Correlation between the Inhibition of the Acto-Heavy Meromyosin ATPase and the Binding of Tropomyosin to F-Actin: Effects of Mg^{2+}, KCl, Troponin I, and Troponin C†

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ABSTRACT: When stoichiometric amounts of tropomyosin (TM) are bound to F-actin in the presence of 2 m*M* ATP, the Mg^{2+}-activated acto-heavy meromyosin (HMM) ATPase is inhibited by about 60% in 5 m*M* $MgCl_2$–30 m*M* KCl. If the concentration of $MgCl_2$ is reduced to 1 m*M*, the inhibition disappears because TM no longer binds to F-actin. Increasing the concentration of KCl to 100 m*M* restores both the binding and the inhibition. Thus, the binding of TM alone to F-actin causes significant inhibition of the ATPase provided that the HMM is saturated with ATP. (When the HMM is not saturated, TM activates the ATPase.) When TM alone can bind stoichiometrically to F-actin, addition of troponin I (TN-I) increases the inhibition from 60% to about 85%, but the TM binding to F-actin is not affected. Under conditions such that TM alone neither inhibits the acto-HMM ATPase nor binds to F-actin, the inhibition caused by TN-I plus TM still approaches 100%. Direct binding studies under these conditions show that TN-I induces binding between TM and F-actin. A dual role for TN-I is proposed: first, TN-I can induce TM to bind to F-actin, causing inhibition of the ATPase; and second, TN-I can itself enhance the inhibition of the ATPase in a cooperative manner. The addition of TN-C in the absence of Ca^{2+} has only a limited effect on the first role, but seems to be able to block completely the cooperative inhibition caused by TN-I such that the residual inhibition is a function only of the TM which remains bound.

The dual regulatory effect of TM[1] on the ATPase (EC 3.6.1.3; ATP phosphohydrolase) of actomyosin prepared from purified actin and myosin was first demonstrated by Katz (1964). He showed that the presence of TM inhibits the Mg^{2+}-activated ATPase during the initial clearing phase, thus delaying the rapid increase in turbidity which accompanies superprecipitation. Following the onset of superprecipitation, a potentiating effect of TM on the Mg^{2+}-activated ATPase was observed. These observations on the effects of TM alone were made at about the same time that Ebashi and Kodama (1965) discovered a second regulatory protein, troponin, which, when combined with TM and added to reconstituted actomyosin, was able to restore the Ca^{2+} sensitivity of desensitized actomyosin. During the next 7 years, many laboratory groups worked to identify the subunits of troponin, and by 1972 (Ebashi et al., 1973; Hartshorne and Dreizen, 1973; Greaser et al., 1973; Drabikowski et al., 1973; Perry et al., 1973; see also review by Weber and Murray, 1973) there was general agreement with the original proposal of Greaser and Gergely (1971) that troponin from rabbit skeletal muscle contains three subunits which in addition to TM are all required for full Ca^{2+}-sensitive regulation of the actomyosin, acto-HMM, or acto-subfragment 1 Mg^{2+}-activated ATPase.

Recently, Wilkinson et al. (1972) demonstrated that TN-I, the troponin subunit having a mol wt of 24,000, inhibits the Mg^{2+}-activated actomyosin ATPase even in the absence of TM, and that TM enhances this inhibition. They also reported that TM itself has no effect on the ATPase, a result which is inconsistent with the earlier report by Katz (1964) that TM alone exerts a dual regulatory effect on the Mg^{2+}-activated actomyosin ATPase. The effects of TM alone on the ATPase were therefore reinvestigated. The results which will be described in this paper clarify the following points: first, TM alone is probably not bound to F-actin under the conditions chosen by Wilkinson et al. (1972), and this may explain why they observed no effect of TM alone on the ATPase; second, when conditions are such that TM alone does bind to F-actin, the acto-HMM ATPase is inhibited by a maximum of about 60% provided that the HMM is saturated with ATP; and third, TM is able to enhance TN-I's inhibition of the ATPase when conditions do not permit TM alone to bind to F-actin because TN-I induces binding between TM and F-actin. These results show that TM is itself a strong inhibitor of the Mg^{2+}-activated ATPase, not merely a transmitter of the inhibition due to TN-I from one actin monomer to its neighbors in the thin filament as has been suggested by Perry et al. (1973).

The inhibition of the Mg^{2+}-activated ATPase by TM alone as described above occurs only at ATP concentrations higher than 10^{-3} *M*. Several groups (Katz, 1964; Shigekawa and Tonomura, 1972, 1973; Bremel et al., 1973) have found that TM also activates the ATPase if the ATP concentration is below 10^{-4} *M*. In the present study, this result is confirmed and shown to be cooperative as proposed by Bremel et al. (1973).

A preliminary account of some of these findings has been presented (Eaton et al., 1974).

Materials and Methods

Preparation of the Proteins. All proteins used in this

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[1] Abbreviations used are: TM, tropomyosin; TN-I, subunit of troponin which inhibits the actomyosin ATPase; TN-C, subunit of troponin which binds Ca^{2+}; TN-T, subunit of troponin which binds TM; HMM, heavy meromyosin, a proteolytic fragment of myosin; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

study were prepared from rabbit skeletal muscle. Myosin was obtained using a modification of the procedure of Weber and Portzehl (1952), as described by Kielley and Bradley (1956), except that treatment of the ground muscle with the Waring Blendor was eliminated (Kielley and Harrington, 1960). Myosin was stored up to 2 weeks at 0–4° in 50% ammonium sulfate solution. No difference between the stored myosin and fresh myosin was ever observed. HMM was prepared from myosin by trypsin digestion (Lowey and Cohen, 1962) in 0.5 *M* KCl–2 m*M* EDTA–2 m*M* imidazole buffer (pH 7.0), at 25° for 5 min. Myosin concentrations ranged from 22 to 26 mg/ml, and 0.20% trypsin solution was added to provide 1.0 mg of trypsin for 250 mg of myosin. An equal volume of 0.40% soybean trypsin inhibitor was added to stop digestion. The digest was dialyzed overnight at 0–4° against 0.05 *M* KCl–2 m*M* imidazole buffer (pH 7.0); the light meromyosin and undigested myosin were removed the following day by centrifugation for 45 min at 30,000 rpm using a 30 rotor in a refrigerated Spinco Model L preparative ultracentrifuge. The supernatant was clarified by a second identical centrifugation before a second overnight dialysis at 0–4° in 2 m*M* imidazole buffer (pH 7.0). F-Actin was prepared as described by Spudich and Watt (1971) with the following modification: the actin was treated twice with 0.8 *M* KCl rather than just once with 0.6 *M* KCl (Eisenberg and Kielley, 1974). TM and the troponin subunits were prepared according to the method of Eisenberg and Kielley (1974). Protein concentrations were determined by ultraviolet (uv) absorption using the following extinction coefficients: 647 cm^2/g at 280 nm for HMM (Young et al., 1964); 1149 cm^2/g at 280 nm for F-actin (Eisenberg and Moos, 1967); 290 cm^2/g at 278 nm for TM (Hartshorne and Mueller, 1969); 596 cm^2/g at 280 nm for TN-I (Margossian and Cohen, 1973); 193 cm^2/g at 280 nm for TN-C (Margossian and Cohen, 1973). The following molecular weights were used for calculating molar concentrations: 42,000 for actin (Elzinga et al., 1973); 68,000 for TM (Woods, 1967); 20,000 for TN-C (Greaser et al., 1973); 24,000 for TN-I (Greaser et al., 1973).

Iodination of TM. The iodination of TM was catalyzed by lactoperoxidase (Marchalonis, 1969) at room temperature. The reaction beaker contained a mixture of 85 mg of TM dissolved in 3.7 ml of 0.37 *M* KCl–0.063 *M* phosphate buffer (pH 6.9), 0.10 mg of lactoperoxidase (Sigma Chemical Co.) dissolved in 0.1 ml of water, and 0.35 μg of $Na^{125}I$ (Amersham-Searle) with a radioactivity of 0.5 mCi, dissolved in 30 μl of water. The reaction was initiated by adding 20 μl of 0.03% hydrogen peroxide. Five additional 20-μl aliquots of 0.03% hydrogen peroxide were added at 10-min intervals. Following the fourth aliquot of hydrogen peroxide 10 μl of 0.01 *M* KI was added as a carrier. Ten minutes after the addition of the sixth aliquot of hydrogen peroxide the reaction was stopped by adding 50 μl of 0.05 *M* dithiothreitol. The mixture was dialyzed against 0.3 *M* KCl–0.5 m*M* dithiothreitol with several changes of dialysate until the dialysate was free of significant radioactivity. Approximately 45% of the label was incorporated into the protein giving a specific activity of 1.8×10^6 cpm/mg of TM.

Acto-HMM ATPase Determinations. A method similar to that of Green and Mommaerts (1953) was used to measure ATPase rates at 25° and pH 7.0 with an automatic pH-Stat apparatus as described by Eisenberg and Moos (1967). The titrant, KOH (50–90 m*M*), was added through Teflon tubing to the reaction mixture which was constantly stirred with a magnetic stirrer. The proteins were added in the following order: TN-I, TN-C, F-actin, TM, and HMM which initiated the reaction (Eisenberg and Kielley, 1974). Because the rate of the acto-HMM ATPase is quite sensitive to ionic strength, the HMM concentration was adjusted for each set of conditions to give ATPase rates which could be measured accurately. These ATPase rates were then converted to rates per milligram of HMM and found to be much slower than V_{max}, indicating that almost all of the HMM and F-actin present were dissociated (Eisenberg and Moos, 1968). Varying the HMM concentration between 0.5 and 1.5 mg/ml therefore had no effect on the ATPase rate per milligram of HMM, and any changes found in that rate must be attributed to other variables. The reaction volume was 7.5 ml, and all reaction mixtures except those shown in Figure 6B contained both 1 m*M* EGTA and 2 m*M* ATP.

Binding of [^{125}I]TM to F-Actin. All studies were conducted in the presence of 1 m*M* EGTA and 2 m*M* ATP. The binding of [^{125}I]TM to F-actin was studied using a modification of the method of Kominz and Maruyama (1967). Reagents and protein solutions were mixed in small centrifuge tubes at room temperature. The tubes, each containing a final volume of 2.0 ml, were incubated in a 25° water bath for 15 min and then centrifuged at 36,000 rpm at 25° using a 40 rotor in a Spinco Model L preparative ultracentrifuge. All protein solutions except HMM were added in the same order used for the ATPase determinations; HMM was omitted to prevent rapid depletion of ATP and subsequent formation of rigor bonds between HMM and F-actin. This omission of HMM is unlikely to have affected the binding between TM and F-actin, however, due to the fact that HMM and F-actin were almost completely dissociated under the conditions used in the ATPase experiments (Eisenberg and Moos, 1968). Lowry protein determinations (Lowry et al., 1951) of the supernatants of controls indicated that at least 89% of the F-actin sedimented when no other proteins were present. Samples of 100 μl were taken before and after centrifugation, added to 4 ml of Aquasol (New England Nuclear), and counted in a Mark I liquid scintillation computer (Nuclear-Chicago Corporation) over 10-min intervals. The difference in counts between the samples before and after centrifugation represented the amount of TM bound. At least four 10-min counts were averaged for each sample. Counting efficiency was about 30%.

Results

Effects of TM Alone. Figure 1B shows the effect of TM on the acto-HMM ATPase in 30 m*M* KCl, 2 m*M* ATP, and either 5 or 1 m*M* $MgCl_2$. TM has a negligible inhibitory effect in the low $MgCl_2$; in the high $MgCl_2$ it has a strong effect which increases sharply to 60% inhibition and then seems to level off when 1–2 mol of TM has been added per 7 mol of F-actin monomer. The points marked with the asterisks were obtained using [^{125}I]TM and demonstrate that the iodination process does not affect the TM activity. The results of binding studies of TM to F-actin under the same conditions are shown in Figure 1A. The binding of TM to F-actin is negligible in 1 m*M* $MgCl_2$ compared to that in 5 m*M* $MgCl_2$ where it is almost stoichiometric, approximating one TM per seven F-actin monomers. Comparison of Figures 1A and 1B suggests that when TM binds to F-actin it produces a marked inhibitory effect on the ATPase. To further test this correlation between the binding of TM to F-actin and inhibition of the acto-HMM ATPase, studies at 1 m*M* $MgCl_2$ and 2 m*M* ATP were made at two

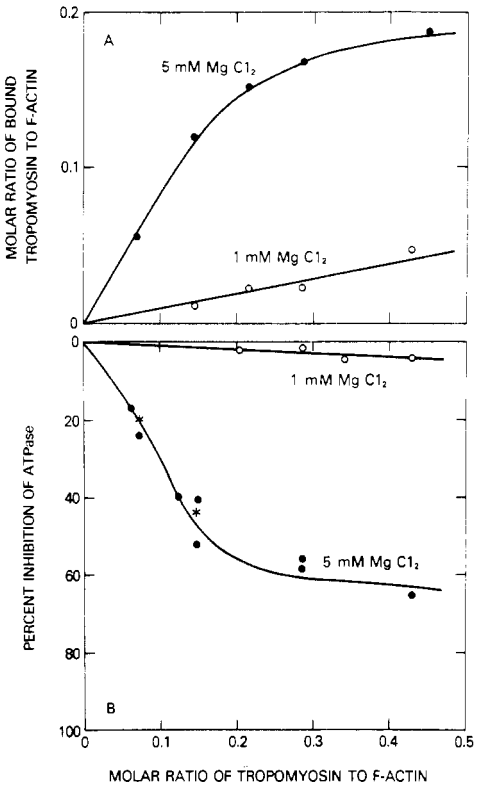

FIGURE 1: Effects of Mg^{2+} on the binding of TM to F-actin and the inhibition of the acto-HMM ATPase. Conditions: 2 m*M* ATP, 30 m*M* KCl, 1 m*M* EGTA, 0.2 mg/ml of F-actin, 1 m*M* imidazole buffer (pH 7.0); (○) 1 m*M* $MgCl_2$, 0.5 mg/ml of HMM in B only; (●, *) 5 m*M* $MgCl_2$, 0.7 mg/ml of HMM in B only. Points designated by an asterisk were obtained using [^{125}I]TM.

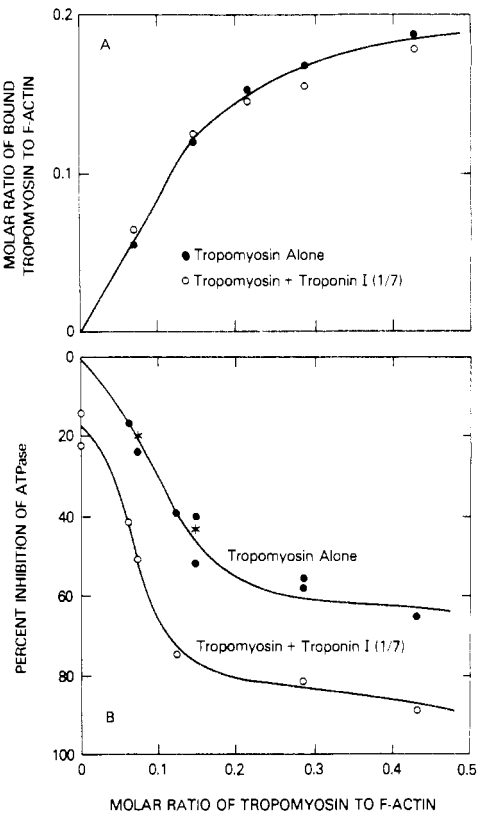

FIGURE 2: Effect of TN-I on binding of TM to F-actin and the inhibition of the acto-HMM ATPase in high Mg^{2+}. Conditions: 5 m*M* $MgCl_2$, 2 m*M* ATP, 30 m*M* KCl, 1 m*M* EGTA, 0.2 mg/ml of F-actin, 1 m*M* imidazole buffer (pH 7.0); (●, *) TM alone added; (○) TM added in presence of 0.016 mg/ml of TN-I; 0.7 mg/ml of HMM in B only. Points designated by an asterisk were obtained using [^{125}I]TM.

Table I: Correlation between the Binding of TM to F-Actin and Inhibition of the Acto-HMM ATPase.[a]

Conditions (m*M*)			
[$MgCl_2$]	[KCl]	% Inhibition	mol Ratio TM Bound/F-Actin
1	12	0	0.02
1	30	3	0.03
1	100	33*	0.085**
5	30	60***	0.16

[a] Conditions: 2 m*M* ATP, 1 m*M* EGTA, 1 m*M* imidazole buffer (pH 7.0); 0.2 mg/ml of F-actin, 0.08 mg/ml of TM, and 0.5 mg/ml of HMM except (*) 0.6 mg/ml of F-actin and 1.5 mg/ml of HMM, (**) both 0.2 mg/ml of F-actin and 0.6 mg/ml of F-actin, and (***) 0.2 mg/ml of F-actin and 0.7 mg/ml of HMM.

additional concentrations of KCl, 100 and 12 m*M*. These conditions were chosen because Tanaka and Oosawa (1971) had shown that in the absence of $MgCl_2$, the binding of TM to F-actin is maximal at about 100 m*M* KCl, and Kominz (1966) had reported that the inhibitory activity of native TM is gradually lost when the ionic strength is lowered from 30 to 2 m*M*. The results are summarized in Table I which compares the binding of TM to F-actin with the inhibition of the acto-HMM ATPase for a ratio of 0.25 mol of TM added per mol of F-actin. In 100 m*M* KCl, TM alone both binds to F-actin and inhibits the acto-HMM ATPase. In 12 m*M* KCl, TM alone neither binds to F-actin nor inhibits the acto-HMM ATPase. Thus, the results under different salt conditions also demonstrate the correlation between the binding of TM to F-actin and the inhibition of the ATPase.

Effects of TM plus TN-I. Figure 2B shows the increasing inhibition of the acto-HMM ATPase in 30 m*M* KCl, 2 m*M* ATP, and 5 m*M* $MgCl_2$ as the concentration of TM increases, in the absence and in the presence of a constant concentration of TN-I (1 mol of TN-I per 7 mol of F-actin monomer). The inhibition by TM alone increases sharply until about 0.2 mol of TM has been added per mol of F-actin monomer and then appears to level off at about 60% as the addition of TM increases. Figure 2A shows that the binding of TM to F-actin follows a similar pattern. It increases sharply until about 0.2 mol of TM has been added per mol of F-actin monomer and then begins to level off as the stoichiometric binding ratio (0.14) is reached. Even though the presence of a stoichiometric amount of TN-I does not affect the binding of TM to F-actin under these conditions (Figure 2A), it does increase the inhibition (Figure 2B). The shape of the inhibition curve is similar to that of TM alone, and a maximum of about 85% is attained. Thus, TN-I significantly enhances the inhibition observed when conditions are such that TM alone binds F-actin and inhibits the acto-HMM ATPase by a maximum of 60%.

If the $MgCl_2$ concentration is reduced from 5 to 1 m*M*, TM alone neither inhibits the ATPase (Figure 1B, Table I) nor binds to F-actin (Figure 1A, Table I). Under these conditions, however, TM does enhance the inhibition of the ATPase when TN-I is present (Wilkinson et al., 1972; Eisenberg and Kielley, 1974). As shown in Figure 3B, the addition of TM to a constant amount of TN-I (1 or 2 mol

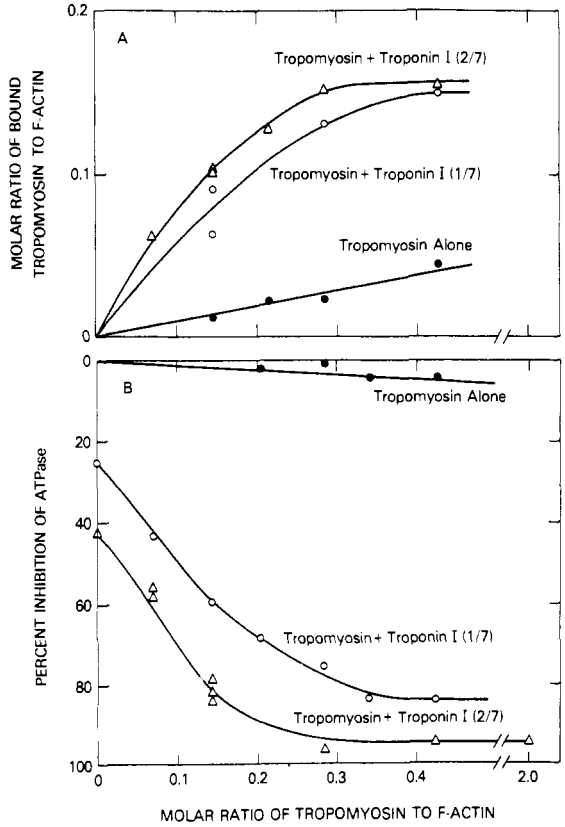

FIGURE 3: Effects of the TN-I on binding of TM to F-actin and the inhibition of the acto-HMM ATPase in low Mg^{2+}. Conditions: 1 m*M* $MgCl_2$, 2 m*M* ATP, 30 m*M* KCl, 1 m*M* EGTA, 0.2 mg/ml of F-actin, 1 m*M* imidazole buffer (pH 7.0); (●) TM alone added; (○) TM added in presence of 0.016 mg/ml of TN-I; (△) TM added in presence of 0.033 mg/ml of TN-I; 0.5 mg/ml of HMM in B only.

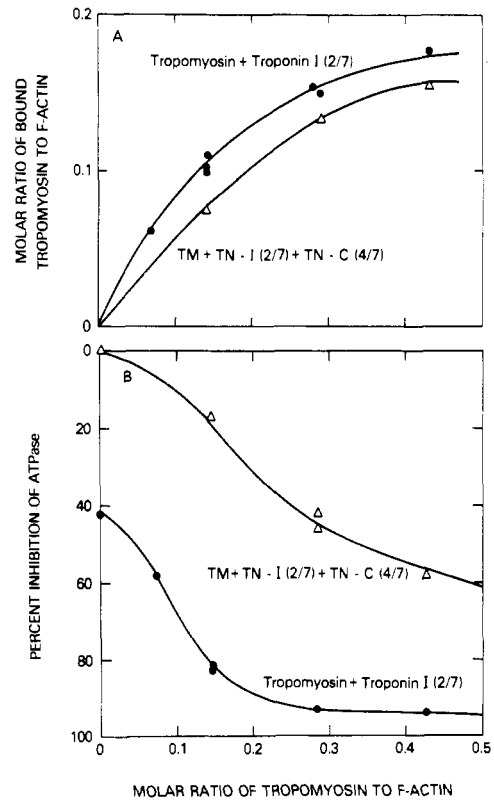

FIGURE 4: Effects of TN-C plus TN-I on binding of TM to F-actin and the inhibition of the acto-HMM ATPase in low Mg^{2+}. Conditions: 1 m*M* $MgCl_2$, 2 m*M* ATP, 30 m*M* KCl, 1 m*M* EGTA, 0.2 mg/ml of F-actin, 1 m*M* imidazole buffer (pH 7.0); (●) TM added in the presence of 0.033 mg/ml of TN-I; (△) TM added in the presence of 0.033 mg/ml of TN-I plus 0.054 mg/ml of TN-C; 0.5 mg/ml of HMM in B only.

Table II: Effects of TN-I on the Binding of TM to F-Actin and the Inhibition of the ATPase.[a]

mol Ratio TN-I/ F-Actin	1 m*M* $MgCl_2$; 12 m*M* KCl		1 m*M* $MgCl_2$; 30 m*M* KCl		5 m*M* $MgCl_2$; 30 m*M* KCl	
	TM Bound	Inhibition, %	TM Bound	Inhibition, %	TM Bound	Inhibition, %
None	0.02	0	0.03	3	0.16	60*
0.14	0.04	10	0.12	75	0.16	82*

[a] Conditions: 2 m*M* ATP, 1 m*M* EGTA, 1 m*M* imidazole buffer (pH 7.0); 0.2 mg/ml of F-actin, 0.08 mg/ml of TM, and 0.5 mg/ml of HMM except (*) 0.7 mg/ml of HMM.

Table III: Effects of TN-I plus TN-C on the Binding of TM to F-Actin and the Inhibition of the Acto-HMM ATPase.[a]

mol Ratio TN-I/F-Actin	mol Ratio TN-C/F-Actin	% Inhibition	mol Ratio TM Bound/F-Actin
None	None	3	0.03
0.29	None	90	0.15
0.29	0.57	40	0.12

[a] Conditions: 1 m*M* $MgCl_2$, 30 m*M* KCl, 2 m*M* ATP, 1 m*M* EGTA, 1 m*M* imidazole buffer (pH 7.0); 0.2 mg/ml of F-actin, 0.08 mg/ml of TM, and 0.5 mg/ml of HMM.

per 7 mol of F-actin monomer) causes the initial inhibition of 20 or 40% by TN-I alone to approach 100% as the TM concentration rises, even though the inhibition by TM alone under these conditions is negligible. This behavior can be explained by binding studies (Figure 3A) which show that TN-I greatly enhances the binding of TM to F-actin until a stoichiometric ratio of TM to F-actin (0.14) is reached. It appears that TN-I can induce TM to bind to F-actin and to inhibit the acto-HMM ATPase when TM alone can do neither. If the KCl concentration is reduced to 12 m*M*, however, even TN-I cannot induce TM to bind to F-actin and the ATPase is inhibited by only 10% (Table II).

Figure 3B also demonstrates that under these conditions the combination of TN-I and TM inhibits the acto-HMM ATPase maximally even when the molar ratio of TM/actin is increased to 2/1. This observation is in disagreement with Wilkinson et al. (1972) who found that in some cases large amounts of TM decreased the extent of inhibition by their inhibitory factor.

Effects of TM plus TN-I and TN-C in the Absence of Ca^{2+}. When conditions are such that TN-I induces stoichiometric binding between TM and F-actin, the inhibition of the acto-HMM ATPase by TM plus TN-I approaches 100% (Figure 3). Further addition of TN-C reduces the inhibition (Figure 4B) and the binding of TM to F-actin (Figure 4A) to different extents. The molar concentration of TN-C was always twice that of TN-I to ensure that the TN-I was fully saturated (Eisenberg and Kielley, 1974). Table III shows the effects of TN-C for a given molar ratio of one TM added per four F-actin monomers. The inhibition is reduced from 90 to 40% but the binding is reduced

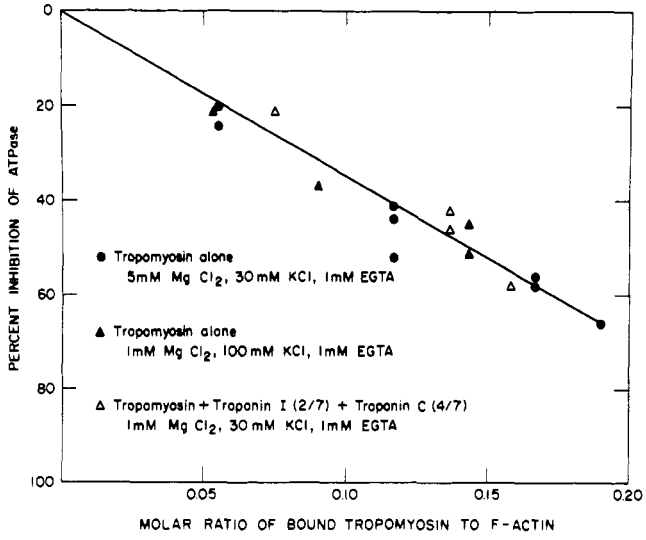

FIGURE 5: Effects of TN-C plus TN-I on the inhibition of the acto-HMM ATPase as a function of the binding of TM to F-actin. Conditions: 2 mM ATP, 1 mM EGTA, 1 mM imidazole buffer (pH 7.0); (●) 5 mM $MgCl_2$, 30 mM KCl, 0.2 mg/ml of F-actin, 0.7 mg/ml of HMM; (▲) 1 mM $MgCl_2$, 100 mM KCl, 0.6 mg/ml of F-actin, 1.5 mg/ml of HMM; (△) 1 mM $MgCl_2$, 30 mM KCl, 0.2 mg/ml of F-actin, 0.5 mg/ml of HMM, 0.033 mg/ml of TN-I, 0.054 mg/ml of TN-C.

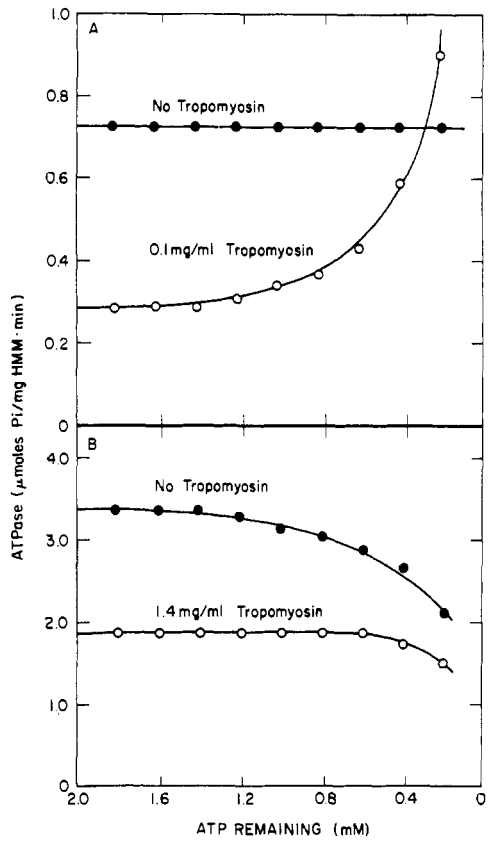

FIGURE 6: Effects of ATP concentration and TM on the acto-HMM ATPase in high Mg^{2+}. Conditions: 5 mM $MgCl_2$; (A) 1 mM EGTA, 0.2 mg/ml of F-actin, 0.27 mg/ml of HMM; (●) no TM; (○) 0.1 mg/ml of TM; (B) 2.8 mg/ml of F-actin, 0.11 mg/ml of HMM; (●) no TM; (○) 1.4 mg/ml of TM.

only from 0.15 to 0.12. If the residual inhibition under these conditions is plotted as a function of the molar ratio of TM bound per F-actin monomer, the points follow the linear relationship that exists when TM alone both binds to F-actin and inhibits the ATPase (Figure 5). Since it has been reported that the complex of TN-I plus TN-C binds to F-actin only in the presence of TM and the absence of Ca^{2+} (Hitchcock et al., 1973; Potter and Gergely, 1974), the above result (Figure 5) suggests that the complex of TN-I plus TN-C, like TN-I alone, can induce TM to bind to F-actin but this same complex, unlike TN-I alone, does not enhance the inhibition caused by TM.

Dual Effect of TM on the Mg^{2+}-Activated ATPase. Figure 6A shows the acto-HMM ATPase activity as a function of decreasing ATP (and increasing ADP) concentration under conditions such that TM alone binds to F-actin. In the absence of TM the rate is constant over the whole range of ATP concentration. Addition of TM to the system produces either of two effects. At high ATP concentrations, as shown above, TM inhibits the ATPase, whereas at very low concentrations of ATP, TM activates the ATPase. Figure 6B shows data similar to that in 6A except that the ratio of HMM to F-actin is drastically reduced. Although the inhibition of the acto-HMM ATPase still occurs at high ATP concentration, the activation by TM at low ATP concentration disappears, suggesting that the activation is cooperative. When this experiment is repeated in 1 mM $MgCl_2$ and 2 mM ATP, addition of TM neither inhibits at high ATP (Figure 1B) nor activates the ATPase as the ATP concentration falls (data not shown), presumably because TM does not bind to F-actin under these conditions.

Discussion

Inhibitory Effect of TM Alone. The results summarized in Table I demonstrate the following points. First, when purified TM is bound to F-actin, the Mg^{2+}-activated acto-HMM ATPase is inhibited provided that the HMM is saturated with ATP. Second, a maximum inhibition of about 60% occurs when 1 mol of TM is bound per 6–7 mol of F-actin monomer. Third, the occurrence of inhibition and binding by TM alone is critically dependent on either high ionic strength if the free Mg^{2+} concentration is lower than 10^{-4} M or on millimolar concentrations of free Mg^{2+} when the KCl concentration is low. These results with acto-HMM are consistent with observations made on actomyosin by Katz (1964) and Shigekawa and Tonomura (1973). They showed that purified TM can inhibit the Mg^{2+}-activated ATPase of actomyosin in the presence of 0.1 or 0.04 M KCl, 1 mM ATP, and 2 mM Mg^{2+}. For a ratio of 1 mol of TM added per 6.5 mol of actin monomer, Katz (1964) obtained a maximum inhibition of 50–70% depending on whether the TM was added to F-actin or G-actin prior to polymerization. The results in Table I may explain why Schaub et al. (1967), Schaub and Ermini (1969), Wilkinson et al. (1972), and Cummins and Perry (1973) have been unable to demonstrate any inhibitory effect of TM alone on the Mg^{2+}-activated ATPase of actomyosin. Their experimental conditions of 2.5 mM ATP, 2.5 mM $MgCl_2$, and no added salt probably preclude the binding of TM alone to F-actin.

The conditions under which TM binds to F-actin have been studied by a number of investigators. Martonosi (1962) discovered that TM does not bind to actin polymerized in 0.7 mM Mg^{2+} and 0.2–0.4 mM ATP. Maruyama (1964) showed that TM binding to F-actin in 0.5 mM ATP commenced at 1.6 mM Mg^{2+} or 30 mM KCl and was complete above 3 mM Mg^{2+} or 60 mM KCl. Recently Tanaka (1972) found that addition of 2.5 mM Mg^{2+} raised the dis-

sociation temperature of the TM–F-actin complex by 5°. Tanaka suggested that Mg^{2+} strengthens the structure of F-actin, making it more rigid and able to bind TM even when the latter has lowered helix content at elevated temperatures. The complementary conclusion that bound TM makes F-actin more rigid has been reached by Kawamura and Maruyama (1970) using electron microscopy, and by Fujime and Ishiwata (1971) using quasielastic scattering of laser light. These studies suggest a correlation between the rigidity of F-actin and its ability to bind TM. Since Ishiwata and Fujime (1972) subsequently found that in Ca^{2+}-free solution addition of troponin further increased the rigidity of TM-containing actin filaments, it is possible that the mechanism by which TN-I induces TM to bind to F-actin may involve a stiffening of the actin filament.

Effects of TM plus TN-I. In the presence of millimolar free Mg^{2+}, binding of 1 mol of TM per 6–7 mol of F-actin monomer takes place, and no increase in this binding is induced in the presence of the same ratio (1/7) of TN-I (Figure 2A), although the inhibition of the ATPase does increase from 60% to about 85% (Figure 2B). Assuming that one molecule of TM inhibits seven F-actin monomers by 60% each, one would expect an increased inhibition of only about one-seventh of the remaining 40% activity if one molecule of TN-I affects only one of seven F-actin monomers. The fact that 85% is considerably more than 66% implies that this TN-I effect must be cooperative in nature. It follows, therefore, that either one molecule of TN-I must affect several F-actin monomers or TN-I must work in concert with TM to induce cooperativity in F-actin. Since TM consists of rod-shaped molecules which can lie in the groove of the actin filament (Hanson and Lowy, 1963; O'Brien et al., 1971), it is quite possible that seven adjacent F-actin monomers may be directly influenced by one molecule of TM. TN-I could work in concert with TM by influencing the way TM interacts with F-actin, thus affecting the amount of inhibition which occurs. This type of cooperative effect by troponin and TM was originally suggested by Ebashi et al. (1968). On the other hand, the recent finding by Eisenberg and Kielley (1974) that 1 mol of TN-I plus 1 mol of TN-T per 4 mol of F-actin monomer can inhibit the ATPase by 80% suggests that cooperativity in the actin filament in the absence of TM still remains a possibility. Therefore, the exact mechanism of the cooperative TM–TN-I effect remains to be determined.

When ATP is in excess of Mg^{2+} so that the free Mg^{2+} concentration is less than 10^{-4} *M*, TM alone does not bind to F-actin. The presence of a stoichiometric amount of TN-I, however, can dramatically increase both the binding of TM to F-actin and the ATPase inhibition (Figure 3; Table II). The induction of TM binding by TN-I which occurs under these conditions is complementary to the recent report by Potter and Gergely (1974) that TM enhances the binding of TN-I to F-actin. Yamaguchi et al. (1974) have suggested that TN-I and TM can interact directly based on their observation that the presence of TN-I drastically decreases the amount of TM paracrystals produced in 50 m*M* $MgCl_2$. In contrast, direct interaction between TN-I and TM has not been demonstrable using sodium dodecyl sulfate gel electrophoresis (van Eerd and Kawasaki, 1973; Potter and Gergely, 1974), ultracentrifugation (Greaser et al., 1973), addition of TN-I to preformed TM paracrystals (Greaser et al., 1973), or studies of paracrystals formed from mixtures of TN-I plus TM (Margossian and Cohen, 1973). If TN-I and TM do not interact directly, then the enhancement by TM and TN-I of each other's binding to F-actin must be mediated by the actin filament. This would mean that bound TN-I can cause a conformational change in F-actin, allowing TM to bind, and that bound TM can also induce a similar conformational change in F-actin, allowing TN-I to bind. This explanation is valid at 30 m*M* KCl; at 12 m*M* KCl, however, even the addition of TN-I does not induce TM to bind to F-actin so it cannot inhibit the ATPase (Table II).

The fact that TN-I both induces TM to bind to F-actin (Figure 3A) and enhances the inhibition of the acto-HMM ATPase (Figure 3B) suggests a dual role for TN-I when conditions do not allow TM alone to bind to F-actin. First, it induces TM to bind to F-actin, thus causing TM to inhibit the ATPase. Second, it can itself enhance the inhibition of the ATPase in a cooperative manner. It appears that TN-C can block the second of these roles but not the first.

Figure 5 shows that the inhibition exerted on the acto-HMM ATPase by a mixture of TM, TN-I, and TN-C corresponds to the inhibition exerted by TM alone when conditions are such that TM alone both binds to F-actin and inhibits the ATPase. This relationship strongly suggests that the effect of TN-C on the ATPase is to reduce the inhibition of TM plus TN-I to a level which can be produced by the bound TM alone. Thus, Figure 5 shows that TN-C completely reverses the cooperative inhibitory effect of TN-I in the presence of TM even though it has only a limited effect in reversing TN-I's ability to induce TM to bind to F-actin under these conditions (Figure 4A). This result is consistent with the finding of Hitchcock et al. (1973) that the complex of TN-I plus TN-C does bind to F-actin when TM is present and the Ca^{2+} concentration is very low. Although additional experiments will have to be performed in the presence of Ca^{2+} and under conditions which allow TM alone to bind to F-actin before a general conclusion about the role of TN-I plus TN-C can be drawn, our tentative conclusion based on the above data is that the complex of TN-I plus TN-C can induce TM to bind to F-actin but has no additional effect on the Mg^{2+}-activated acto-HMM ATPase.

Dual Effect of TM on the Mg^{2+}-Activated ATPase. The data shown in Figure 6A demonstrate that TM exerts a dual effect on the acto-HMM ATPase similar to the effect on the actomyosin ATPase reported 10 years ago by Katz (1964): inhibition at high levels of ATP followed by an activation of the ATPase when most of the ATP has been hydrolyzed. This activation does not occur when the ratio of HMM to F-actin is reduced drastically (Figure 6B), suggesting that cooperativity between HMM and TM-containing actin filaments is required. Such cooperative activation has been reported with actomyosin and TM (Shigekawa and Tonomura, 1972, 1973), acto-HMM and native TM (Eisenberg and Kielley, 1970), and acto-subfragment 1 and TM (Bremel et al., 1973). The dual biochemical role of TM can be related to recent X-ray and optical diffraction studies (Spudich et al., 1972; Hanson et al., 1973; Haselgrove, 1973; Huxley, 1973; Parry and Squire, 1973) which indicate that there may be two binding positions for TM on F-actin: one position associated with relaxation (analogous to the inhibition of the ATPase in biochemical terms) and a second position, closer to the center of the groove of the actin filament, which is associated with both contraction and rest length rigor. Thus, there is a correlation between the structural evidence showing that TM has two different positions on the thin filament and the biochemical evidence showing that TM has either an inhibitory or an activating

effect on the acto-HMM ATPase, depending on the conditions.

To explain the cooperative nature of the activation effect of TM, Bremel and Weber (1972) postulated that formation of "rigor bonds" between nucleotide-free HMM and F-actin releases the inhibition of adjacent actin monomers, allowing them to interact with any HMM still containing bound nucleotide. This theory is supported by X-ray evidence (Haselgrove, 1973) that the attachment of myosin cross-bridges to the thin filaments in Ca^{2+}-free rigor links may cause TM to move from the position associated with relaxation toward the center of the groove in the thin filament.

The experimental evidence reviewed above has been incorporated into a model (Huxley, 1973) in which TM physically obstructs the interaction between myosin and actin when it is in the relaxation position. One problem with this model, however, is that the data shown in Figures 1B and 2B suggest that TM may have more than an all or none effect. In the absence of TN-I, TM inhibits the acto-HMM ATPase by a maximum of about 60%, and it is not clear how this partial inhibition can be explained by the steric blocking model (Huxley, 1973). Possibly TM can occupy intermediate positions, but further work will be necessary to determine if this is in fact the case.

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On Neutral Fucoglycolipids Having Long, Branched Carbohydrate Chains: H-Active and I-Active Glycosphingolipids of Human Erythrocyte Membranes†

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ABSTRACT: H-Active ceramide heptasaccharide (H_2-glycolipid) and ceramide decasaccharide (H_3-glycolipid) were isolated from blood group O human erythrocyte membranes. Their structures have been determined by conventional methylation analysis, enzymatic degradation, and direct total mass spectrometry of the enzymatic degradation products after permethylation and reduction with sodium bis(2-methoxyethoxy)aluminum hydride. The branched sugar residue in the structure of H_3-glycolipid was unambiguously determined by a new method with the combination of enzymatic degradation and comparison of the total mass spectrogram of the reduced product of the enzyme-degraded compounds. The proposed structures are as follows:

H_2 component: α_L-Fuc(1→2)βGal(1→4)βGlcNAc(1→3)βGal(1→4)βGlcNAc(1→3)βGal(1→4)Glc–ceramide

H_3 component: α_L-Fuc(1→2)βGal(1→4)βGlcNAc(1→$^{3)}_{6)}$$\beta$Gal(1→4)$\beta$GlcNAc(1→3)$\beta$Gal(1→4)Glc–ceramide
α_L-Fuc(1→2)βGal(1→4)βGlcNAc(1→

The fourth component of H-active glycolipid (H_4-glycolipid) was also isolated in chromatographically heterogenous form, but chemical analysis and methylation study indicate heterogeneity of the fraction. Both H_3- and H_4-glycolipids inhibit I-hemagglutination, whereas H_1- and H_2-glycolipids do not inhibit I-hemagglutination.

Recent studies on blood group ABH glycolipid antigens of human erythrocyte membranes indicate the presence of multiple forms of glycolipids carrying A, B, and H determinants (see, for a review, Hakomori, 1974). Chemically all these are fucoglycosphingolipids with internal variance in the carbohydrate chain. Although H activity in the glycolipid fraction of human erythrocytes was previously claimed to be difficult to demonstrate (Koscielak et al., 1970), the presence of H-active glycolipids has been proven in recent studies (Stellner et al., 1973; Koscielak et al., 1973).

At least four types of H-active glycolipids have been distinguished according to their migration rates on thin-layer chromatography. The fastest migrating component (H_1-glycolipid) was characterized unambiguously as L-Fucα(1→2)Galβ(1→4)GlcNAcβ(1→3)Galβ(1→4)Glc→ ceramide, i.e. lacto-*N*-fucopentaosyl(IV)ceramide (Stellner et al., 1973). Koscielak et al. (1973) also reported the isolation of a ceramide pentasaccharide with a structure identical with "H_1-glycolipid" and of a ceramide heptasaccharide having a straight carbohydrate chain bearing the H-active determinant. Among a number of oligosaccharides liberated from a ganglioside mixture of human spleen by ozonolysis–alkaline degradation method, an H-active fucosylsialyl oligosaccharide was isolated whose structure was identified as αL-Fuc(1→2)Galβ(1→3)GalNAcβ(1→3)[NeuNG(2→3)]Galβ(1→4)Glc (Wiegandt, 1973). The presence of sialylhexaose was also noticed, whose desialylated residue was chromatographically indistinguishable from "lacto-*N*-neohexaose" (Wiegandt, 1973), the branched milk oligosaccharide previously reported by Kobata and Ginsburg (1972).

The presence of a third H-active component (H_3-component) with a slower migration rate on thin-layer chromatography was found in this laboratory, but further purification was necessary, and its chemical structure has been awaiting elucidation. The presence of a fourth H-active component was recently recognized and has been partially characterized.

Interestingly, H_3 and H_4 components clearly inhibit I-hemagglutination, in contrast to H_1 and H_2 components, which do not. The H_3 and H_4 components may represent a major recognition site on human erythrocyte membranes for anti-I ("Ma") of Feizi et al. (1971).

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