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Splitting of Inorganic Tripolyphosphate (TPP) by Myosin and its Effect on Glycerinated Muscle Fibre

By

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(With 4 Text-figures)

Recently, Friess and Morales ('55) have indicated in the study of myosin that there is a remarkable agreement in function between tripolyphosphatase (TPPase) and adenosinetriphosphatase (ATPase). They found a weak shortening of actomyosin thread by TPP. On this basis, they concluded that the ATP contraction of actomyosin thread needs binding of actomyosin with ATP but not the splitting of ATP. This conclusion is in disagreement with that of Nagai who emphasized the importance of ATP-splitting in the contraction of actomyosin system. The present paper gives some information concerning the enzymic property and the reaction mechanism of myosin TPPase, with a discussion on the view of Friess and Morales ('55).

Before going further the present authors desire to express thanks to Professor Torao Nagai for suggesting this investigation as well as for expert guidance in the course of this work. Thanks are also due to Mr. Uchida and Dr. Satô for their valuable advices.

Material and methods

Myosin solution : Crystalline myosin from rabbit muscle prepared after the method of Szent-Györgyi, and dissolved in 0.6 M KCl was used for study. Total N of this stock solution was measured by micro-Kjeldahl method; its factor was 6.25. Thus the concentration of stock solution was calculated to be 15-20 mg protein/ml.

TPP solution : Inorganic Na-salt of TPP was recrystallized twice with 70% alcohol. A stock solution was obtained dissolving refined crystals in dist. water at about $1.4\text{-}1.1 \times 10^{-2}$ M. This solution was adjusted to pH 7.0.

Buffer solution : Michaelis' veronal acetate buffer solution without NaCl was used at pH 6.6-9.0.

Procedure of Reaction : The substrate medium was made by mixing the stock solution mentioned above in the following manner; the final concentrations of TPP, KCl and myosin were 1×10^{-3}M, 0.6M and 2.5mg/ml, respectively.

Myosin	(18.4mg/ml)	0.4ml
KCl	3M	0.5ml
KCl	1M	0.1ml

Jour. Fac. Sci. Hokkaido Univ. Ser. VI, Zool. 13, 1957 (Prof. T. Uchida Jubilee Volume).

Buffer solution	1.0ml
Dist. water	0.5ml

On experiment, the test tube filled with the mixture (2.5ml) was preincubated in a water bath (20°C) for 3 minutes and then 0.5ml of TPP solution was poured into this tube.

Measurement of TPPase activity: The reaction was stopped by adding 2.0ml of 5% trichlor acetic acid after 25 minutes. The filtrate obtained from the mixture was measured colorimetrically for free inorganic P according to Bodansky's method. TPPase activity was shown by the following formula: free γP/mg myosin/25 min.

For determination of Michaelis' constant (Km) in myosin TPPase, the TPPase activity after 4 minutes' reaction was considered as TPPase velocity.

Glycerinated muscle: Clycerinated psoas muscle fibres of the rabbit were isolated following Szent-Györgyi's method. Usually they were immersed in glycerine for more than 3 weeks. They were isolated into fine muscle bundles, about 0.2mm in diameter and 20 mm in length, and used for the experiment after washing with 0.16 M KCl. For study on the contraction of glycerinated muscle, chymographic curve was recorded using isotonic lever with 200mg. The chymographic study was performed at 14°C or 17°C.

Results

1) *Michaelis' constant (Km) and maximum velocity (Vmax)*: In the time course of TPPase activity, there occurred a 'break' at about 4 minutes after the addition of TPP. The activity was higher in the reaction prior to the break than in that after the break. Thus the velocity (V) recorded as the activity value at 4 minutes after the addition of TPP. Each final concentration (S) of TPP was 2.2×10^{-5} M, 3.3×10^{-5} M, 6.7×10^{-5} M and 8.9×10^{-5} M. As shown in Figure 1, it is possible to read off the values of Km and Vmax. Then the following results were obtained: Km≒1.5×10^{-4} mol/1, Vmax≒0.1 γ P/mg myosin/min.

2) *The effect of TPP on glycerinated muscle fibre*: The contraction of glycerinated muscle fibre was recorded on the smoked paper using a chymograph, with the results as shown in Figures 2 and 3. The figures indicatet hat, when the fibres were treated with 1.5×10^{-3} M TPP alone, the curve showing the grade of the contraction is almost equal to the base line, but slightly above after 20 minutes (Fig. 3, Curve 1). When the fibres were treated with TPP solution containing 2.5×10^{-3}M $MgCl_2$, the curve was below the Curve 1 (Figs. 1 and 3, Curve 2). In the control experiment, the fibres were treated with the mixture of 4×10^{-3}M ATP and 4×10^{-3} M $MgCl_2$ or with 1×10^{-3} M pyrophosphate solution. In the treatment with the ATP and $MgCl_2$ mixture, the fibres showed a typical ATP-contraction (Fig. 2, Curve 3). For pyrophosphate solution, the glycerinated muscle fibres reacted with a slight extension (Fig. 3, Curve 3). Further experiments were carried out using 0.3 M KCl with the results illustrated in Figure 4 that glycerinated muscle fibres were extended in the addition of 5×10^{-3} M TPP solution or pyrophosphate solution alone (Fig. 4, Curves 1 and 2). The effect of TPP is therefore stronger than that of pyrophosphate in the absence of Mg ions. In the presence of Mg ions, however, the effect of TPP was weaker than that of pyrophosphate.

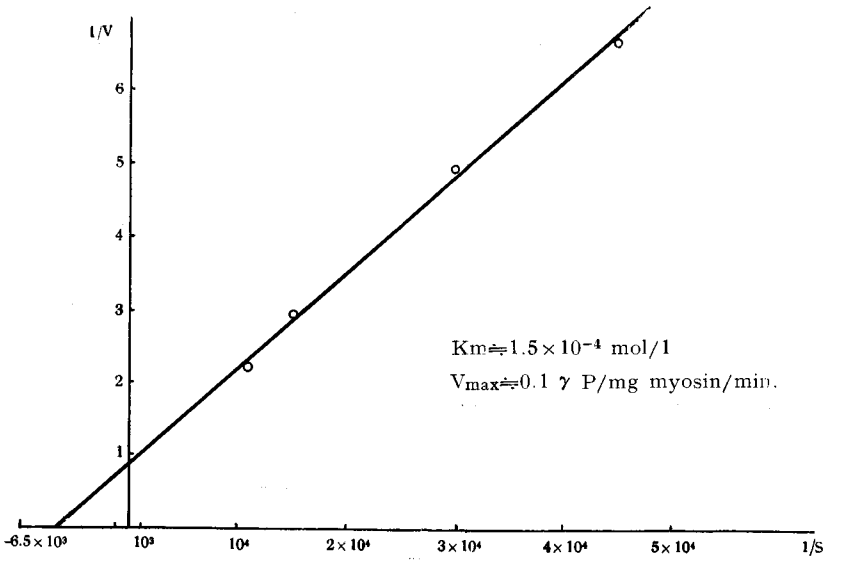

Fig. 1. 1/S–1/V curve of myosin tripolyphospl.atase in 0.6M KCl, pH 7.0, Temp. 20°C.

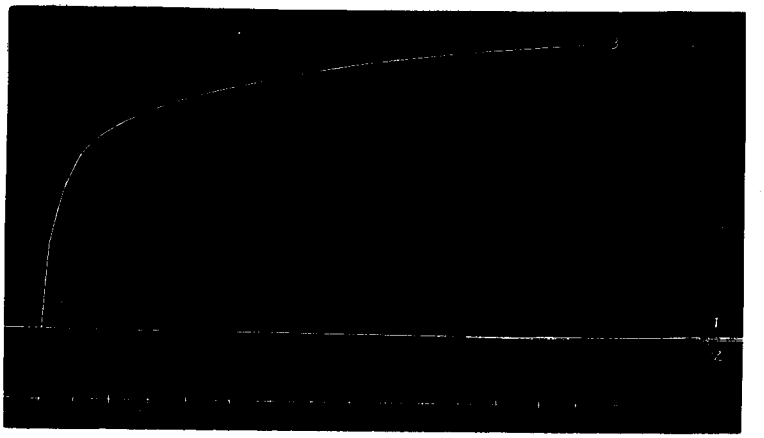

Fig. 2. The effect of tripolyphosphate and adenosinetriphosphate on the glycerinated muscle fibre in 0.16M KCl. Curve 1 : tripolyphosphate (1.5×10^{-3}M). Curve 2 : tripolyphosphate (1.5×10^{-3}M)+$MgCl_2$(2.5×10^{-3}M). Curve 3 : ATP (4×10^{-3}M)+$MgCl_2$(4×10^{-3}M). Temp. : 14°C. Load : 200mg. Time marks : 1 min.

It is then evident that Mg ions promote strikingly the effect of pyrophosphate, without strengthening the effect of TPP (Fig. 4, Curves 3 and 4).

Fig. 3. The effect of tripolyphosphate and pyrophosphate on glycerinated muscle fibre in 0.16 M KCl. Curve 1 : tripolyphosphate (1.5×10^{-3} M). Curve 2 : (on the base line) tripolyphosphate (1.5×10^{-3}M)+$MgCl_2$ (2.5×10^{-3} M). Curve 3 : pyrophosphate (10^{-3} M). Temp. : 17°C. Load : 200mg. Time marks : 1 min.

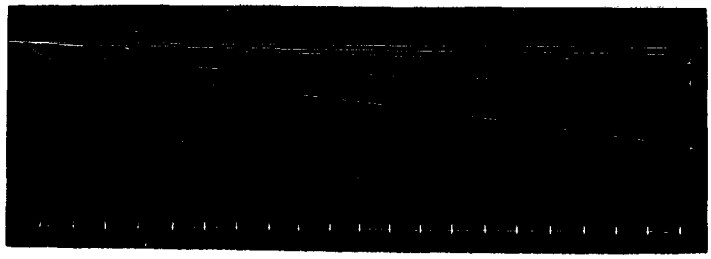

Fig. 4. The effect of tripolyphosphate and pyrophosphate on the glycerinated muscle fibre in 0.3 M KCl. Curve 1 : pyrophosphate (5×10^{-3}M). Curve 2 : tripolyphosphate (5.2×10^{-3}M). Curve 3 : tripolyphosphate (5.2×10^{-3}M)+$MgCl_2$(10^{-3}M). Curve 4 : pyrophosphate (5×10^{-3}M)+$MgCl_2$(10^{-3}M). Temp. : 12°C. Load : 200mg. Time marks : 1 min.

Discussion

1) *Michaelis' constant (Km) and Vmax of TPPase* : It was shown above that the present experiments yielded the following results; Km≒1.5×10^{-4} mol/1, and Vmax≒0.1 γ P/mg myosin/min, (Fig. 1 and shamet). Concerning the values of these constants, the data reported by Friess and Morales seem only to be available. They measured the reciprocal value ($\overline{K}$) of Km in the presence of Ca ions and showed $\overline{K}=5\times10^{-2}$ 1/mol and Vmax=1×10^{-6} M/sec./gr. myosin. If one represents these values with the dimensions used in this paper, Km is equal to 2×10^{-3} mol/1, and Vmax is equal to 1.92 γ P/mg myosin/min. These values seem to be very different from those obtained in the present experiments.

On the other hand, Gergely ('53) obtained the value, Km<10^{-4}, using H-meromyosin for the Michaelis constant of ATPase. According to Friess and

Morales $\overline{K}$ of ATPase is 150 times $\overline{K}$ of TPPase. When that value is compared with $\overline{K}$ of other nucleotide triphosphates, the following order, ATP>UTP>ITP>TPP, is obtained. Further Friess and Morales stated that the order, ITP>UTP>ATP>TPP, is also obtained for Vmax. Watanabe and Tonomura ('53) measured Km in the presence of Ca ions using myosin B which was also used in the experiment of Friess and Morales, and they obtained the value, $Km=1.0\text{–}1.9\times10^{-4}$. But they noted that Ca ions changed the value of Km. Recently Kielly *et al.* ('56) measured the value of Km in various nucleotide triphosphates and obtained the value, $Km<3\times10^{-5}$. But they failed to recognize the primary relation, reported by Friess and Morales, in other compounds. On the other hand, Mommaerts ('54) has pointed out that the process of splitting of ATP(k_3) can not be neglected in the measurement of the value of Km, because Ca ions promote remarkably the activity of ATPase. Recently, Uchida ('57) has emphasized that the reaction prior to the break should be distinguished from that after the break in the measurement of ATPase activity of various incubations prior to the break under the condition of absence of Ca ions. The values are 2.4×10^{-4} mol/l and 14.7 γP/mg myosin/min. respectively. The former value nearly approximates that obtained in the present study.

Although a question remains with regard to determination of Km in the actomyosin system, the value of Km of TPPase obtained in the present study differs from that reported by Morales and approximates the value of Km of ATPase measured by Watanabe and Tonomura and by Uchida. The value of Vmax is, however, about 1/140 that of the value obtained by Uchida. On this point, the present results reverse those reported by Morales in comparison. The fact may be due to the presence of Ca ions.

The present data record different values whether k_3 is neglected or not.* If k_3 is neglected, $\overline{K}$ $(=k_1/k_2)$ of TPPase is the same as that of ATPase but the value of Vmax of TPPase is about 1/140 that of the value of ATP. These results may support the view expressed by Nagai, but not that by Morales. If k_3 is not neglected, $\overline{K}$ of TPPase must be remarkably smaller than that of ATPase, since the value of Km of TPPase is the same as that of ATPase. Accordingly, the

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* $TPP+M \underset{k_2}{\overset{k_1}{\rightleftarrows}} TPP.\ M \xrightarrow{k_3} PP+P$

M: myosin. PP: pyrophosphate. P: inorganic orthophosphate.

k_1, k_2, k_3: velosity constant.

$\overline{K}=\frac{1}{km}=\frac{k_1}{k_2+k_3}$, when k_3 is negligibly small.

$\overline{K}\fallingdotseq\frac{k_1}{k_2}$, equilibrium constant.

values of both $\overline{K}$ and Vmax of TPPase are considerably smaller than those of ATPase, therefore it could not be concluded at present which reaction, binding or splitting, contributes to the shortening of muscle fibres.

2) *Action of TPP on the glycerinated muscle fibre*: Contrary to the case of ATP, 1.5×10^{-3} M TPP can not induce the contraction of the glycerinated muscle fibre in 0.16 M KCl solution. This is not controlled by the presence of absence of Mg ions in the medium. Also, this result approximates that obtained by the use of pyrophosphate. It was shown on the other hand that the fibres are extended by TPP or pyrophosphate in 0.3 M KCl solution. In the latter case, the presence of Mg ions in the medium promoted the action of pyrophosphate more intensely than that of TPP. It has been known that TPP does not induce the contraction of actomyosin under ordinary conditions, but there has been published no information as to the contraction of glycerinated muscle fibre by TPP. On this point, Weber ('52) has considered that inorganic phosphate compounds including TPP may not serve as a contracting agent but act as a plasticizing agent. Szent-Györgyi ('50), and Maruyama and Ito ('54) reported that the action of pyrophosphate upon the glycerinated muscle fibres leading to their extension increased with increase of the concentration of KCl in the medium. Furthermore, it is known that pyrophosphate and TPP cause reduction in the viscosity of actomyosin solution in the presence of Mg ions. Based on these facts and referring to the views of Nagai and Bendall, it may be stated that TPP possesses the extending action on glycerinated muscle fibre. Recent studies have yielded evidence to support this consideration.

As above mentioned, the present experiments support and Friess and Morales's conclusion that the action of TPPase is identical with that of ATPase. But presently obtained data seems to be against their concept as to the mechanism of contraction of actomyosin and to support the view of Weber and Nagai. The different conclusion may be due to the absence of Ca ions in the present material, and further study is needed to solve the final mechanism of the contraction caused by the actomyosin system.

Summary

1) The present paper gives some information concerning the enzymic property of myosin tripolyphosphatase (TPPase), and the effect of TPP on glycerinated muscle fibre. The opinions expressed by Friess and Morales on enzymic property of myosin TPPase are discussed.

2) The results of the present study yield $Km\fallingdotseq1.5\times10^{-4}$ mol/l, and $Vmax\fallingdotseq0.1\ \gamma$ P/mg myosin /min. for Michaelis' constant (Km) and Vmax of TPPase.

3) TPP failed to induce contraction of glycerinated muscle fibre in 0.16 M KCl. This is not controlled by the presence or absence of Mg ions in the medium. In 0.3 M KCl, the fibres were extended by both TPP and pyrophosphate. In the

latter case the presence of Mg ions in the medium promoted the action of pyrophosphate more intensely than that of TPP.

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