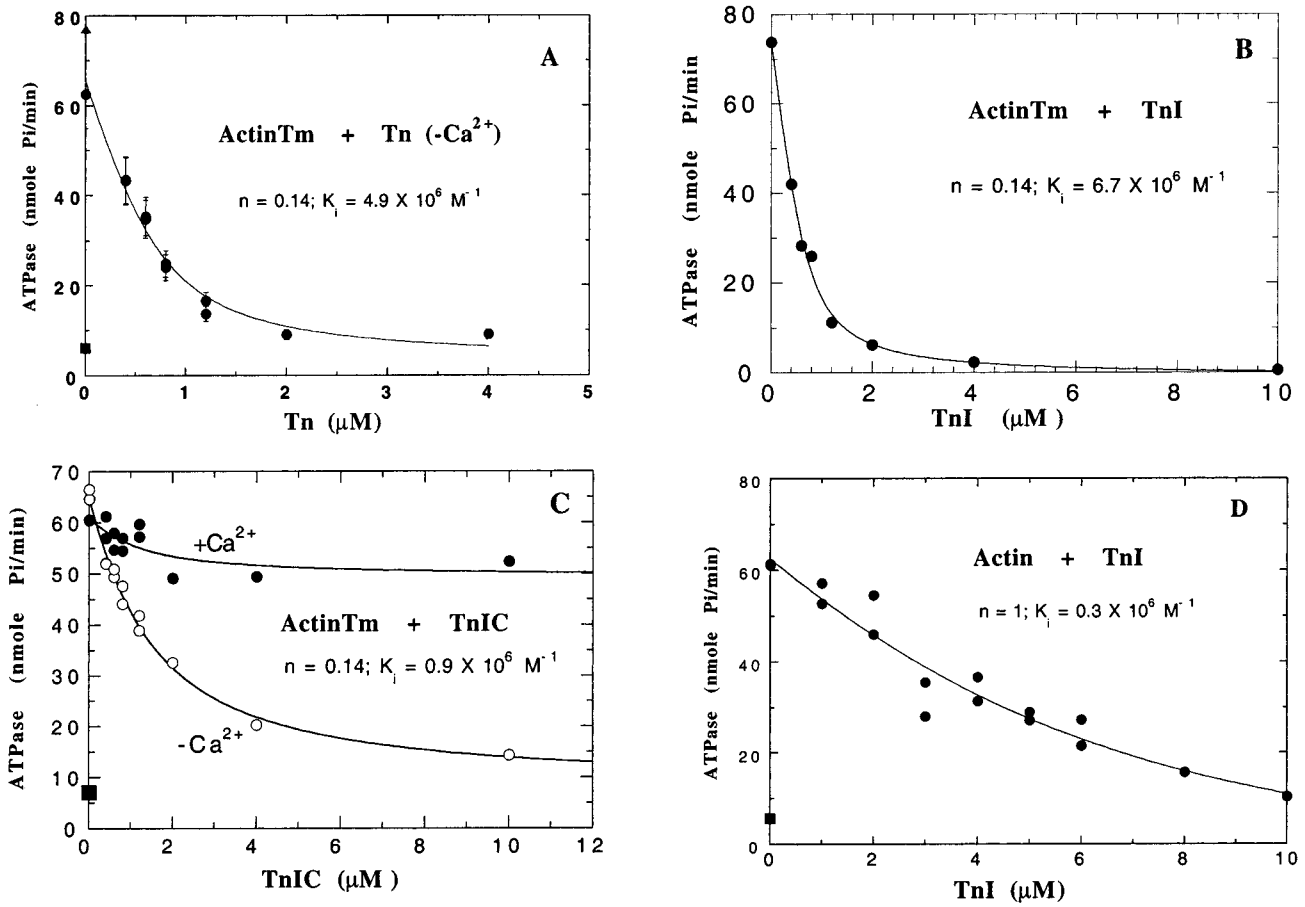

FIGURE 1: Inhibition of actin-activated S1 ATPase by Tn, TnI, and TnIC binding to actin and actinTm. (**A**) Tn in the absence of Ca^{2+} binding to actinTm (●). S1 alone (■). S1 and actin (▲). (**B**) TnI binding to actinTm. S1 alone background subtracted. (**C**) TnIC binding to actinTm (with Ca^{2+}, ●). S1 alone (■). (**D**) TnI binding to actin (●). S1 alone (■). Conditions were as follows: 5 μM F-actin, 1.12 μM Tm, and 9 μM S1 in 37 mM NaCl, 6 mM $MgCl_2$, 3 mM ATP, 10 mM Hepes buffer (pH 7.6), 1 mM DTT, and 1 mM EGTA (without Ca^{2+}) at 25 °C or 0.2 mM $CaCl_2$ (with Ca^{2+}). S1 binding conditions were as follows: 5 μM F-actin, 1 μM Tm, and 1 μM S1 in 60 mM NaCl, 5 mM $MgCl_2$, and 20 mM Mops buffer at pH 7.0 and 20 °C.

standard methods (*24*). S1 was prepared by chymotryptic digestion of myosin (*25*). Concentrations were determined spectrophotometrically using the following absorption values (per milligrams per milliliter) at the indicated wavelengths: 0.63 (290 nm, G-actin), 0.74 (280 nm, S1), TnI (0.40, 280 nm), and TnC (0.18, 280 nm). Pyrene-labeled actin was prepared as decribed previously (*26*).

Sedimentation studies were performed by mixing TnI and TnIC with actinTm, spinning at 250000*g* for 30 min or at 100000*g* for 40 min at 20 °C, homogenizing the pellets containing increasing amounts of TnI and TnIC, and running the samples on 4 to 20% gradient SDS−PAGE gels (Novex) as indicated in the figure legends. The relative amounts of protein present in the stained gels were determined by scanning the region of interest and using a densitometric analysis program (NIH Image). The areas of the gel bands were normalized to the area of the Tm or TnC bands whose concentrations were constant.

ATPase measurements were performed as outlined previously (*27*), and stopped-flow experiments were used to monitor the pyrene monomer fluorescence of actin (*28*).

ATPase titration profiles were fit to the quadratic binding equation (*8*) to give inhibition constants and stoichiometries using the nonweighted nonlinear least-squares program in Kaleidagraph (Synergy).

RESULTS

Effects of TnI and TnIC on ActinTm−S1 ATPase. In the presence of Tm, the plot of actin-activated S1 ATPase activity versus S1 concentration is sigmoidal with inhibition at low S1 concentrations and activation at intermediate S1 concentrations (*22*) as a result of the S1-induced shift in the actinTm equilibrium from the off-activity to the on-activity state (*4*). The V_{max} in the on-state is, however, the same as in the absence of Tm and TmTn (*29*). At very low S1 concentrations, the actinTm−S1 ATPase activity is quite low. However, to document the inhibition of actinTm−S1 ATPase by Tn components, a sufficient S1 concentration was chosen to allow a sufficiently high activity so that accurate inhibition titrations could be performed (Figure 1).

In Figure 1A, the ATPase values under our conditions for S1 alone (6 nmol/min), for actin−S1 (77 nmol/min), and for actinTm−S1 (62 nmol/min) are indicated before the Tn titration. Titrations with Tn in the absence of Ca^{2+} resulted in the inhibition of actin-activated S1 ATPase activity close to background levels of S1 ATPase alone, in agreement with previous data from several laboratories. Similar inhibition profiles were obtained for TnI alone (Figure 1B), and for TnIC in the absence of Ca^{2+} (Figure 1C), indicating a similar mechanism for the inhibition. Essentially no inhibition was observed for TnIC in the presence of Ca^{2+} (Figure 1C), as

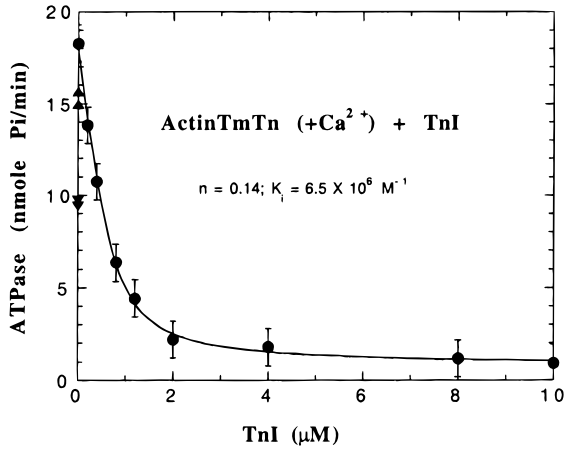

FIGURE 2: ATPase inhibition by TnI binding to actinTmTn (with Ca^{2+}). Conditions were as described for Figure 1 except [Tn] = 2 μM and [S1] = 2 μM.

expected since, under these conditions, TnIC is essentially dissociated from actinTm. From the titrations, we obtained inhibition constants ($K_i = 5-7 \times 10^6$ M^{-1}) for both Tn and TnI, with a 5-fold smaller inhibition constant for TnIC, and a stoichiometry of one TnI per one Tm and seven actins in all cases. In contrast, addition of TnI to actin in the absence of Tm required high concentrations to yield appreciable inhibition (Figure 1D). The analysis indicated an approximate value for K_i of 0.3×10^6 M^{-1} and a stoichiometry of one TnI per actin subunit. This is consistent with a much weaker binding of TnI to each actin subunit.

Effects of TnI on ActinTmTn−S1 ATPase. The binding of Ca^{2+} to the actinTmTn-reconstituted thin filament results in the local dissociation of the TnI component from actin (*11*), exposing the TnI binding site on the thin filament. The TnI remains tethered to the thin filament via the TnT component. To study the properties of the Ca^{2+}-exposed TnI binding site, TnI titrations were performed on actinTmTn in the presence of Ca^{2+} by monitoring the ATPase (Figure 2). A lower S1 concentration was used for this titration (2 μM) compared to the concentration of 9 μM used for Figure 1, since there was a higher ATPase activity in the presence of Tn and Ca^{2+}. It was found that TnI addition to actinTmTn in the presence of Ca^{2+} resulted in the inhibition of ATPase to a degree similar to that of addition of TnI to actinTm. The inhibition data also gave a similar inhibition constant (6.5×10^6 M^{-1}) and stoichiometry (one TnI per one Tm and seven actins). Thus, in the presence of Ca^{2+}, the local dissociation of TnI from actin is extensive enough not to interfere with the additional binding of another molecule of TnI to produce a thin filament with the composition $actin_7 \cdot Tm_1 \cdot TnT_1 \cdot TnI_2 \cdot TnC_1$. In the absence of Ca^{2+}, the system was largely inhibited, but additional inhibition of ATPase could be produced on addition of extra TnI (Table 2). This was most probably due to the weak binding to free actin sites as observed for actin alone (Figure 1D).

Direct Assessment of TnI Binding. To verify that the inhibition of ATPase was the result of stoichiometric binding of TnI to actinTm and to actinTmTn, the composition of pellets was investigated after sedimenting actinTm mixtures with Tn components (Figure 3). It can readily be seen that the same amount of TnI was bound to actinTm (lane 1) as

Table 1: Stoichiometry of TnI Bound to Reconstituted Thin Filaments As Determined by Densitometric Analysis of Pellets

sample[a]	Ca^{2+}	A_I/A_{Tm}[b]	TnI/Tm	A_I/A_C[b]	TnI/TnC
Tm and TnI (unspun control)		0.63	(1.0)		
actin, Tm, and TnI		0.66	1.05		
actin, Tm, TnI, and S1		0.50	0.79[c]		
Tn (unspun control)				3.1	(1.0)
Tn and TnI (unspun control)				6.0	(1.94)
actin, Tm, and Tn	+			3.3	1.06
actin, Tm, Tn, and TnI	+			5.6	1.8
actin, Tm, Tn, TnI, and S1	+			4.25[c]	1.4
actin, Tm, and Tn	−			3.2	1.0
actin, Tm, Tn, and TnI	−			8.3	2.7
actin, Tm, Tn, TnI, and S1	−			6.4[c]	2.0

[a] Unspun samples and homogenized pellets were loaded on gels as indicated in the legend of Figure 3. Equimolar concentrations were used for unspun controls. Reconstituted thin filaments contained 7 μM actin, 2 μM Tm, 2 μM Tn, 2 μM TnI, and 1.4 μM S1. The unspun controls were used to obtain correction factors (see footnote b) for the stained gel band area ratios. [b] Ratio of the absorbance of stained gel band areas of TnI to Tm or to TnC. [c] Corrected for the overlapping LC1 band with a control sample without TnI.

Table 2: TnI Inhibition of ATPase and the Rate of S1 Binding to Actin, ActinTm, and ActinTmTn and Comparison with the Inhibition Caused by the Removal of Ca^{2+} from ActinTmTn

	S1 binding rate[a]		
system	k_{obs} (s^{-1})	% inhibition	ATPase % inhibition[b]
actin, Tm, and Tn with Ca^{2+}	30.0		
actin, Tm, and Tn without Ca^{2+}	12.6	58	>95
actin and Tm	40.0		
actin and TnI	11.9	72	>95
actin, Tm, and Tn with Ca^{2+}	30.0		
actin, Tm, Tn, and TnI	8.3	72	91
actin, Tm, and Tn with Ca^{2+}	12.6		
actin, Tm, Tn, and TnI	8.8	30	≈50
actin	34.6		
actin and TnI	26.0	25	≈50

[a] [actin*] = 5 μM. [Tm] = 1.1 μM. [S1] = 1 μM. [Tn] = 2 μM. [TnI] = 3.35 μM. The buffer included 60 mM KCl, 5 mM Mg^{2+}, and 20 mM Mops at pH 7 and 20 °C. [b] [actin] = 5 μM. [Tm] = 1.1 μM. [S1] = 2−9 μM. [Tn] = 2 μM. [TnI] = 3.35 μM. The buffer included 36 mM NaCl, 6 mM Mg^{2+}, 3 mM ATP, and 10 mM Hepes at pH 7.5 and 25 °C.

was present in the control Tm/TnI mixture (lane 3). In addition, considerably more TnI sedimented with actinTmTn than is intrinsically present (lane 7 vs lane 6), but the amount was about the same as a control Tn sample that contains twice as much TnI (lane 5). Densitometric analyses used unspun mixtures of Tn components as controls (1/1 TnI/Tm or TnI/TnC) to determine staining ratios (Table 1). The data showed that one TnI bound to $actin_7Tm$ and an additional TnI bound to actinTmTn in the presence of Ca^{2+}. Thus, the observed inhibition of ATPase is due to the binding of TnI. In the absence of Ca^{2+}, TnI in addition to that present in Tn can be bound to actinTmTn. Although the data for the binding of TnI to actinTmTn($-Ca^{2+}$) are semiquantitative due to solubility problems in the absence of strong binding sites, they were included to confirm that some weak binding does take place. We kept the TnI concentration low to minimize precipitation, but in the absence of strong binding sites, some precipitation may have taken place to account for the larger amount of TnI found in the actin pellet for actinTmTn($-Ca^{2+}$) than of that with Ca^{2+}.

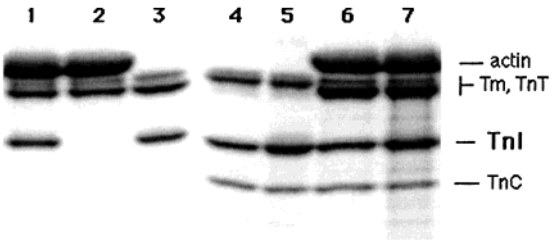

FIGURE 3: Binding of TnI to actin as assessed by sedimentation and gel electrophoresis. Pellets were obtained by ultracentrifugation (250000*g* for 30 min at 20 °C) of actin-containing solutions (200 μL) at the indicated concentrations and homogenized in 50 μL of buffer, and 20 μL of each sample was loaded on gels. If stoichiometric amounts of Tm and Tn pelleted with actin (1/7), the Tm and Tn bands on the gel would match the control mixtures: lane 1, 7 μM actins, 2 μM Tm's, and 2 μM 2Tn's spun; lane 2, 7 μM actins and 2 μM Tm's spun; lane 3, 4 μM Tm's and 4 μM TnI's in unspun control; lane 4, 4 μM Tn's in unspun control; lane 5, 4 μM Tn's and 4 μM TnI's in unspun control; lane 6, 7 μM actins, 2 μM Tm's, and 2 μM TnI's spun; lane 7, 7 μM actins, 2 μM Tm's, 2 μM Tn's, and 2 μM TnI's (with Ca^{2+}) spun. The buffer included 50 mM NaCl, 5 mM $MgCl_2$, 10 mM Hepes, and either 0.15 mM $CaCl_2$ (with Ca^{2+}) or 0.66 mM EGTA (without Ca^{2+}).

The data also show that the binding of small amounts of S1 to actin (one S1 per seven actins) released TnI from the thin filament. The data for the S1-induced dissociation are also less precise because of the problem with correcting for the overlapping of LC1 with TnI. These data were included to show the general effect of S1, which was more clearly studied in a previously published paper (*18*).

TnI Inhibition of the S1 Binding Rate. We have previously shown that for an actinTmTn filament removal of Ca^{2+} shifts the open−closed−blocked equilibria to the blocked state of the thin filament, defined by the fraction of total actin sites that are unavailable to bind S1. The kinetics of S1 binding to an excess of actin sites is the most convenient way to assess the available actin sites since the observed exponential rate constant (k_{obs}) of S1 binding is directly proportional to the concentration of available actin sites ($k_{obs} = k_1$[A], where k_1 is the second-order rate constant for S1 binding). In the presence of Ca^{2+}, k_{obs} is identical to that for pure actin filaments which defines k_1 and the maximum actin concentration. Removal of calcium reduces k_{obs} to $^1/_3$ and is consistent with 67% of the actin being blocked by TmTn. Addition of TnI to actinTm caused an up to 3−4-fold inhibition of the observed rate constant of S1 binding which is similar to the reduction observed on removal of Ca^{2+} from actinTmTn (Table 2). This is consistent with the formation of a similar level of blocked actin sites and requires one TnI per seven actins. The rate reduction also parallels the inhibition of ATPase to background values (data not shown). The addition of TnI to actinTmTn in the presence of Ca^{2+} also decreased the k_{obs} for S1 binding to a similar degree (Figure 4 and Table 2). Additions of TnI to actinTmTn in the absence of Ca^{2+} or to actin alone resulted in a 25−30% smaller inhibition of the S1 binding rate. Thus, as for the ATPase and the sedimentation study, TnI binds readily to actinTm and actinTmTn in the presence of Ca^{2+} to inhibit the interaction with S1. TnI binds much more weakly to actin alone or to actinTmTn in the absence of Ca^{2+}. The specific binding of TnI therefore causes a parallel inhibition of the observed rate constant of S1 binding and loss of actinTm−S1 ATPase.

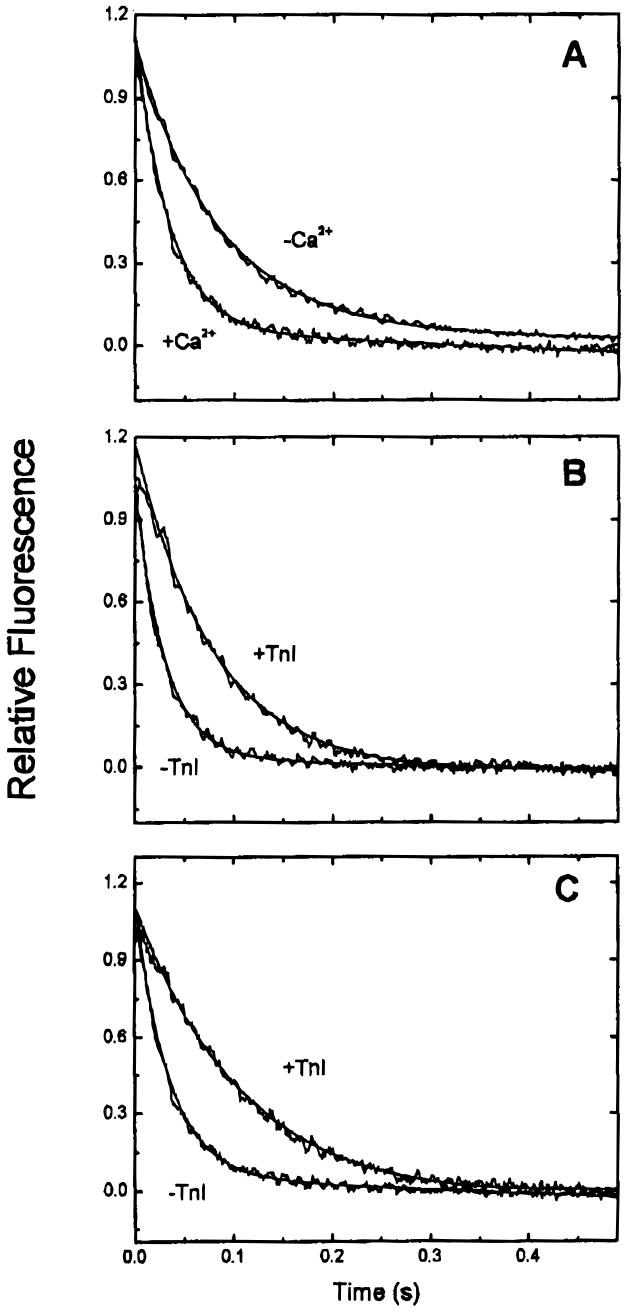

FIGURE 4: Comparison of the inhibition of the S1 binding rate caused by Ca^{2+} removal from actinTmTn (A), with TnI addition to actin*Tm (B), and with TnI addition to actin*TmTn (with Ca^{2+}) (C). [actin*] = 5 μM. [Tm] = 1.1 μM. [S1] = 1 μM. [Tn] = 2 μM. [TnI] = 3.35 μM. The buffer include 60 mM KCl, 5 mM $MgCl_2$, and 20 mM Mops at pH 7 and 20 °C (actin* is pyrene-labeled actin).

DISCUSSION

Inhibition of ATPase by TnI and TnIC. The observation that TnI inhibits actinTm−S1 ATPase activity agrees with early and recent studies (*9*). Our titration data, however, allow for a more careful comparison of effects of TnI with whole Tn in the absence of Ca^{2+} and also with TnIC. The data indicate that TnI inhibits the ATPase activity to the same degree and with the same stoichiometry and inhibition constant as Tn in the absence of Ca^{2+}, suggesting that the degree of inhibition is solely determined by TnI, with little contribution from TnC and TnT. The fact that TnT apparently

does not contribute to the affinity of Tn in view of the strong binding of TnT to Tm may be due to a compensatory effect of a reduced contribution from TnI in the Tn complex.

The ATPase inhibition that we observed with TnIC appears to be somewhat controversial. Early studies reviewed recently (*30*) and more recent studies have indicated either that TnIC in the absence of Ca^{2+} does not inhibit or that the extent of inhibition of TnI is reduced by the addition of TnC even though TnI is thought to remain bound to actinTm. The discrepancy with our results appears to be related to the necessity to include 2.5−3 mM Mg^{2+} in excess over the ATP concentration to obtain complete inhibition by Tn in the absence of Ca^{2+} (*31*). Indeed, when such amounts were included, TnC did not appreciably relieve the inhibition of TnI (*9*); i.e., TnIC inhibited actinTm−S1 ATPase. It appears that the Ca^{2+} or Mg^{2+} high-affinity sites on TnC need to be fully occupied (*31*). The fact that TnIC can inhibit ATPase data to the same level with the same stoichiometry as Tn and TnI indicates that a similar mechanism is involved.

Our data and previous data (*12*) have shown that in the absence of Tm, TnI can bind weakly to each actin subunit to inhibit ATPase. Thus, although TnI does not apparently bind to Tm alone, it binds strongly and specifically to a site on actinTm, involving one Tm molecule since the stoichiometry is one TnI per one Tm and seven actins. Either Tm increases the affinity for one actin site out of the seven, or Tm forms a joint site involving both an actin subunit and a region of Tm. The latter explanation seems more reasonable and is supported by the observation of the environmental change of a fluorescence probe on Cys 190 of Tm produced by the binding of TnI and TnIC (*32*).

Configuration of TnI in the ActinTmTn Thin Filament at High Ca^{2+} Concentrations. The distance between donor and acceptor fluorescence labels on TnI and actin increases upon Ca^{2+} binding to the complete thin filament (actinTmTn) (*11*), supporting the concept of local dissociation of TnI which remains anchored to the thin filament via TnT. Our data show that an additional TnI can be bound to this system with the same inhibition constant as Tn in the absence of Ca^{2+} or TnI binding to actinTm. Thus, it appears that the intrinsic TnI is sufficiently dissociated locally and the TnI binding site is sufficiently exposed so that there is no interference with the binding of another TnI. These data suggest that for the complete thin filament, Ca^{2+} binding to the TnC complex dissociates TnI not only from its site on actin but also from a Tm site. This further emphasizes the importance of TnT in retaining the TnIC complex on the filament surface. The fact that native thin filaments can strongly bind an extra TnI per seven actin subunits suggests that the extra density might be useful for structural studies.

Relationship of ATPase Inhibition to Thin Filament States. We recently proposed that Tm can be considered to be the regulatory component which sets up a two-state equilibrium for the actinTm system, resulting in on- and off-activity states (*4*). In this cooperative and allosteric model, Tn in the absence of Ca^{2+} was considered to be an allosteric inhibitor because it shifted the equilibrium to the off-activity state. However, it was recognized that Tn in the absence of Ca^{2+} had another inhibitory property; it produced substantial blocking of the initial binding of S1. This blocked state is one of the three S1-binding states of the thin filament (blocked, closed, and open) with the indicated equilibrium constants, K_B and K_T (*19*)

	K_B		K_T	
S1 binding state	Blocked	↔	Closed	↔ Open
ATPase state	Off		Off	On

In the blocked state, the initial binding of S1 to actin to form the S1−A complex with actin is blocked. In the closed state, the initial binding of S1 to actin is possible but the isomerization of actoS1 from the A state to the R state is inhibited. Only the open state results in actin-activated ATPase.

The current observation that the k_{obs} for S1 binding to the three systems (actinTmTn in the absence of Ca^{2+}, actinTmTnI, and actinTmTnTnI in the presence of Ca^{2+}) was inhibited to the same extent, compared to that in the presence of Ca^{2+} in the first case and the absence of TnI in the last two cases, indicated that a similar occupation of blocked state is produced in all three cases. Thus, TnI is as effective as whole Tn in inducing the blocked state.

Since TnI behaves like Tn in the absence of Ca^{2+}, the influence of TnI on the ATPase activity can be explained in terms of recent data on the influence of Tn on the three states of the thin filament (*28*). This recent work gave the following values: $K_T = 0.14$ and $K_B \geq 10$ in the presence of Ca^{2+} compared to $K_T = 0.033$ and $K_B = 0.5$ in the absence of Ca^{2+}. This predicts a fractional occupancy of the blocked, closed, and open states of <0.08, 0.87, and 0.13 in the presence of Ca^{2+} (where closed + open = 1.00−0.92 when blocked = 0−0.08) and 0.67, 0.32, and ∼0.01 in the absence of Ca^{2+}, respectively. As the ATPase primarily reflects the actin sites in the open or on-activity state, at the limit of very low S1 concentrations, removal of Ca^{2+} will reduce the fraction of on-activity states from 0.13 to ≤ 0.01. Although we do not yet have an independent value of K_T for actinTmTnI, it is expected to be similar to that for actinTmTn in the absence of Ca^{2+} because K_B is about the same and the ATPase activity is inhibited to the same degree, indicating a similar amount of the open state.

The TnI inhibition of ATPase can therefore be explained by the increased occupancy of the blocked and closed states, resulting in a reduction in the occupancy of the open or on-activity state of the thin filament. Our previous binding studies (*32*) indicated that TnI and TnIC in the absence of Ca^{2+} bind to actinTm in the blocked and closed states (*18*). This provides additional evidence that Tn in the absence of Ca^{2+} and TnI and TnIC in the absence of Ca^{2+} stabilize actinTm in the closed and blocked states (off-activity states) both sterically and allosterically.

Relationship of the Three Thin Filament States to Structure. The data presented here along with the previous solution kinetic data for the three states of the thin filament (*28*) do not distinguish between structural models of regulation. However, the correlation between the three regulatory states of actin filaments (blocked, closed, and open) and the three positions of Tm observed in EM image reconstructions of thin filaments ($-Ca^{2+}$, $+Ca^{2+}$, and +S1) (*33*) suggests that the simplest explanation involves a steric blocking model (*34*−*36*).

In the closed state, Tm is in a position on actin to directly inhibit the isomerization of the S1−actin complex into its

strong-binding R state (*37*), thereby inhibiting activity. However, once an S1 is in the strong binding R state, the S1 traps Tm in the open state which then allows activation of ATPase. Since *n* actin subunits become available, the activation is cooperative. The value of *n* depends on the components present on the thin filament. In the skeletal actinTm filament, the presence of Tn increases *n* for the closed to open transition from 5−6 to 10−12 (*38*). The role of the individual Tn components in this change in *n* remains to be resolved, but a large part of the increase can be attributed to TnT1, the N-terminal fragment of TnT (*39*).

The cooperativity in the blocked to closed transition appears to be smaller than that for the closed to open transition. The limit of the information transfer along the filament in this case may be due to TnI tying down Tm using a joint site with actin in a ternary complex, actinTmTnI. This occurs every seven actins and limits the cooperativity to $n = 7$ at maximum. The steric blocking must involve Tm since in the absence of Tm our kinetic data indicate that TnI can only inhibit S1 binding to a single actin site without transmission of the information to adjacent actins. It is possible that Tm facilitates actin−actin communication indirectly (*5*), but the simplest model is that it is the Tm itself which directly blocks the S1 binding site as suggested in the EM reconstructions. However, it remains clear that TnI is binding to a site on actin in which the TnI itself can inhibit S1 binding to that particular site. Thus, it appears that both TnI and Tm are involved in steric blocking, with the major effect being due to Tm.

The inhibition of the ATPase of the actinTm regulatory system interacting with S1 ATP only requires TnI and only one TnI per Tm. To remove the inhibition, TnC and Ca^{2+} are required. To prevent the TnIC components from diffusing away in the presence of Ca^{2+}, the TnT component is also necessary. Evidence for other roles for TnT in regulation has been obtained (*39*−*42*). Clearly, further studies are required to determine the precise changes in the molecular arrangement within the actinTmTn thin filament associated with the three regulatory states and of the relationship to Ca^{2+} regulation in muscle.

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