

Effects of deletion of tropomyosin overlap on regulated actomyosin subfragment 1 ATPase

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The role of the overlap region at the ends of tropomyosin molecules in the properties of regulated thin filaments has been investigated by substituting nonpolymerizable tropomyosin for tropomyosin in a reconstituted troponin–tropomyosin–actomyosin subfragment 1 ATPase assay system. A previous study [Heeley, Golosinka & Smillie (1987) *J. Biol. Chem.* **262**, 9971–9978] has shown that at an ionic strength of 70 mM, troponin will induce full binding of nonpolymerizable tropomyosin to F-actin both in the presence and absence of calcium. At a myosin subfragment 1-to-actin ratio of 2:1 ([actin] = 4 μ M) and an ionic strength of 50 mM, comparable levels of ATPase inhibition were observed with increasing levels of tropomyosin or the truncated derivative in the presence of troponin ($-Ca^{2+}$). Large differences were noted, however, in the activation by Ca^{2+} . Significantly lower ATPase activities were observed with nonpolymerizable tropomyosin and troponin ($+Ca^{2+}$) over a range of subfragment 1-to-actin ratios from 0.25 to 2.5. The concentration of subfragment 1 required to generate ATPase activities exceeding those seen with actomyosin subfragment 1 alone under these conditions was 3–4-fold greater when nonpolymerizable tropomyosin was used. Similar effects were seen at the much lower ionic strength of 13 mM and are consistent with the reduced ATPase activity with nonpolymerizable tropomyosin observed previously [Walsh, Trueblood, Evans & Weber (1985) *J. Mol. Biol.* **182**, 265–269] at low ionic strength and a subfragment 1-to-actin ratio of 1:100. Little cooperativity in activity as a function of subfragment 1 concentration with either intact tropomyosin or its truncated derivative was observed under the present conditions. Further studies are directed towards an understanding of these effects in terms of the two-state binding model for the attachment of myosin heads to regulated thin filaments.

INTRODUCTION

Tropomyosin (TM) together with the troponin (Tn) complex is responsible for the Ca^{2+} regulation of the actomyosin ATPase systems of skeletal and cardiac muscles (for reviews, see refs. [1–3]). Situated in the grooves of the F-actin structure, each TM molecule spans seven actin monomers on each of the two actin strands and interacts with contiguous TM molecules through head-to-tail contacts at their ends. Tn, composed of the three subunits, Tn-C, Tn-I and Tn-T, is asymmetrically arranged by multiple attachments through the Tn-I and Tn-T components to actin and tropomyosin (see Fig. 8 of ref. [4]). In particular, that part of Tn-T represented by its T1 fragment (residues 1–158) is now known to interact with TM at its head-to-tail overlap [5–8], while fragment T2 (residues 159–259) binds in the central region of TM some 13–15 nm from its C-terminal end. This latter fragment also binds to Tn-I Tn-C and probably actin [4,8–14]. In the absence of Ca^{2+} , the Tn–TM complex exerts inhibitory constraints on the actomyosin ATPase activity. Conformational changes in this complex are triggered by the binding of Ca^{2+} to Tn-C, resulting in the facilitated release of products from actomyosin [15] and a shift in the position of TM in the grooves of F-actin [16–19] and hence force generation.

The cooperative aspects of this regulatory system have been the subject of numerous investigations in recent years. Thus, while the binding of Ca^{2+} to the regulatory

sites of isolated Tn-C appears to show little cooperativity [20–22], the ATPase activities of myofibrils and of regulated actomyosin systems do respond to varying Ca^{2+} concentration in a cooperative manner [23–27]. In addition, the ATPase activity of the regulated actomyosin system has been shown to be markedly influenced by the binding of myosin heads. Thus at low ATP concentrations, the formation of rigor links between myosin subfragment 1 (S1) and regulated actin turns on the ATPase activity in a cooperative manner [28–32]. Associated with this phenomenon is the ability of myosin heads to increase the binding of TM [33] and an effect of TM on the binding of S1 [28,30]. These and other observations have been incorporated into an allosteric model in which the regulated actin complex exists as an equilibrium of strong and weak S1 binding forms [30,31,34]. The effects of increasing $[Ca^{2+}]$ and/or $[S1]$ are to shift this equilibrium in the direction of the strong binding form.

One of the structural features considered to be responsible for at least some aspects of these cooperative phenomena is the head-to-tail overlap of tropomyosin molecules. This region, believed to consist of eight to eleven amino acid residues at both the N- and C-terminal ends of the molecule, is known to be required for the polymerization of TM and for the cooperative binding of TM to F-actin [35]. By treating TM with carboxypeptidase A under controlled conditions, a truncated form can be prepared in which eleven amino acid residues

are quantitatively removed from its C-terminal end. The product, designated nonpolymerizable tropomyosin (NPTM), no longer aggregates head-to-tail and fails to bind to F-actin [6,36,37]. Full binding to F-actin can be induced however by the addition of Tn both in the absence and presence of Ca^{2+} under appropriate ionic strength conditions [4,37].

In previous experiments to test the importance of the TM overlap in this regulatory system, Walsh *et al.* [26] reported that the substitution of TM by NPTM had two effects: first, to decrease the Ca^{2+} activated ATPase, and secondly, to shift the range of activating Ca^{2+} concentrations to slightly higher values. However, no change in the cooperative response of the ATPase to increasing Ca^{2+} concentration was detected.

To further explore the importance of this structural feature, we have in the present work investigated the effects of replacing TM by NPTM on the ATPase activities of the regulated system as a function of TM (or NPTM) and S1 concentration. Conditions were chosen such that the dual effects (inhibition and potentiation [38–42]) of TM alone and of TM plus Tn ($+\text{Ca}^{2+}$) on the actomyosin S1 activities could be compared to those in which NPTM replaced TM. Although there was little effect on the inhibition of activity in the absence of Ca^{2+} , large differences were observed in the activation by Ca^{2+} over a range of S1 concentrations and at both low and intermediate ionic strength.

MATERIALS AND METHODS

Actin [43], Tn [44], $\alpha\alpha\text{TM}$ [45] and NPTM [4] were prepared from rabbit skeletal and cardiac muscles as previously described. The preparation of myosin S1 was according to Weeds & Taylor [46], with the exception that the chymotryptic digestion was terminated by adding di-isopropyl fluorophosphate to a final concentration of 15 mM. Concentrated stock solutions of TM, NPTM and S1 were dialysed against 30 mM-KCl/2 mM-Tris, pH 7.8/6 mM- MgCl_2 /0.1 mM-EGTA/2 mM-dithiothreitol/0.01% sodium azide (Buffer A). Following a centrifugation step to remove any insoluble material, TM and NPTM (50–160 μM) were stored frozen at -20°C while S1 was kept at 4°C . S1 had an EDTA-activated ATPase activity of 8.5–12 s^{-1} similar to previously reported values [47]. It was found to be stable over a period of two months with only a 10% decrease in activity, coincident with a small amount of degradation as detected by SDS/polyacrylamide-gel electrophoresis. A stock solution of Tn was prepared in 5 \times concentrated Buffer A (minus EGTA), clarified by centrifugation and then dialysed overnight against 1 \times Buffer A (minus EGTA). The Tn (20–30 μM) was used immediately after a second centrifugation. Gel electrophoresis indicated that stoichiometry of the three subunits was not altered by these procedures. Actin (250 μM) was converted to the F form by dialysis against Buffer A plus 1 mM-ATP and diluted to 100 μM with the same buffer. A stock solution of ATP (0.2 M) was prepared by dissolving the disodium salt in 1.0 M-KOH to a final pH of 7.8 and dilution with Buffer A (minus dithiothreitol). Protein concentrations were determined by absorbance at 280 nm and/or amino acid analyses.

ATPase measurements were made with an automated pH-stat (Radiometer TTT-2 titrator, SBR2C Titrigraph, SBU1 syringe burette and GK2321C electrode). For all

experiments the electrode was soaked overnight in 0.1 M-HCl, the saturated KCl replaced and the electrode equilibrated for 2 h in standard pH 8.0 buffer. The electrode calibration was checked after each experiment and did not normally change over the course of a day. The order of addition of reactants to the stirred glass vial at 25°C was Buffer A (minus dithiothreitol), S1, ATP, actin, TM (or NPTM) and Tn, followed by CaCl_2 as appropriate. Final concentrations are given in the Figure legends. The total ionic strength was 50 mM. The final reaction volume was 1.6–2.0 ml and the titrant was 10–20 mM-standardized KOH. The concentrations of TM, NPTM and Tn were such that their addition did not increase the total volume by more than 8%. In every experiment the ATPase activity was monitored over periods of 2.5 min following the stepwise addition of components to a mixture of S1 and ATP. Reaction rates were only calculated from linear records. The effects of TM and TM (or NPTM) + Tn ($-\text{Ca}^{2+}$) on the actomyosin S1 ATPase were therefore investigated by the successive addition of TM and then Tn, 2.5 min apart, to the same reaction mixture. It was not possible, however, to assess the effects of Ca^{2+} addition during the same experiment, since the reaction rate did not remain linear for a further 2.5 min. This phenomenon, due to ATP depletion, was particularly pronounced at high S1:F actin ratios. Linear rates for the $+\text{Ca}^{2+}$ condition were thus obtained by the successive addition of TM (or NPTM), Tn ($-\text{Ca}^{2+}$) and Ca^{2+} , 30 s apart, to a mixture of myosin S1 and F-actin whose activity had been determined immediately prior to these additions. Since in all experiments the S1 and F-actin concentrations were far removed from conditions of V_{max} , it was necessary to establish the background MgATPase activity of S1 alone at each S1 concentration. Control experiments showed that NPTM alone did not significantly alter the ATPase rate for mixtures of S1 and F-actin when the S1 concentration was equal to or below 10 μM .

Experiments were also carried out at the lower ionic strength of 13 mM using Buffer B. This was composed of 2 mM-Tris, pH 7.8, 4.0 mM- MgCl_2 /0.1 mM-EGTA/2 mM-dithiothreitol. The various components were dialysed against the appropriate Buffer B in an identical fashion to what has been described above for the respective Buffer A solutions. Rigor complexes were formed by the sequential addition to Buffer B (minus dithiothreitol) of F-actin, TM (or NPTM), Tn, Ca^{2+} and finally S1 and the ATPase activity was then initiated by the delivery of 1 mM-ATP into the reaction vial (total volume = 1.6 ml). Under these conditions linear records could be obtained for a duration of 30 s using 40–70 mM-standardized KOH as titrant, depending upon the concentration of S1.

RESULTS

Effects of TM and NPTM \pm Tn ($\pm \text{Ca}^{2+}$) at a constant S1:F-actin ratio of 2:1

Under the assay conditions described in the Materials and methods section and in the legend to Fig. 1, the addition of F-actin (4 μM) to myosin (8 μM) resulted in a 6-fold activation of the myosin S1 Mg^{2+} -ATPase activity. This small activation is due to the low concentration of actin relative to that of S1 (far from V_{max} conditions). When TM alone was titrated into this system, the activity increased by almost 50%, reaching a maximum

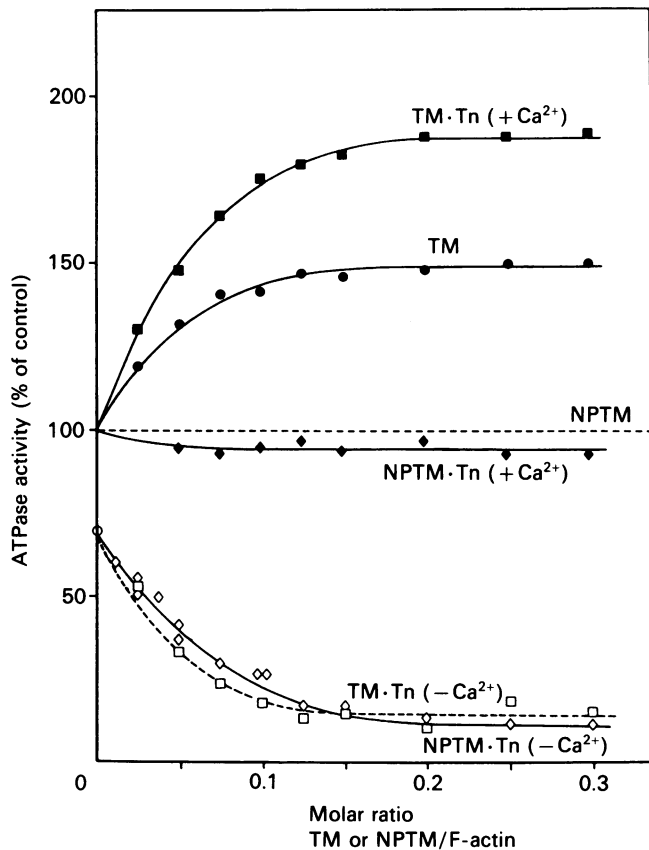


Fig. 1. The effects of TM and NPTM on the actomyosin S1 MgATPase activity

The concentrations of S1, F-actin and Tn were 8, 4 and 1.14 μM respectively, while the concentration of TM (or NPTM) was varied between 0 and 1.2 μM . All rates are expressed relative to the ATPase activity of S1 and F-actin alone (100% = 3.2 nmol/s). The activities were corrected for the rate of S1 alone (0.5 nmol/s). NPTM, in the absence of Tn, had no effect on the 100% value (see dotted line). Assay conditions were 30 mM-KCl/6 mM-MgCl₂/2 mM-Tris, pH 7.8/0.01% Na azide/0.1 mM-EGTA/2.5 mM-ATP and 0.3 mM-CaCl₂ (when added). Total ionic strength was 50 mM.

at a molar ratio of TM:actin slightly greater than 1:7. This potentiation by TM is consistent with previous observations [38,39] under similar conditions. When NPTM, rather than TM, was added to the reaction, there was no effect on the ATPase activity. This was expected since previous work [4,36] has shown that NPTM alone does not bind to F-actin in the micromolar concentration range.

When either intact TM or NPTM is added to the actomyosin S1 system in the presence of Tn ($-\text{Ca}^{2+}$) the inhibition ($\sim 30\%$) seen with Tn alone (see data point on ordinate of Fig. 1) is enhanced, to give an ATPase value of 10–15% of that seen with actomyosin alone. There was no apparent difference in the abilities of these two proteins to function in this manner. However, in the presence of Tn and Ca^{2+} , the effects of adding TM and NPTM were very different. Whereas the addition of TM led to an almost doubling of the activity, addition of NPTM led to a marginal decline below that seen with actomyosin alone. This minimal effect of NPTM was not due to a lack of binding since it has previously been

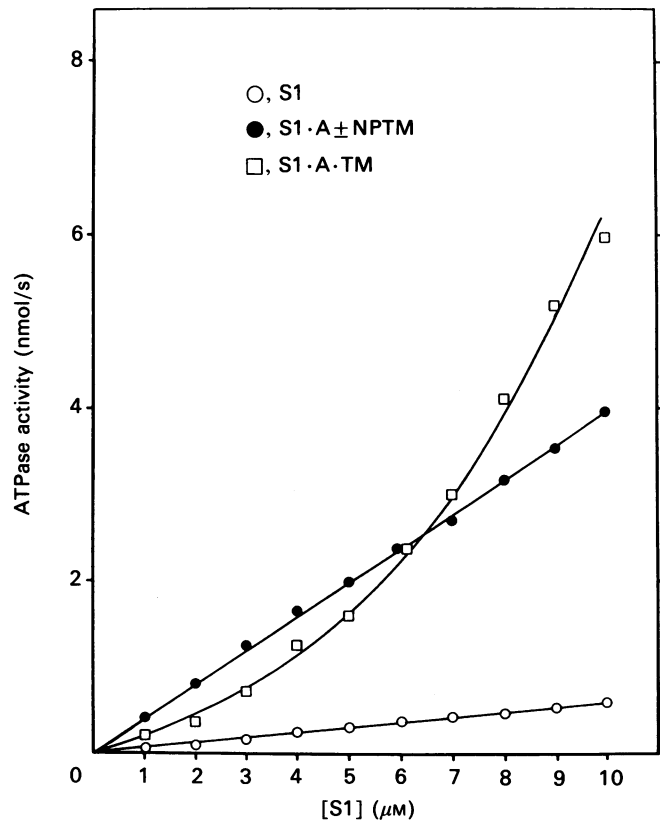


Fig. 2. The actomyosin S1 MgATPase activity with F-actin containing either TM or NPTM, as a function of S1 concentration

Assay conditions were as described in the Materials and methods section. The concentration of S1 was increased from 0 to 10 μM , while the concentrations of F-actin (A) and TM (or NPTM) were maintained at 4 and 1.14 μM respectively. Multiple measurements at identical S1 concentrations were $\pm 5\%$ of the mean. Assay conditions were as in Fig. 1.

shown that under conditions of slightly higher ionic strength, Tn is able to induce stoichiometric binding of NPTM to actin, even in the presence of Ca^{2+} [4].

Effects of varying S1 concentration on ATPase activity at constant regulatory protein concentrations

Previous work [29,38,39] has demonstrated that when the S1 concentration is progressively increased at saturating ATP concentrations in a regulated actin system in which the concentrations of actin, TM and Tn ($+\text{Ca}^{2+}$) are held constant, there is a cooperative activation of ATPase attributable to a higher proportion of regulatory units being in the 'turned-on' state. This has been ascribed to the formation of ATP-resistant rigor complexes arising from trace contamination of the S1 with a non-functional denatured fraction and/or to the two-fold stronger binding of S1 to the 'turned-on' state of the regulatory complex [31,34,40]. To assess the importance of the TM overlap in this phenomenon and to expand on the observations reported above, we have compared the enzymic properties of such a system reconstituted with either TM or NPTM, with and without Tn and in the absence and presence of Ca^{2+} . The results of these experiments in which the S1 concentration was varied from 1 to 10 μM while maintaining the TM (or NPTM),

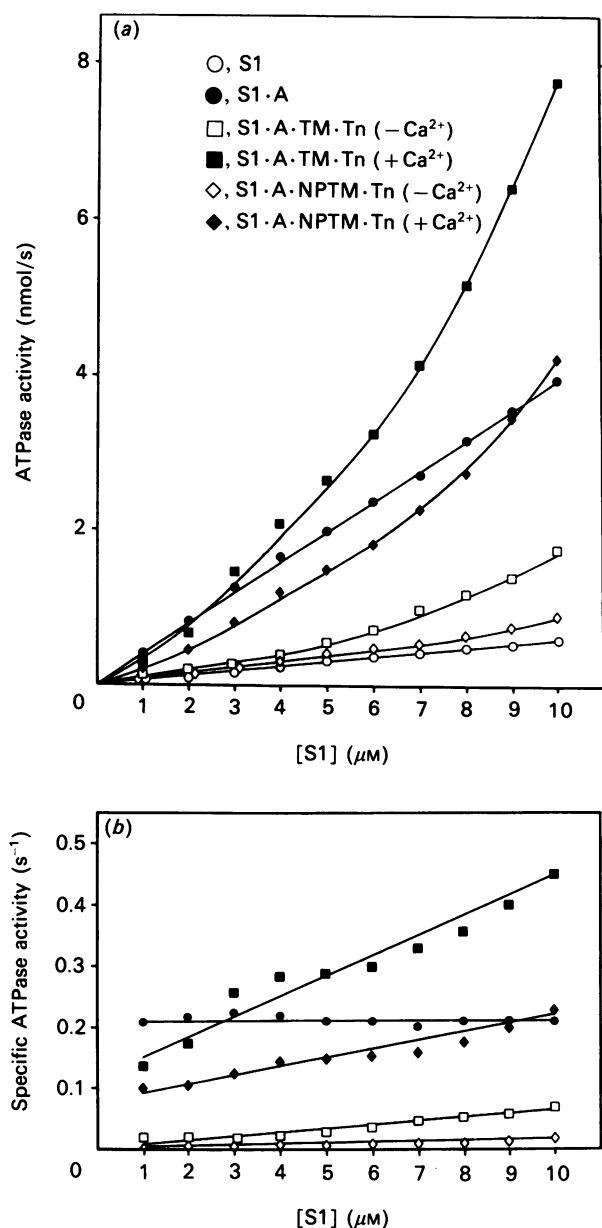


Fig. 3. The actomyosin S1 MgATPase activity with regulated F-actin containing TM (or NPTM) and whole Tn, as a function of S-1 concentration

Assay conditions were as in Fig. 1. The concentration of S1 was increased from 0 to 10 μM while the concentrations of F-actin (A), Tn and TM (or NPTM) were maintained at 4, 1.14 and 1.14 μM respectively. The ionic strength was 50 mM. (B) Data shown in (A) are expressed as specific activity and corrected for the rate of S1 alone (0.038 s^{-1}).

Tn (when present) and F-actin concentrations at 1.14, 1.14 and 4 μM , respectively are presented in Figs. 2 and 3. In the absence of Tn, but with TM present (Fig. 2), the addition of S1 led to an inhibition of activity at S1 concentrations below 5.5 μM and to activation at higher [S1] above that seen with actomyosin S1 alone. Similar dual effects of TM alone have been observed by others [39–42]. When NPTM replaced TM, no effect on the activity of actomyosin S1 alone was observed, consistent with its inability to bind to actin in the absence of Tn [4,36].

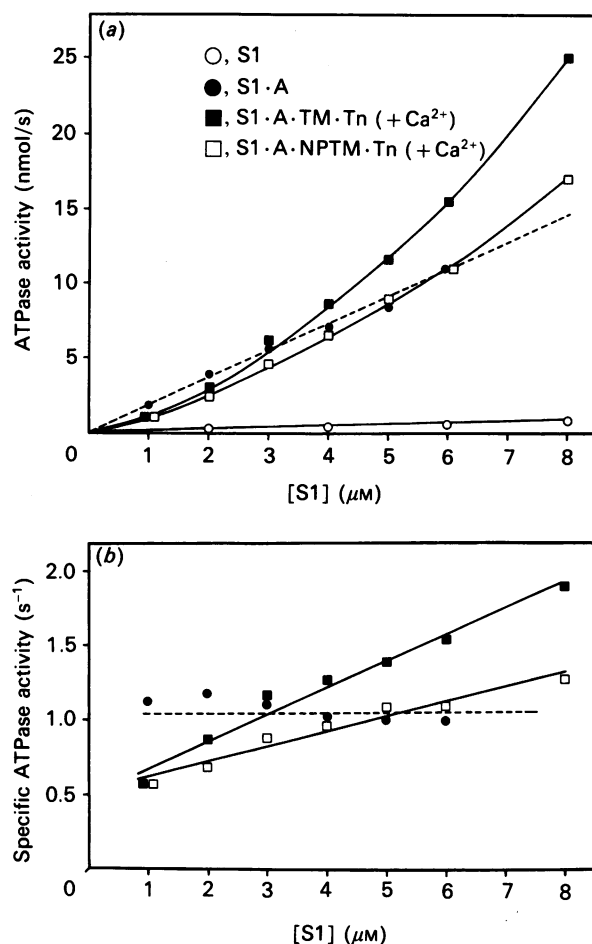


Fig. 4. The actomyosin S1 MgATPase activity with regulated F-actin containing TM (or NPTM) and whole Tn, as a function of S1 concentration at an ionic strength of 13 mM

The concentration of S1 was varied between 0 and 8 μM , while the concentrations of F-actin (A), Tn and TM (or NPTM) were maintained at 4, 1.14 and 1.14 μM respectively. (B) Data shown in (A) are expressed as specific activity and corrected for the rate of S1 alone (0.063 nmol/s). Assay conditions were 2 mM-Tris, pH 7.8/4.0 mM-MgCl₂/1.0 mM-ATP/0.1 mM-EGTA and 0.3 mM-CaCl₂ (when added).

In the presence of Tn and TM (or NPTM) and in the absence of Ca²⁺, inhibition was observed at all concentrations of S1 up to 10 μM . At the higher S1 concentrations the inhibition observed with NPTM appeared to be marginally more complete. When the ATPase measurements were carried out in the presence of TM, Tn and Ca²⁺, the effects of increasing the S1 concentration were to markedly potentiate this activity from values below that of actomyosin S1 alone at low S1 concentrations to much higher levels when the S1 concentration was in excess of the actin concentration (Figs. 3a and 3b). On the other hand, when NPTM replaced TM in the same system, the ATPase activity remained lower than that observed with actomyosin S1 alone, and only reached a value equal to the latter at the highest S1 concentration examined (10 μM). To eliminate the possibility that these results were due to incomplete binding of NPTM to actin in the presence of Tn, data were also collected at 8 μM -TM (and NPTM) as compared with

1.14 μM . The results (not shown) were very similar to those of Fig. 3. Finally, since the above results were obtained under conditions far removed from V_{max} (50 mM ionic strength), the assays were repeated at a much lower ionic strength (13 mM) where the interaction between S1-ATP and actin is enhanced and where the measured ATPase would represent a more significant proportion of the maximal activity. Inspection of the results (Figs. 4a and 4b) reveals that while the differences observed when TM is substituted by NPTM are less dramatic than those at higher ionic strength, they are still highly significant.

DISCUSSION

In the present work we have examined the extent to which NPTM molecules, lacking the head-to-tail overlap region of intact TM, can function in the actomyosin S1 ATPase system in the absence and presence of Tn ($\pm \text{Ca}^{2+}$). The conditions chosen for this study were such that the dual effects (inhibition and potentiation) of TM alone and of TM plus Tn ($+\text{Ca}^{2+}$) [38–42] as a function of S1 concentration could be compared with those in which NPTM replaced TM. These conditions differ significantly from those of Walsh *et al.* [26]. The results with intact TM were similar to those described by others and to be expected on the basis of the known stoichiometries and activities of the several components. The observations with NPTM alone were also not unexpected since its inability to bind to F-actin in the absence of Tn should preclude any effects on the actomyosin S1 ATPase activity. When TM was replaced by NPTM in the presence of Tn ($-\text{Ca}^{2+}$), there was little difference in the abilities of the two proteins to accentuate the inhibition by Tn. Inhibition in each case was 85–90% complete at a molar ratio of TM (or NPTM): actin of about 1:7 (Fig. 1). Similar results were obtained over a range of S1 concentrations (Figs. 3a and 3b). Very different results, however, were obtained in the presence of Ca^{2+} and Tn. Over the entire range of S1 concentrations examined in the present work, the ATPase rates observed with NPTM were significantly lower than with TM.

The present observations are in agreement with Walsh *et al.* (26) who, working at a low ratio (1:100) of S1:actin and at low ionic strength, also observed a significant decrease in ATPase activity of the regulated actin system under $+\text{Ca}^{2+}$ conditions when NPTM replaced TM. These effects of the deletion of the head-to-tail overlap of TM on the Ca^{2+} -activated ATPase are thus seen to be manifested over a broad range of S1:actin ratios and at both low and intermediate ionic strengths. However, in the current study, regulated actin filaments containing NPTM and Tn ($+\text{Ca}^{2+}$) were found to potentiate the ATPase activity at high ratios (10:4) of S1:F-actin (Figs. 3 and 4). Therefore, in the presence of Tn, the truncated molecule can still exhibit the dual regulatory characteristics of intact TM.

The available information on the role of TM overlap in this regulated system is still fragmentary. Previous studies [35] have shown that the interaction of intact TM to F-actin is highly cooperative and that this binding is reduced dramatically when the overlap is eliminated as with NPTM [6,36]. Full NPTM binding can be induced however by the addition of whole Tn ($\pm \text{Ca}^{2+}$) under appropriate ionic conditions [4,37]. This binding retains

a degree of cooperativity both in the presence and absence of Ca^{2+} . These studies also showed that the inductive effect of whole Tn could be ascribed to the Tn-T and Tn-I components and that both the T1 (residues 1–158) and T2 (residues 159–259) regions of Tn-T participate in this phenomenon. The role of the T1 region in this induction was attributed to its binding to the residual binding sites for this region of Tn-T at the intact *N*-terminus and truncated *C*-terminus of NPTM. This interpretation provided a possible mechanism for the cooperativity of binding of NPTM induced by whole Tn ($\pm \text{Ca}^{2+}$) [4].

How these binding studies may be related to the role of the TM overlap and its associated T1 region of Tn-T in the cooperative aspects of the regulated actin S1 ATPase is presently unclear. However, the observation by Walsh *et al.* (26) that the replacement of TM by NPTM in such a system containing whole Tn had no effect on the cooperativity of the ATPase in response to Ca^{2+} concentration suggests a possible role for the T1 region of Tn-T in the mediation of such effects. An assessment of its importance relative to conformational changes transmitted through the underlying F-actin should now be experimentally feasible.

The present observations that the Ca^{2+} -activated ATPase activities of the reconstituted regulated actomyosin S1 system are significantly reduced when NPTM replaces TM may be considered in light of the proposed allosteric model in which the regulated actin complex exists as an equilibrium of two forms [30–32,34]. In the strong S1-binding form, the ATPase activity is maximally turned on while in the weak S1-binding state, the ATPase is not significantly activated. The proportion of regulatory units in the turned-on or strong binding form is dependent on several parameters including L' , the equilibrium constant between the two forms; Y , a value that takes into account the interaction between neighbouring tropomyosin molecules; and two binding constants K_{strong} and K_{weak} describing the affinities of S1 for the strong binding and weak binding forms respectively. While the deletion of the head-to-tail overlap as in NPTM would be expected to markedly reduce the interaction between TM molecules and thus alter the Y parameter, it is presently not at all clear how important this structural feature is relative to that of the T1 region of Tn-T as discussed above. It must also be appreciated that the deletion of the contact region between adjacent TM molecules could have significant effects on the structural organization of the regulatory unit as a whole since changes in the conformation of one part of the TM molecule can be expected to be transmitted over long distances in its coiled-coil structure [48–51]. Such structural alterations could of course alter the equilibrium (and thus L') between weak and strong binding forms and/or the strength of binding of S1 to these two forms (K_{strong} and K_{weak}).

The question of whether some degree of cooperativity is lost in this system when NPTM replaces TM cannot be assessed from the present data. Although the upward curvature evident in Figs. 3a and 4a suggests some degree of cooperativity in response to increasing S1 concentration, this is seen to be minimal when the activities are corrected for free S1 ATPase and expressed in terms of specific activity as in Figs. 3b and 4b. Such minimal cooperativity is consistent with the conclusion of Greene *et al.* (31) that S1. ATP (or S1.ADP.P) binds with (at most 2-fold) greater affinity to the turned-on than to the

turned-off form of regulated actin and thus exhibits little cooperativity. It also suggests that the degree of contamination of our S1 preparation with a denatured fraction, capable of forming ATP-resistant cross-bridges with actin, is negligible. An assessment of the extent to which the head-to-tail overlap of TM molecules may participate in the cooperative binding of S1.ADP to the regulated thin filament [31] and how the several parameters describing this complex system are affected by its deletion await further studies.

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