THIAMINE TRIPHOSPHATASE ACTIVITY OF MYOSIN AND ACCELERATING EFFECT OF THIAMINE DI- AND TRI-PHOSPHATES ON SUPERPRECIPITATION OF ACTOMYOSIN

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Summary TTP accelerated ATP-induced superprecipitation of actomyosin in as low a concentration as 30 μM and decreased light scattering by actomyosin. These effects could also be observed in the same way, but to a lesser degree, by addition of TDP. Myosin was able to hydrolyze TTP to TDP, but some important differences were confirmed between myosin TTPase and ATPase. Myosin TTPase was inhibited by actin and showed a much larger $K_m$ than that of ATPase. TTP significantly inhibited myosin B ATPase and ATP greatly inhibited myosin B TTPase. These findings suggest that the accelerating effect of TDP and TTP may be due to the binding of thiamine phosphate to the regulatory site of myosin followed by a change in its physical chemical property, rather than due to the competitive binding of thiamine phosphate to the catalytically active site of myosin.

In 1964 we first discovered a remarkable inotropic action of thiamine and its synthetic derivatives except phosphate compounds on the isolated toad heart (1, 2). In our later study to account for the mechanism of this action, glycerinated muscle fiber was taken as a simple model of muscle contraction and it was concluded that a muscle contraction was never induced by any of these substances including thiamine phosphates, but that two of their phosphate compounds i.e., TDP and TTP, enhanced ATP-induced contraction (3). This observation necessarily led us to examine whether TDP and TTP were able to accelerate ATP-induced superprecipitation of actomyosin because it was generally accepted that ATP-induced superprecipitation of actomyosin associated with increased ATPase activity corresponded just to muscle contraction.

The following abbreviations are used: TDP, thiamine diphosphate; TTP, thiamine triphosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; GEDTA, glycoletherdiamine tetraacetic acid; pCMB, p-chloromercuric benzoate.

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With respect to TTPase activity of myosin, there are contradictory reports. According to VELLUZ et al., potato apyrase hydrolyzes TTP but ATPase preparation obtained from guinea pig muscle is devoid of any hydrolyzing activity towards TTP (4). On the contrary, GREILING et al. observed that myosin prepared from rabbit muscle hydrolyzed TTP to liberate terminal orthophosphate and this myosin TTPase activity was activated by Ca\(^2+\) and inhibited by Mg\(^2+\) as well as ADP (5).

The present study was carried out with the aim to investigate thoroughly the property of myosin TTPase and to compare it with myosin ATPase to account for the mechanism of the accelerating effect of thiamine phosphates and the functional role which they may play in muscle contraction.

**EXPERIMENTAL**

**Preparation of actomyosin.** Myosin B was prepared from rabbit skeletal muscle and heart muscle according to the prescriptions of EBASHI (6) and BENSON et al. (7), respectively. Myosin and actin were prepared from rabbit skeletal muscle according to the prescriptions of PERRY (8) and SZENT-GYÖRGYI (9), respectively.

**Superprecipitation of actomyosin.** Superprecipitation of myosin B was measured by following the increase in turbidity (6). The optical density at 660 m\(\mu\) was successively determined using a Shimazu D40S spectrophotometer.

**Light scattering by actomyosin.** Using a Shimazu GSF-16 spectrofluorophotometer, the scattered light at an angle 90° was followed after adding the reagent. The wavelength of exciting light was adjusted to 420 m\(\mu\) and scattered light was measured at the same wavelength.

**Determination of TTPase and ATPase activities.** TTPase and ATPase activities of myosin were determined by measuring the liberated orthophosphate by the method of NAKAMURA (10). When TTP\(^{32}\)P or ATP\(^{32}\)P was used, the liberated orthophosphate was extracted with an aliquot of an isobutanolbenzene mixture (11) and then enzyme activity was determined by counting radioactivity with a Nuclear Chicago 181A gas flow counter.

**Determination of protein.** Protein was determined by the Biuret method in which crystalline bovine serum albumin was taken as a standard.

**Materials.** TTP and TTP\(^{32}\)P were generously supplied by the Sankyo Co., Japan, and a part of the TTP was also supplied by the Takeda Chemical Industries Ltd., Japan. ATP\(^{32}\)P was a gift of Dr. S. Nakamura. Disodium ATP was purchased from the Wako Pure Chemical Industries Ltd., Japan, GEDTA from Dojindo Laboratories, Japan, and Tris (Trizma, Sigma 121) from the Sigma Co., U.S.A. All other chemicals used were of analytical grade. We tried to avoid contamination by extraneous Ca\(^2+\) as much as possible. Therefore, only Hario glassware and polyethylene or polypropylene laboratory ware were used. Deionized water treated with a mixed bed resin was distilled with all glass apparatuses. Tris-maleate buffer, TDP, TTP and ATP stock solutions were passed
through a Chelex 100 column. Residual Ca$^{2+}$ contents were measured but no Ca$^{2+}$ was detected.

RESULTS

1. Accelerating effect of TDP and TTP on ATP-induced superprecipitation of myosin B

When superprecipitation of myosin B was induced by addition of ATP in the presence and absence of thiamine phosphate, it was noted that both TDP and TTP shortened the clearing phase and remarkably increased the speed of superprecipitation but did not change its extent. While the effect of TDP was observed only in concentrations higher than 740 µM, TTP was able to exert the accelerating effect in as low a concentration as 30 µM in the case of skeletal myosin B (Fig. 1).

Fig. 1 Effect of TTP on the superprecipitation of myosin B initiated by the addition of ATP. Basal medium: 0.38 mg/ml skeletal myosin B, 73 mM KCl, 20 mM Tris-maleate buffer pH 7.4, 1 mM MgCl$_2$, 4 µM GEDTA, at room temperature. The final concentration of ATP was 0.5 mM. Broken lines indicate the control curves without TTP. Solid lines indicate the curves in the presence of TTP: upper, 300 µM TTP; lower, 30 µM TTP.

Fig. 2. Effect of ATP, TDP and TTP on light scattering by myosin B. Basal medium: 1.08 mg/ml skeletal myosin B (Δ, □, ■, ○, ●) or 0.13 mg/ml cardiac myosin B (×), 0.6 mM KCl, 20 mM Tris-maleate buffer pH 7.4, 1 mM MgCl$_2$, at room temperature. The final concentrations of phosphate compounds added were: 0.5 mM ATP (Δ), 1.2 mM TDP (□), 10 mM TDP (■), 1.2 mM TTP (○), 10 mM TTP (●, ×). Scattered light is expressed as percent of original value.
On the other hand, this effect on cardiac myosin B was detected only in the presence of more than 900 μM TTP (not shown).

2. *Effect of TDP and TTP on light scattering by myosin B*

When ATP was added to skeletal myosin B solution, scattered light rapidly decreased to a constant level corresponding to 47% of the original value. The addition of TDP or TTP also decreased the scattered light, but the speed as well as extent of decrease were significantly less than those produced by ATP, indicating the lower affinity of TDP and TTP for myosin B than that of ATP. This affinity was much lower in cardiac myosin B than in the skeletal one (Fig. 2).

3. *TDPase and TTPase activities of skeletal myosin*

Myosin hydrolyzed both TDP and TTP at the rates of 0.67 and 34.2 mmol/min/mg protein, respectively, under the following condition: 0.15 M KCl, 20 mM Tris-maleate buffer pH 7.4, 1 mM MgCl₂, 1 mM CaCl₂, 0.5 mM TDP or TTP, and 25°C.

(a) *Effect of metal ions.* Both K⁺ and Rb⁺ activated myosin ATPase but Na⁺ inhibited it. On the other hand, myosin TTPase was activated by Rb⁺, while it was rather inhibited by K⁺ and Na⁺ (Fig. 3). As the concentration of Mg²⁺ was increased, myosin ATPase was strikingly inhibited, but myosin TTPase was strikingly activated both in the presence and absence of Ca²⁺ (Fig. 4). As the concentration of Ca²⁺ was increased, myosin ATPase was activated both in the presence and absence of Mg²⁺, especially in the latter, but the effect of Ca²⁺ on TTPase was reversed by Mg²⁺. Ca²⁺ activated myosin TTPase in the absence...
Fig. 4. Effect of Mg$^{2+}$ on myosin TTPase and ATPase activities. Basal medium: 0.44 mg/ml (TTPase) or 0.22 mg/ml (ATPase) skeletal myosin, 0.15 M KCl, 20 mM Tris-maleate buffer pH 7.4. The final concentration of TTP and ATP was 0.5 mM and incubation was carried out for 5 min at 25°C. Open symbols indicate no addition of Ca$^{2+}$ and solid symbols indicate the presence of 1 mM Ca$^{2+}$.

Fig. 5. Effect of Ca$^{2+}$ on myosin TTPase and ATPase activities. Basal medium: 0.44 mg/ml (TTPase) or 0.088 mg/ml (ATPase) skeletal myosin, 0.15 M KCl, 20 mM Tris-maleate buffer pH 7.4. The final concentration of TTP and ATP was 0.5 mM and incubation was carried out for 5 min at 25°C. Open symbols indicate no addition of Mg$^{2+}$ and solid symbols indicate the presence of 1 mM Mg$^{2+}$.

of Mg$^{2+}$ but inhibited it in the presence of 1 mM Mg$^{2+}$ (Fig. 5).

(b) pH-Dependency. pH-dependency of myosin ATPase and TTPase activities was determined in various concentrations of Ca$^{2+}$. True optimal pH was observed around 6.5 in each case (12) (Fig. 6).
Fig. 6. pH-dependency of myosin TTPase and ATPase activities. Basal medium: 0.27 mg/ml (TTPase) or 0.014 mg/ml (ATPase) skeletal myosin, 0.15 M KCl, 50 mM Tris-maleate and glycine-NaOH buffer, 0.5 mM TTP or ATP. Incubation was carried out for 5 min at 25°C. Circle indicates TTPase activity in the presence of 25 mM Ca²⁺ and triangle indicates ATPase activity in the presence of 5 mM Ca²⁺.

Fig. 7. Km of myosin TTPase. Basal medium: 0.15 M KCl, 20 mM Tris-maleate buffer pH 7.4, at 25°C. The concentrations of skeletal myosin were 0.52 mg/ml in the case of 0.1 and 0.2 mM TTP and 0.33 mg/ml in the case of 1 mM TTP. Single, double and solid circles indicate Km in the presence of 1 mM Mg²⁺, 0.1 mM Mg²⁺ and 1 mM Ca²⁺, respectively.

(c) Michaelis constant. Km of myosin ATPase was within the range of 0.04 to 0.09 mM under the conditions used. On the other hand, Km of myosin TTPase was 0.17 mM in the presence of 1 mM Mg²⁺ without the addition of Ca²⁺, and 1.8 mM in the presence of 1 mM Ca²⁺ without the addition of Mg²⁺ (Fig. 7).

(d) Substrate inhibition. When the concentration of TTP was raised to 1 mM or more in the presence of 1 mM Mg²⁺ and Ca²⁺, the reaction was significantly inhibited (Fig. 8).

(e) Effect of SH reagent. pCMB activated myosin ATPase in 10 μM or lower concentration but inhibited it in 30 μM or higher concentration. As the concentration of pCMB was increased, myosin TTPase activity was successively inhibited and no activity was detected in 0.1 mM or higher concentration (Fig. 9).

(f) Effect of aging. Although myosin preparation had been kept at 0°C, both ATPase and TTPase activities gradually diminished as the time elapsed. The rate of decrease was much higher in TTPase than in ATPase and hence the ratio of TTPase to ATPase activity decreased with aging. This tendency was confirmed until 80 days after sacrifice of the animal (Fig. 10).

(g) Early burst in TTP hydrolizing reaction by myosin. Analysis of the early phase of this reaction within one minute after addition of TTP clearly revealed the phenomenon of early burst which was considered to indicate the formation of phosphorylated myosin with TTP (Fig. 11). Calculating the extent of phosphoryl-
Fig. 8. Substrate inhibition of myosin TTPase. Basal medium: 0.3 mg/ml skeletal myosin, 0.15 M KCl, 20 mM Tris-maleate buffer pH 7.4, 1 mM MgCl₂, 1 mM CaCl₂, at 25°C. Under this condition $K_m$ was 0.3 mM.

Fig. 9. Effect of pCMB on myosin TTPase and ATPase activities. Basal medium: 0.52 mg/ml skeletal myosin, 0.15 M KCl, 20 mM Tris-maleate buffer pH 7.4, 1 mM MgCl₂, 1 mM CaCl₂, 0.5 mM TTP or ATP. Myosin was preincubated with pCMB and then the reaction was initiated by the addition of TTP or ATP. Incubation was carried out for 5 min at 25°C. Circle indicates TTPase and triangle indicates ATPase.

ation on the basis of extra liberation of orthophosphate, about one-tenth of the myosin molecules were phosphorylated with TTP.

(h) Effect of actin. It is well known that actin activates myosin ATPase activity, but on the other hand, actomyosin immediately dissociates into actin and myosin provided the concentration of KCl is increased, and at the same time the low concentration of polyphosphate is present. In the present study, myosin ATPase activity was enhanced in proportion to the amount of actin added. In contrast, myosin TTPase activity was inhibited linearly in proportion to the amount of actin added and no activity could be detected if the ratio of actin to myosin exceeded 5/18 (Fig. 12). As the concentration of KCl was increased, ATPase activity of myosin B was rapidly decreased but TTPase was gradually activated (not shown).

4. Effect of TTP on skeletal myosin B ATPase activity and ATP on skeletal myosin B TTPase activity

To ascertain whether the accelerated superprecipitation shown in Fig. 1 was accompanied with increased ATPase activity, liberated orthophosphate was determined in the course of this phenomenon. The presence of 900 μM TTP brought about a significant increase of the rate of orthophosphate liberation
Fig. 10. Effect of aging on myosin TTPase and ATPase activities. Basal medium: 1.7 mg/ml (TTPase) or 3.5 mg/ml (ATPase) skeletal myosin, 0.15 M KCl, 20 mM Tris-maleate buffer pH 7.4, 1 mM MgCl₂, 1 mM CaCl₂, 0.5 mM TTP or ATP, at 25°C. Circle and triangle indicate TTPase and ATPase, respectively.

Fig. 11. Early burst in TTP hydrolysis by myosin. Basal medium: 1.5 mg/ml skeletal myosin, 0.15 M KCl, 20 mM Tris-maleate buffer pH 7.4, 10 μM MgCl₂, at 25°C. The reaction was initiated by the addition of TTP-³²P, the final concentration of which was 100 μM.

which was considered to be attributable exclusively to the increased ATPase activity (Fig. 13). Since the liberation of orthophosphate was nearly parallel to the increase in turbidity, it was concluded that ATPase was activated by TTP during superprecipitation. To separate the effect of TTP on myosin B ATPase activity from the possible contribution of TTPase activity, both TTP and ATP-³²P were simultaneously added to start the reaction and an aliquot was drawn to determine the amount of ³²P liberated. Figure 14 indicates that ATPase activity was significantly inhibited by TTP. It should be taken into account that this reaction was carried out under a condition corresponding to the clearing phase. In fact, no superprecipitation could be observed during this experiment. It is of interest that the result thus obtained is clearly in contrast to the finding that myosin B ATPase is activated by TTP during superprecipitation as shown in Fig. 13. If the reaction was carried out under a condition corresponding to the clearing phase, it would be expected that the total orthophosphate liberated by myosin B would be less in the presence of both TTP and ATP than in the presence of ATP alone. This was realiz-
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Fig. 12. Effect of actin on myosin TTPase and ATPase activities. Basal medium: 0.46 mg/ml (TTPase) or 1.8 mg/ml (ATPase) skeletal myosin, 0.5 M KCl, 20 mM Tris-maleate buffer pH 7.4, 1 mM MgCl₂, 1 mM CaCl₂, 0.5 mM TTP or ATP. Incubation was carried out for 5 min at 25°C. Circle and triangle indicate TTPase and ATPase, respectively.

Fig. 13. Effect of TTP on ATPase activity of myosin B. Basal medium: 0.81 mg/ml skeletal myosin B, 92 mM KCl, 20 mM Tris-maleate buffer pH 7.4, 1 mM MgCl₂, 8 μM GEDTA, at room temperature. The final concentration of ATP was 0.5 mM. Open triangle indicates the control curve without TTP. Solid triangle indicates the curve in the presence of 900 μM TTP.
Fig. 14. Effect of TTP on myosin B ATPase activity. Basal medium: 1.2 mg/ml skeletal myosin B, 0.15 M KCl, 20 mM Tris-maleate buffer pH 7.4, 1 mM MgCl₂, 48 μM GEDTA, at 25°C. Open triangle indicates the reaction in which the substrate was 0.5 mM ATP-³²P. Solid triangle indicates the reaction in which the substrates were 0.5 mM ATP-³²P and 1.9 mM TTP.

Fig. 15. Effect of TTP on myosin B ATPase activity. Total amount of orthophosphate was determined under the condition corresponding to the clearing phase. Basal medium: 1.3 mg/ml skeletal myosin B, 0.15 M KCl, 20 mM Tris-maleate buffer pH 7.4, 1 mM MgCl₂, 25 μM GEDTA, at 25°C. Triangle indicates the control experiment in which ATP (0.5 mM) was added into basal medium at the fifth minute. Circle indicates the experiment in which TTP (2 mM) was added into basal medium at the beginning and then ATP (0.5 mM) was added at the fifth minute. At the time shown in the figure an aliquot was drawn to determine total orthophosphate liberated.

The results obtained in the present study clearly demonstrated that TTP becomes bound to the myosin molecule followed by a change of its physical chemical property and at the same time it is hydrolyzed to TDP. It was also demonstrated that TDP could be bound to myosin even if not so tightly. It is,
Fig. 16. Effect of ATP on myosin B TTPase activity. Basal medium: 2.2 mg/ml skeletal myosin B, 0.15 M KCl, 20 mM Tris-maleate buffer pH 7.4, 1 mM MgCl₂, 485 μM GEDTA, at 25°C. Open circle indicates the reaction in which the substrate was 0.5 mM TTP-32P. Solid circle indicates the reaction in which the substrates were 0.5 mM TTP-32P and ATP, respectively.

however, yet in doubt whether TDP is actually hydrolyzed with myosin or not because the TDPane activity was too much lower than the TTPase activity to confirm the reaction and the possibility of contaminant TTP remained to be elucidated.

GREILING et al. first described myosin TTPase activity which was greatly activated by Ca²⁺, inhibited by Mg²⁺ and ADP, and showed two optimal pH around 6.5 and 9 (5). Their description is consistent with our result with respect to pH-dependency, but there is disagreement between them regarding the effect of Mg²⁺. It seems likely that this disagreement is attributable to the difference of pH adopted, which was 9.1 in the former and 7.4 in the latter.

Some differences were shown between myosin ATPase and TTPase. Among these different properties, it is of interest that actin activated myosin ATPase but inhibited myosin TTPase, and furthermore, the $K_m$ of TTPase was much larger than that of ATPase. These observations suggest that myosin TTPase activity may be almost completely inhibited by actin in vivo and that TTP is not available for the energy liberating reaction in place of ATP, though TTP contains an energy-rich phosphate bond. Therefore, it appears that TTP accelerates ATP-induced superprecipitation of actomyosin neither by supplying high energy required for superprecipitation nor by transferring high energy phosphate to ADP, but rather by binding to the myosin molecule and subsequently changing its physical chemical property.

Even at present our knowledge is not enough to understand the precise mechanism of myosin ATPase reaction and the interaction of ATP and actomyosin. Accordingly, it is impossible in the strict sense to analyze the mechanism of the
accelerating effect of TTP on superprecipitation. Myosin B TTPase activity is apparently inhibited by ATP and myosin B ATPase activity is also apparently inhibited by TTP. These findings allow us to assume two possibilities accounting for the effect of TTP. (1) ATP and TTP are competitively bound to the common site of the myosin molecule at which both ATP and TTP undergo enzymic hydrolysis. Although ATP is bound to the myosin molecule more strongly than TTP, the latter can competitively inhibit the binding of ATP to myosin. It is conceivable that such an inhibitory effect of TTP brings about an acceleration of superprecipitation of actomyosin. (2) TTP is bound to the regulatory site of the myosin molecule at which TTP is not hydrolyzed but changes ATPase activity. If this is the case, TTP should be considered as an allosteric effector. There are many reports that ADP enhances ATP-induced contraction of glycerinated muscle fiber and ATP-induced flocculation of myofibril (13, 14, 15). EISENBERG and Moos postulated that the binding of ADP to the relaxing site raises the threshold ATP level at which contraction was elicited (16). PUSZKIN et al. demonstrated that ADP clearly accelerated ATP-induced superprecipitation of platelet actomyosin and suggested that ADP might form part of a control mechanism of platelet contractile activity (17). TTP evidently shortened the clearing phase in spite of the inhibitory effect of TTP on myosin B ATPase and strikingly activated ATPase once superprecipitation began. Therefore, it seems most likely that both TDP and TTP accelerates superprecipitation of actomyosin in a fashion similar to the effect of ADP. However, at present it is very difficult to approach this control mechanism and there is as yet no direct evidence supporting the mechanism.

Since TDP and TTP are able to accelerate ATP-induced superprecipitation of actomyosin, they might augment the muscle contraction if sufficient amounts of TDP and TTP are produced in muscle cell. Many studies have shown that synthetic derivatives of thiamine easily penetrate into the cell and are accumulated to increase the intracellular concentration of thiamine phosphates (18). FUJIWARA et al. demonstrated that TDP in heart muscle occupies 93% of the total thiamine after repeated injections of thiamine propyldisulfide (18). NAKAMURA and KATSURA, one of the present authors, estimated the amounts of de novo synthesis of TDP and TTP in the heart muscle of rat killed 30 min after a single intravenous injection of thiamine propyldisulfide-14C at a dose of 1.5 mg per 100 g body weight. In their study the amounts of TDP and TTP produced were 5.4 and 0.09 µg per g wet weight, respectively, which were expressed as the amount of thiochrome (19). This permits us to calculate the approximate intracellular concentration of TDP and TTP to be 29 and 0.5 mM, respectively. On the other hand, the present authors measured total thiamine incorporated into isolated toad heart which was perfused with 0.42 mM thiamine propyldisulfide and showed that it was 510 µg per g wet weight (2). Consequently, it seems likely that if only an experiment with isolated heart is under consideration, large amounts of TDP and TTP are produced in heart muscle and raises their intracellular concentration to a level sufficient for
enhancing muscle contraction. However, it would be impossible that such large amounts of TDP and TTP are produced in heart muscle in an experiment with a whole animal.

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