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PHYSICO-CHEMICAL STUDY ON THE ACTOMYOSIN-ADENOSINE TRIPHOSPHATE SYSTEM*)

By

Yuji TONOMURA**)

(Received January 20, 1956)

Introduction

Since ENGELHARDT *et al.* (1, 2) demonstrated that actomyosin catalyzes the hydrolysis of ATP and, at the same time, ATP changes in turn the physico-chemical properties of actomyosin, this interaction has been studied by many investigators. It has thus been established that this phenomenon is the fundamental mechanism of muscular contraction (3, 4, 5, 6). Quantitative studies on ATPase action and changes by ATP in the properties of actomyosin solution have already been carried out by MOMMAERTS (7, 8, 9) and MORALES *et al.* (10, 11). But much remains yet to be clarified, because of the difficulties involved in the quantitative estimation of these reactions and of the complexity of their mechanism.

The present author has investigated the kinetics of ATPase action of myosin B (natural actomyosin) (12, 13) and has studied, using a special apparatus, transient kinetics on the light-scattering drop by ATP of KCl solution of the latter (12, 13, 14, 15): From the results of the study, it has been suggested that a intimate relation exists between these two interactive reactions (12, 13). Recently, the present author has also demonstrated that guanidine-kinase plays a fundamental role in the reaction of the actomyosin-ATP system (16, 17).

The present report deals with the analysis of the experimental results obtained and with a consistent reaction formula deduced for the interaction between actomyosin and ATP.

*) In this paper, the following abbreviations are used: ATP=adenosine triphosphate, ATPase=adenosine triphosphatase, ~P=energy rich phosphate, PPi=inorganic pyrophosphate, EDTA=ethylene diamine tetraacetate.

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Experimental

Myosin B (natural actomyosin) was obtained by extracting fresh muscle for 24 hours with the WEBER-EDSALL solution and purified by means of the dilution-precipitation procedure which was repeated successively 3 times. Throughout the present study, myosin B extracted from rabbit muscle was mainly used, if otherwise not stated. ATPase action was determined by estimation of the inorganic orthophosphate liberated from ATP, according to the YOUNGBURG-YOUNGBURG method (18). Relative intensity of the light scattered by myosin B solution was measured at 45, 90 or 135° by means of an electron multiplier-electromagnetic oscillograph or of a μ-ammeter system (12). Viscosity was determined by means of a viscosimeter of the OSTWALD type.

Adenosine Triphosphatase

It is well known that at higher KCl concentrations (above 0.15 M) actomyosin-ATPase is activated by Ca^{++} and inhibited by Mg^{++}. Under various ionic conditions, the relation between the reaction velocity (v) of ATPase and the concentration of substrate ($[S]$) is given by MICHAELIS-MENTENS' formula:

$$v=\frac{V_{\max}}{1+\frac{K_m}{[S]}} \quad \text{or} \quad \frac{1}{v}=\left(1+\frac{K_m}{[S]}\right)\frac{1}{V_{\max}}.$$

Accordingly, the following reaction mechanism may be given:

$$M+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} MS,$$

$$MS \xrightarrow{k_3} M+P.$$

Hence, $K_m=\frac{k_{-1}+k_3}{k_1}$, $V_{\max}=k_3[\sum M]$.

Here, M, $[\sum M]$, S and P represent actomyosin, its total concentration, ATP and the product, respectively, and k_i the velocity constant of each reaction.

The MICHAELIS constant K_m was slightly increased by the addition of Ca^{++}. Its values were 3.6×10^{-4} and 1.6×10^{-4} mol./ℓ. in 0.15 M KCl, at pH 6.5 and at 25°C, in the presence as well as in the absence of 10 mM Ca^{++} respectively. On the other hand, $V_{\max}$, the reaction velocity in the presence of a sufficient amount of ATP, was enhanced from 2.2 to 17–38 μM P/sec./g. protein. As the functional unit weight of rabbit myosin B was calculated to be 1.85×10^5 g., as will be mentioned below,

V_{max} per one unit, k_3, were 0.4 and 3.1–9.0 1/sec. respectively. As will be shown in the next section, Ca^{++} has no effect on the binding reaction of ATP with actomyosin. In other words, k_1 and k_{-1} are not influenced by the addition of Ca^{++}. Accordingly, k_1 and k_{-1} can be calculated from the values of k_3 and K_m given above. Under the above experimental conditions:

$$k_1 = 1.4\text{–}3.3 \times 10^4 \quad \ell./\text{mol./sec.}$$

$$k_{-1} = 1.8\text{–}4.9 \quad 1/\text{sec.}$$

The MICHAELIS constant was almost of the same value in the presence of Ca^{++} as well as in the presence of Mg^{++} and Ca^{++} (12). Considering that both Mg^{++} and Ca^{++} combine to the same site of actomyosin (13) and that ATPase activity in the presence of Mg^{++} is much smaller than in the presence of Ca^{++}, it can be concluded that neither has Mg^{++} any effect on the binding reaction but it decreases the velocity constant (k_3) of disintegration of the actomyosin-ATP complex*). K_m and V_{max} of myosin B from various muscles are listed in Table 1.

OUELLET *et al.* (10) reported that when Ca^{++} was replaced by Mg^{++} at the same concentration (1 mM), V_{max} was reduced to one twentieth, while K_m remained constant. This is considered to be due to the fact that, in their experiment, k_3 could be neglected against k_{-1}, because of the low concentration of Ca^{++} used. Recently, GREEN and MOMMAERTS

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*) $$M_{ca} + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} MS_{ca} \xrightarrow{k_3} M_{ca} + P,$$

$$M_{mg} + S \underset{k'_{-1}}{\overset{k'_1}{\rightleftarrows}} MS_{mg} \xrightarrow{k'_3} M_{mg} + P,$$

$$M_{ca} + Mg^{++} \underset{K}{\rightleftarrows} M_{mg} + Ca^{++},$$

$$k_3 \gg k'_3 .$$

Then,

$$v \sim \frac{k_3([M_{ca}]) + ([MS_{ca}])}{1 + \frac{k_{-1}+k_3}{k_1[S]}} .$$

When Mg^{++} does not change the combination of ATP with M, i.e.,

$$\frac{k_{-1}+k_3}{k_1} \sim \frac{k_{-1}}{k_1} = \frac{k'_{-1}}{k'_1} \sim \frac{k'_{-1}+k'_3}{k'_1},$$

$$v \sim \frac{k_3[\sum M]}{\left(1 + \frac{k_{-1}+k_3}{k_1[S]}\right)\left(1 + \frac{[Mg^{++}]}{K[Ca^{++}]}\right)},$$

that is, ATPase inhibition by Mg^{++} is competitive with Ca^{++} and the Mg^{++} addition scarecely changes the MICHAELIS constant.

TABLE 1. K_m and V_{max} of Myosin B-ATPase
0.15 M KCl, 10 mM Ca^{++}, pH 6.8-7.0, 12°C

Muscle	K_m (mM)	V_{max} (μM P/sec./g. protein)
Rabbit, striated (12)	0.15	4–6
Swine, oesophagus smooth (19)	0.53	3.9
Pecten, fast adductor (16)	0.41	9.7
Pecten, slow adductor (16)	0.45	3.2

(20) performed kinetic studies on myosin-ATPase, and reported that K_m of myosin-ATPase changed proportionally to V_{max} (k_3) by the addition of Ca^{++} or Mg^{++} at pH 6.4. Therefore, it can be concluded that in contrast to actomyosin-ATPase, k_3 of myosin-ATPase is much greater than k_{-1} and its K_m is almost equal to k_3/k_1 at this pH value.

Interaction with Adenosine Triphosphate

Viscosity, flow birefringence and light scattering of myosin B solution are strikingly changed by the addition of ATP (3, 21, 22). When ATP is added, the light scattered by myosin B decreases rapidly in intensity; this is followed by the period of "splitting time", during which the intensity of light scattering remains constantly in the reduced state; and finally, when ATP is split to a certain extent, it returns slowly to the original value.

From the results of the studies performed by the use of viscosimetry and ultracentrifugation, many investigators supposed that these changes might have resulted from the dissociation of myosin B into actin and myosin (23). Afterwards, after having made, according to the more elaborate "extrapolation method" of ZIMM, light scattering studies at a low concentration of myosin B (below 0.02%) where interaction between the particles can be neglected, BLUM and MORALES (11) have proved that the change in the light scattering by ATP is due to the change in the shape of the myosin B particle. Therefore, the ratio (Δ) between the decrease of the light-scattering intensity in the presence of a certain amount of ATP (I_0-I_s) and that at a sufficient amount of ATP (I_0-I_∞) is equal to the ratio between the concentration of the deformed particles and that of the total myosin B (24). Under our experimental conditions, the concentration of myosin B was about 0.1–0.2 per cent. At this concentration interaction may take place between

the particles, and, furthermore, the functional unit weight (1.8×10^5 g) of myosin B which combines with 1 mole of ATP is much smaller than its molecular weight ($4\text{–}60 \times 10^6$ g) estimated by BLUM and MORALES. For these reasons, the previous assumption can not be said to be admissible for the functional unit. However, as, with this assumption, reasonable conclusions can be drawn from the present experimental data, as will be discussed below, it seems to be reasonable to assume that the particles of functional unit may be independent of each other. The molecular basis for this assumption will be discussed in the last section.

Introductory Remarks: Before entering the discussion on the mechanism of the change produced by ATP in the physico-chemical properties of actomyosin, it must be said that this change does occur following combination of ATP with actomyosin, which can take place without ATPase.

Samples of myosin B without ATPase activity were in some cases obtained from *carassius* skeletal muscle. Even these preparations presented a light scattering drop in the presence of ATP, quite similar to that of myosin B with active ATPase (25). Accordingly, ATPase action can be said unnecessary for the incidence of this change. The intensity of viscosity drop of pecten myosin B in the presence of ATP is decreased by the addition of EDTA. When the concentration of EDTA was more than 5 mM, the viscosity changed only slightly (16). Recently, FRIESS *et al.* (26, 27) showed that EDTA unites with the metal fixed to myosin and that, in 0.6 M KCl, it serves as an accelerator for myosin B-ATPase. They demonstrated thus that EDTA does not intercept the union between ATP and myosin B. Therefore, it may be concluded that a certain reaction other than union with ATP is necessary for the deformation of actomyosin and that this reaction is inhibited by the chelation of EDTA with the metal involved.

Results of Study on the Transition of the Reaction: As described above, the change brought about by the addition of ATP in the properties of myosin B solution is so rapid that viscosimetry, frequently used for the follow-up of this change, can not exactly pursue its transition, because it takes 20–30 sec. for one measurement of viscosity to be achieved. In the present experiments, the transition of change in the light scattering was recorded by usiug a photomultiplier-electromagnetic oscillograph. In this procedure, the accuracy in time was dependent on the rapidity of mixing ATP with myosin B solution. By blowing out a small amount of ATP into the myosin B solution, using

a pipet with its tip cut rectangularly to its axis, it was possible to reduce the mixing time to a time shorter than 0.05-0.1 sec.

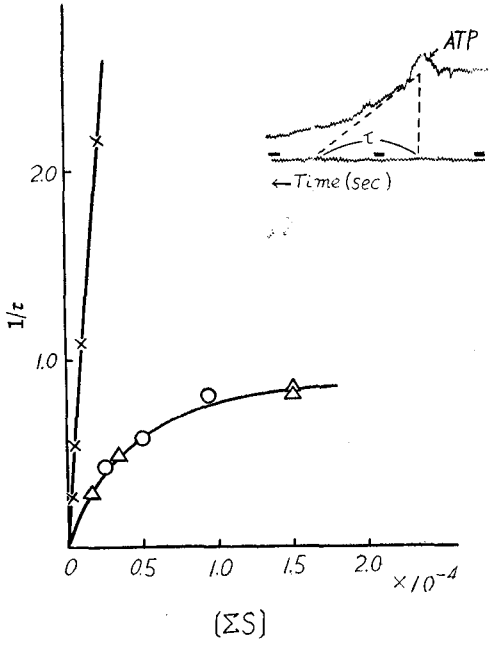


Fig. 1.

Relation between $1/\tau$ and Concentration of ATP Added ($[\Sigma S]$). Myosin B from rabbit striated muscle, 0.48 M KCl, pH 6.4, 20°C.

1. in the presence of 10 mM Mg^{++}, (×), 2. in the presence (○) or the absence (△) of 6.7 mM Ca^{++}. The right upper figure is inserted as an example of oscillograms of the light scattering change in the presence of 10 mM Mg^{++}. The arrow indicates the addition of 11 μM ATP. The base line corresponds to the intensity following the addition of a sufficient amount of ATP.

Fig. 1 shows the relation between the ATP concentration and the initial velocity of the light scattering drop obtained by this method. In this figure, τ denotes the time interval necessary for the light scattering drop to be completed with the initial velocity, i. e., to reach the minimum value of intensity following the addition of a sufficient amount of ATP. Then, in the presence of Mg^{++}, the relation between the reciprocal of τ and the concentration of ATP added ($[\Sigma S]$) was linear for the substrate concentration used. In the absence of Mg^{++}, when $[\Sigma S]$ was more than 10^{-4} M, $1/\tau$ became independent of $[\Sigma S]$. The addition of Ca^{++} had no effect on relation $1/\tau - [\Sigma S]$. When the quantity of ATP was so large that its adsorption to and decomposition by myosin B could be neglected, the transition of the light scattering change was logarithmic, that is, the relation between the logarithm of change in the light scattering ($\log(1-\Delta)$) and the reaction time after the addition of ATP was linear.

Reaction Mechanism : From the facts that, in the absence of Mg^{++}, the velocity of the light scattering drop became independent of $[\Sigma S]$

when it was of considerably large values, and that a certain reaction other than union of ATP is necessary for this change, although ATPase action is not required, the mechanism of the deformation of myosin B particle (M) in the presence of ATP (S) may be given in the following formulas:

$$M + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} MS,$$

$$MS \xrightarrow{k_2} M^*S,$$

where the asterisk denotes the deformed state.

If the decrease of light scattering is proportional to the concentration of deformed myosin B particles, the velocity of change in the light scattering (v) can be given by the formula:

$$v = \frac{d[M^*S]}{dt} = k_2[MS],$$

and at the steady state

$$\frac{d[MS]}{dt} = k_1[M][S] - (k_{-1}+k_2)[MS] = 0.$$

Then, v at the steady state is given by

$$v = -\frac{d([M]+[MS])}{dt} = \frac{k_2([M]+[MS])}{1+\dfrac{k_{-1}+k_2}{k_1[S]}} \qquad (1)$$

At the initial state all the myosin B particles are not changed, and the concentration of free ATP ($[S]$) is equal to that of ATP added ($[\sum S]$). Hence,

$$v_{\text{initial}} = \frac{k_2[\sum M]}{1+\dfrac{k_{-1}+k_2}{k_1[\sum S]}},$$

$$\text{i.e.,} \quad 1/\tau = \frac{k_2}{1+\dfrac{k_{-1}+k_2}{k_1[\sum S]}}, \qquad (2)$$

where $[\sum M]$ indicates the concentration of the total myosin B *).

*) If the above reaction mechanism is correct, a induction phenomenon should appear as pointed out by Dr. J. GERGELY (personal communication). But under our experimental condition about 0.2 sec. after the mixing of ATP the steady state was already observed (Ref. 15, Fig. 1) and the initial velocity in the above equation is, of course, the one at the steady state.

The addition of Ca^{++} did not change relation $1/\tau - [\sum S]$, that is, Ca^{++} had no effect on k_1, k_{-1} and k_2 and as described in the previous section, neither did Mg^{++} change k_1 and k_{-1}. In the absence of Mg^{++}, when $[\sum S]$ was sufficiently large, $1/\tau$ became independent of $[\sum S]$; in other words it was equivalent to k_2. On the other hand, in the presence of Mg^{++}, the relation between $1/\tau$ and $[\sum S]$ became linear over the range of $[\sum S]$ used. This is due to the situation that, by the addition of Mg^{++}, k_2 becomes very larger than k_{-1} and $k_1[\sum S]$, and so $1/\tau$ becomes $k_1[\sum S]$.

These considerations make one enable to estimate the value of each velocity constant easily from Eq. 2. In 0.5 M KCl, at pH 6.4 and at 20°C:

$$k_1 = 10^5 \quad \ell./\text{mol./sec.}$$

$$k_{-1} = 2.3 \quad 1/\text{sec.}$$

$$k_2 = 1 \quad 1/\text{sec.} \quad \text{(in the absence of } Mg^{++}\text{)}$$

$$\gg 5 \quad 1/\text{sec.} \quad \text{(in the presence of } Mg^{++}\text{)}$$

The solid lines shown in Fig. 1 are the theoretical ones calculated from these k's which coincide well with the observed values *).

When $[\sum S]$ is considerably large and its change in the reaction course can be neglected, the following equation is given by integration of Eq. 1:

$$\ln \frac{[\sum M]}{([M])+[MS])_t} = \frac{k_2}{1+\dfrac{k_{-1}+k_2}{k_1[\sum S]}} \cdot t\,,$$

where $([M]+[MS])_t$ represents the concentration of the unchanged myosin B at t sec. after the addition of ATP. Then, in the presence of sufficiently large $[\sum S]$, the process of the light scattering drop becomes logarithmic; the value of $k_2 \Big/ \left(1+\dfrac{k_{-1}+k_2}{k_1[\sum S]}\right)$, 0.86, observed in the present experiment in 0.15 mM ATP, 0.5 M KCl, at pH 6.4 and at 20°C coincides well with the calculated one, 0.82.

*) Recently, Dr. S. WATANABE (personal communication) has observed that Ca^{++} accerelates slightly the light scattering change by ATP. Ca^{++} may also change k_2 and so our above result may be due to our incidental experimental conditions. However, even if Ca^{++} changes k_2, our arguments in this report would not be affected.

Intimate Relation between ATPase and Light Scattering Change

In the two previous sections, it was implicitly assumed that the binding site of myosin B to ATP is the same both in the case of ATPase action and in the case of the change of light scattering. This assumption is confirmed to be valid by comparing the kinetic constants of ATP-binding reaction calculated from these two experiments. The present results, $k_{-1}/k_1 = 2.3 \times 10^{-5}$ mol./ℓ. obtained by the optical method in 0.5 M KCl, at pH 6.4 and at 20°C coincides well with the data: K_m $(\sim k_{-1}/k_1) = 1.3 - 2 \times 10^{-5}$ mol./ℓ. obtained by the chemical method under almost the same condition (10). Furthermore the data: $k_1 = 10^5$ ℓ./mol./sec. and $k_{-1} = 2.3$ 1/sec. obtained by the light scattering change may be said to coincide well with the values estimated from the chemical experiment, $k_1 = 1.4 - 3.3 \times 10^4$ ℓ./mol./sec. and $k_{-1} = 1.8 - 4.9$ 1/sec., if the difference between the KCl concentrations used in the optical experiments and in the chemical ones is taken into consideration *).

Now, the mechanism of the deformation reaction of the actomyosin-ATP complex is still unclarified, except that this reaction needs not ATP decomposition and is accelerated by $Mg.^{++}$ Recently, FEUER and WOLLEMAN (28) has observed that during the drop of viscosity creatine phosphate is produced from creatine adsorbed on creatine-kinase which forms a complex system with actin, and they supposed that this phosphate transferring reaction is necessary for the viscosity drop of actomyosin. However, as will be described below, it has been observed in the present experiment that ATP does deform myosin B from pecten adductor from which arginine **) has been completely removed. Furthermore, PPi, which cannot be a phosphate donor, induces to myosin B the same change as the one induced by ATP. Therefore, the phosphate transferring reaction cannot be said to be necessary for actomyosin to be deformed by ATP.

Interaction with Pyrophosphate (12)

In the presence of Mg^{++} pyrophosphate (PPi) induces to myosin B

*) These differences may arise partly from the difference in the states of actomyosin between two experiments (cf. p. 100).

**) In the invertebrate arginine-kinase has the same physiological chemical action as creatine-kinase in the vertebrate (29). These two enzyme are generally designated as guanidine-kinase.

solution the same light scattering change as that by ATP, thus inhibiting the ATPase action. The mechanism of these reactions can be investigated more readily than the interaction with ATP, because PPi is not decomposed by myosin B.

Inhibition of ATPase: Myosin B-ATPase was inhibited by PPi. This inhibition was competitive with ATP and, when $[S]$ was much larger than K_m, its inhibition grades were expressed in the form of a sygmoid curve of the second order (30). Accordingly, the mechanism of the inhibition by PPi may be formulated as follows:

$$M + S \rightleftharpoons MS \longrightarrow M + P\,,$$
$$M + 2\mathrm{PPi} \underset{K_1}{\rightleftharpoons} M(\mathrm{PPi})_2\,.$$

Then, when $[S]$ is sufficiently large, that is, when $[MS] \gg [M]$, the inhibition grade H is given by

$$H = \frac{[\mathrm{PPi}]^2}{K + [\mathrm{PPi}]^2}\,, \quad K = \left(1 + \frac{[S]}{K_m}\right) K_1\,.$$

By introducing K_m values into this equation, one can evaluate the dissociation constant K_1 under various conditions. The value of K_1 in 0.48 M KCl, 20 mM Ca^{++} and at pH 6.5, at 15°C was $10^{-6.7}$ (mol./ℓ.)^2.

Change of Light Scattering: The decrease of light scattering produced by (PPi) returns to the original value quite easily by the addition of yeast pyrophosphatase (30 a). The relationship between the grade of the light scattering drop (Δ) and the concentration of (PPi) added was given also in the form of a dissociation curve of the second order. Thus, the mechanism of this reaction may be:

$$M + 2\mathrm{PPi} \underset{K_1}{\rightleftharpoons} M(\mathrm{PPi})_2 \underset{K_2}{\rightleftharpoons} M^*(\mathrm{PPi})_2\,.$$

Hence, if $1/K_2 \gg 1$,

$$\Delta = \frac{[M^*(\mathrm{PPi})_2]}{[\sum M]} = \frac{[\mathrm{PPi}]^2}{K_1 K_2 + [\mathrm{PPi}]^2}\,.$$

The dissociation constant K_1K_2 was found to be $10^{-9.4}$ (mol./ℓ.)^2 in 0.5 M KCl, 10 mM Mg^{++}, at pH 6.4 and at 12°C. If the first binding reaction is independent of divalent ions just as in the case of the ATP-binding *), K_2 comes to about $10^{-2.7}$ in the presence of Mg^{++}, while,

*) Probably, the complex, $Me^{++}(PPi)_2$ (31), of PPi with the divalent ion Me^{++} binds with actomyosin. The apparent velocity constant of the deformation of actomyosin by PPi was found to decrease with rise in temperature (32). This may be due to positive ΔH in the formation of this complex. K_1 may, therefore, depend considerably on divalent ions.

in the absence of Mg^{++}, it is much greater than 10^{-1}.

As the reaction of actomyosin with ATP is quite similar to that with PPi, the second deformation of actomyosin by ATP may be also reversible. In the above analysis of the actomyosin-ATP system, the reverse reaction could be neglected because they were made only on the initial stage.

Necessity of Guanidine-Kinase System for the Recovery Process (16, 17)

As described above, the change produced by PPi in the physico-chemical properties of high KCl solution of actomyosin is similar in every respect to that produced by ATP. At low concentration of KCl, however, ATP "superprecipitates" actomyosin, but PPi does not (3, 30 a). When ATPase action is inhibited, "superprecipitation" by ATP does not take place (33). Recently, WEBER and HASSELBACH (34) found that the rate of the ATP-splitting in the first 15 sec. was 2 times as great as its rate which is observed stationary during the succeeding 100 sec. These results suggest that myosin B is changed by ATPase action to a new state other than the deformed one which is produced without ATPase action.

Using myosin B obtained from fast adductor of pecten, the present writer obtained some experimental results which show that the interaction between actomyosin and ATP must be considered not to be a reversible process but to be a complicated cyclic one. Usual myosin B samples, purified by the dilution-precipitation method, which is usually repeated 2–3 times, used to contain a small quantity of arginine (2.0–0.4 mg./g. protein) and a weak arginine-kinase. But when the purification procedure was repeated more than 4 times, the samples of myosin B became almost completely deprived of arginine. Arginine could also be readily removed from the samples by pre-incubation with arginase. The ATPase activity and the relative viscosity of such preparations of myosin B were almost equal to those of usual ones. Though their viscosity dropped in the same manner as in the control by the addition of ATP, it hardly recovered even after the ATP was completely decomposed (Table 2), and when a small quantity of arginine (ca. 10^{-5} M) was added to them, they regained the ability to recover from the viscosity drop (Fig. 2).

TABLE 2. Reaction Products and Recovery of Viscosity Drop of the Pecten Myosin B-ATP System (17)

Incubation medium ; 0.6 M KCl, pH 6.7, 14.5°C

Incubation of ATP with	Incubation time (min.)	Reaction products (μM/ℓ.)				Recovery of viscosity drop (%)
		AMP	IMP	ADP	ATP	
H_2O	—	32.8	8.7	48.6	508	—
Arginine-free myosin B (2.5 mg./mℓ.)	100	122	9.0	458	2.7	6
Usual myosin B (3.0 mg./mℓ.)	15	40.9	8.9	512	40.5	40

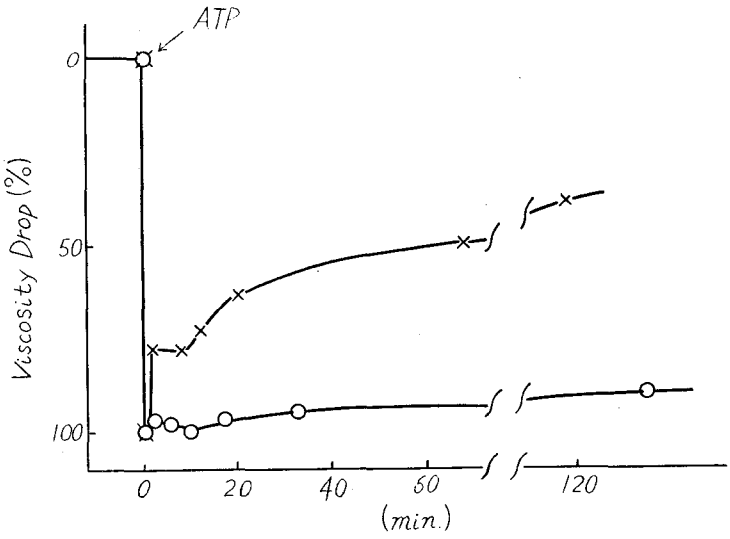


Fig. 2. Change in Viscosity of Myosin B Solution by the Addition of ATP.

The arrow indicates the addition of 3.7×10^{-4} M ATP. 0.6 M KCl, pH 6.7, 14.5°C, 3.0 mg./mℓ. myosin B from pecten fast adductor: ○, arginine-free myosin B: ×, »+3.2 μg./mℓ. arginine.

When arginine was removed from myosin B sufficiently but not completely (*ca.* 0.06 mg./g. protein), the recovery from the drop took place very slowly. When Mg^{++} was added to such a preparation, the recovery of viscosity was considerably accelerated. It was also observed

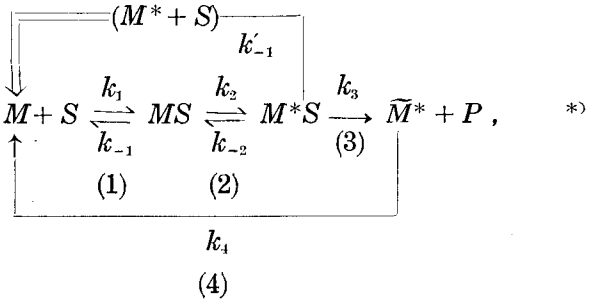
that when myosin B deprived of arginine was kept at 25°C for 24 hours, its viscosity drop caused by ATP did no longer recover, even when arginine was added. It is well known that Mg^{++} is powerful activator for arginine-kinase, and that this enzyme becomes unstable and denatured when deprived of arginine (35). Therefore, it may be deduced that the arginine-kinase system is not necessary for the viscosity of actomyosin solution to drop in the presence of ATP but an indispensable factor for the viscosity once dropped to return to normal. In other words, the deformation of myosin B by ATP is not a reversible process but a cyclic one *).

POLISSAR (36) has proposed a physico-chemical model of the mechanism of muscular contraction. According to this model, contractile units of muscle would be capable of existing in two different states, a long one L and a short one S, the reaction between these two states proceeding in a cyclic process like $L \rightleftarrows S$. The author has been able to interpret, in support of this model, various physiological phenomena of muscle contraction. As mentioned above, the conclusion drawn from the present experiment that the interaction between actomyosin and ATP is a cyclic process supports the validity of this model from the standpoint of molecular biology. Recently FEUER (37) has observed that, during the contraction of glycerinated muscle fibers, ~P is transferred from ATP to creatine and the muscle tension is quite proportional to the content of creatine phosphate produced in the fibers. But this process of transferring phosphate seems not to be necessary for muscular contraction, because the present author has observed that myosin B deprived of arginine is also capable of being superprecipitated by ATP, just like in the case of usual myosin B.

Functional Unit Weight

Putting together the various analyses described above, the following reaction formula may be given for the interaction between ATP and actomyosin;

*) It can not be determined from these results whether the guanidine-kinase system parcipitates directly in the recovery process or whether, in the absence of its action, an active myosin B produced by ATPase action is denatured irreversibly to form a certain state, from which the myosin B no longer recover to the original one. But, as the viscosity, intensity of light scattering and ATPase activity of arginine-free myosin B were held at constant values during the "splitting time", the latter presumption seems rather improbable.



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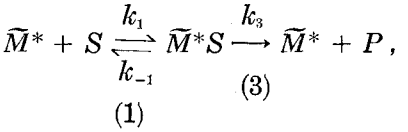

where M, M^* and $\widetilde{M}^*$ denote respectively actomyosin in the original, the deformed and the active, deformed state. The last state is produced by ATPase action. The existance of this particular state can be assumed from the various experimental data described above, though it can not materially be distinguished from M^* by any technique available at present **) ***). The reaction (1) is the union of ATP with the ATPase-active site of actomyosin. It is independent of divalent ions. The deformation reaction (2) is not influenced by Ca^{++} but accerelated by Mg^{++}, and in a KCl concentration higher than 0.15 M, the decomposition of the enzyme-substrate complex (3) is enhanced by Ca^{++} and inhibited by Mg^{++}.

The recovery process (4) from the active, deformed state to the original one does not occur in the absence of guanidine-kinase, suggesting the intervention of a complicated reaction (cf. 17). In usual myosin B preparation which contains a sufficient amount of guanidine-kinase, the rate of the reaction (4) may be proportional to $[\widetilde{M}^*]$.

*) For simplicity's sake, the reaction constants of the binding of ATP with M and $\widetilde{M}^*$ are assumed to be equal to each other.

**) H. H. WEBER (37 a) has recently suggested that the energy of the splitting of ATP is transferred to actomyosin by transphosphorylation. This view is supported by our results (i) that creation of the active state $\widetilde{M}^*$ may be inhibited by the addition of EDTA (37 b) and (ii) that, as stated above, the guanidine-kinase system is a necessary factor for the recovery process ($\widetilde{M}^* \rightarrow M$).

***) As suggested in previous papers (12, 13) the deformed state corresponds to the latency relaxation and the active deformed state to the active state in physiology (38). The latter is a prerequisite condition to muscular contraction.

Then, at the steady state, the relationship between the grade of light scattering drop (Δ) and the concentration of ATP ([S]) is given by the formula:

$$\Delta = \frac{[M^*S]+[\widetilde{M}^*]+[\widetilde{M}^*S]}{[\sum M]} = \frac{1+\frac{k_3}{k_4}\left(1+\frac{[S]}{K_m}\right)}{1+\frac{k_3}{k_4}\left(1+\frac{[S]}{K_m}\right)+\frac{k'_{-1}+k_{-2}+k_3}{k_2}+\frac{k'_{-1}+k_3+\frac{k_{-1}(k'_{-1}+k_{-2}+k_3)}{k_2}}{k_1[S]}}.$$

If $k_3 \gg k_4,\ k'_{-1}\ ;\ k_2 \gg k_{-2}$

$$\Delta \sim \frac{1}{1+\frac{k_3(1+k_{-1}/k_2)}{k_1[S]}\Big/\frac{k_3}{k_4}\left(1+\frac{[S]}{K_m}\right)},$$

and when $[S] \ll K_m$

$$\Delta \sim \frac{1}{1+\frac{\phi}{[S]}}, \qquad \phi = \frac{k_4}{k_1}\cdot(1+k_{-1}/k_2).$$

On the other hand, the velocity of ATPase action (v) is given by the formula:

$$\frac{v}{V_{\max}} = \frac{[M^*S]+[\widetilde{M}^*S]}{[\sum M]} = \frac{1+\frac{k_3}{k_4}\frac{[S]}{K_m}}{1+\frac{k_3}{k_4}\left(1+\frac{[S]}{K_m}\right)+\frac{k'_{-1}+k_{-2}+k_3}{k_2}+\frac{k'_{-1}+k_3+\frac{k_{-1}(k'_{-1}+k_2+k_3)}{k_2}}{k_1[S]}}.$$

As $k_3 \gg k_4,\ k'_{-1};\ k_2 \gg k_{-2};\ k_2 \sim k_{-1}$ or k_3, over a considerable range of [S] the left side of this equation reduces to

$$\sim \frac{1}{1+\frac{K_m}{[S]}},$$

that is, to MICHAELIS-MENTENS' equation without consideration on the deformation of actomyosin.

Curve 1 in Fig. 3 A shows the relation between Δ and $-\log[\sum S]$

of myosin B from pecten fast adductor in the presence of Ca^{++}. Where $[\sum S]$ was small, the curve was represented as a sygmoid curve of the first order (30), but when $[\sum S]$ became large and $[S]/K_m$ could not be

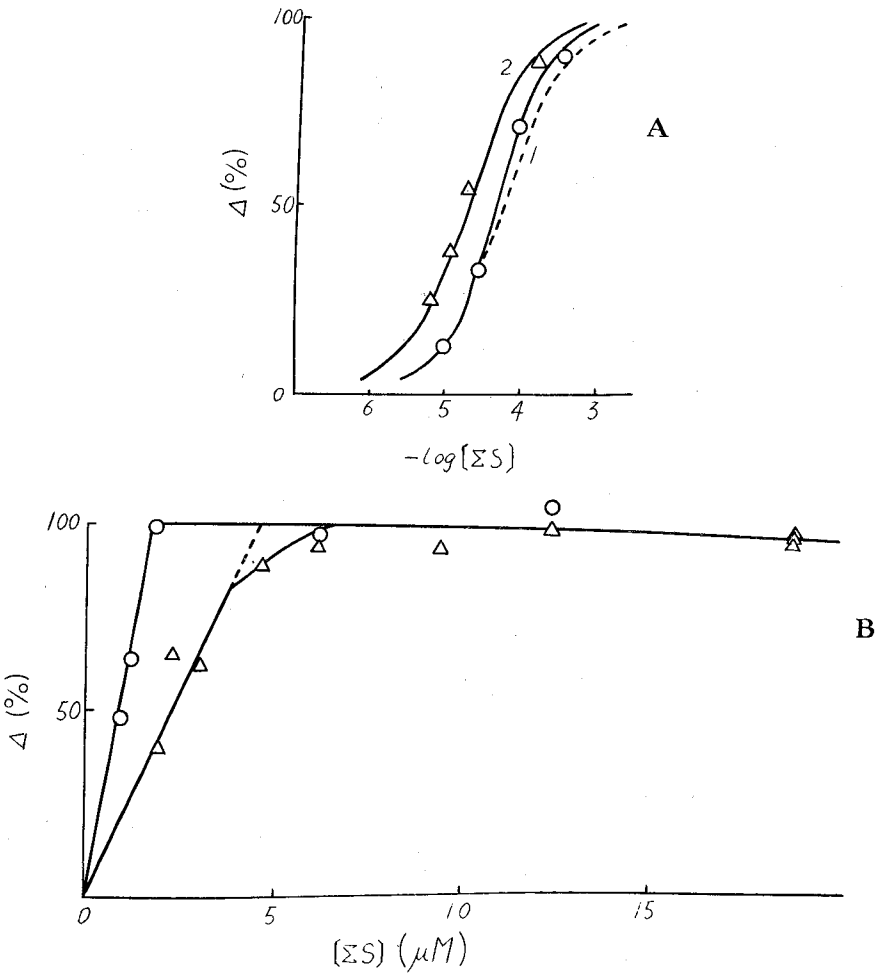

Fig. 3. Relationship between Grade of the Light Scattering Drop (Δ) and Concentration of ATP Added ($[\sum S]$).

Myosin B from pecten fast addutor, 0.6 M KCl, pH 6.7, 20°C.

(A) In the presence (○) and in the absence (△) of 10 mM Ca^{++}, 1.81 mg. protein/mℓ. The dotted line is given by: $\Delta = -\dfrac{1}{1+\dfrac{[S]}{6.3\times10^{-5}}}$.

(B) In the presence of 2.5 mM Mg; ○, 0.6 mg. protein/mℓ.; △, 1.81 mg. protein/mℓ.

neglected against 1, it became steeper than that of the first order. The solid line calculated from $\phi=6.3\times10^{-5}$ and $K_m=3.2\times10^{-4}$ mol./ℓ. coincided with the experimental values. This value of K_m obtained by means of the optical method was almost equal to the value: $K_m=4.5\times10^{-4}$ mol./ℓ. obtained by means of the chemical method at a different KCl concentration.

In the absence of Ca^{++} and Mg^{++}, relation $\Delta-\log[\sum S]$ was given as a sygmoid curve of the first order and its ϕ was 2.1×10^{-5} mol./ℓ. (Curve 2 in Fig. 3 A). As shown in Fig. 3 B, in the presence of Mg^{++}, the drop of light scattering intensity was proportional to the concentration of ATP added, until its concentration increased to a definite value proportional to the concentration of the actomyosin used. This means that Mg^{++} decreases k_4 and increases k_2 and so ϕ becomes much smaller than the concentration of the ATP-binding site, in other words, $[S]\ll[\sum S]$ *).

TABLE 3. Contraction Time of some Muscles and the Functional Unit Weights of their Myosin Bs

Muscle	Contraction time (m sec.) (40)	Functional unit weight (g.)	Active site/10^6 g. (mole)
Rabbit, striated (12)	30	1.85×10^5	5.4
Swine, cardiac (41)	—	1.8×10^5	5.5
Pecten, fast adductor (17)	100–200	3.9×10^5	2.5^6
Swine, oesophagus smooth (19)	—	1.0×10^6	1.0
Pecten, slow adductor (17)	500–5000	1.22×10^6	0.8^2

Thus, the weight of myosin B bound with 1 mole of ATP can be calculated easily from relation Δ-$[\sum S]$ in the presence of Mg^{++}. The functional unit weight of myosin Bs from various muscles is listed in Table 3. K. YAGI (42) in this laboratory has shown that the degree of decrease in the light scattering depends on the amount of actin in the protein preparation, but the quantity of ATP necessary for the change

*) After the manuscript of this paper had been completed, we have received a very interesting paper from Dr. J. J. BLUM (39). He has measured the optical ϕ and the chemical K_m of rabbit myosin B for various nucleotides (ATP, ITP and UTP), especially the ϕ in the presence of Mg^{++} which we could not measure, due to the high concentration of myosin B used in our optical experiments. He has also proposed a reaction scheme which is almost identical to the above one or our previous one, in order to interprete the difference between ϕ and K_m.

is independent of the amount of actin and proportional to the amount of myosin in the myosin B preparation. As for the samples of myosin B listed in Table 3, they are very similar to each other in their protein composition and the greater part (3/5–4/5) of each myosin B is myosin. Therefore, it may be concluded that the differences in their functional unit weights are mostly ascribed to the differences in the functional unit weights of their myosins, although one cannot deny the possibility of the differences being partly due to the contamination of some proteins (mostly actin) other than myosin (17, 19). As myosin B from rabbit striated muscle contains 1 part of actin for 4 parts of myosin (19) *), the unit weight of rabbit myosin is calculated to be 1.5×10^5 g.

Using the chromatographic analysis for the nucleotides, it was also revealed that, in the presence of Mg^{++}, the quantity of ATP remaining in the reaction mixture in the early stage of the recovery phase was only about 1/5 of the amount of the functional unit of (pecten) myosin B (30 a). As in the presence of Mg^{++} hardly any trace of free ATP could be left in the reaction mixture in that stage, the following conclusion can be deduced from the above estimation:

$$[\widetilde{M}^*]/[M^*S] = k_3/k_4 \sim 4 \ .$$

Thus, one of the above assumptions is verified.

Concluding Remarks

In his flow birefringence studies on "myosin" (myosin B) solution, EDSALL (43) has shown that the length of his "myosin" was about 12,000 Å. Recently, by means of the ZIMM method, BLUM and MORALES (11) have elucidated the shape of myosin B particles to be a rod about 7,000 Å long and demonstrated that its molecular weight was not changed but it was lengthened by the addition of ATP. As was shown in Fig. 3, Δ was proportional to the amount of ATP combined with myosin. Therefore, if BLUM's model is correct, this means that the unit of myosin is independent of each other and the length of myosin B is changed with a constant value, every time when one ATP molecule combines with one myosin unit, because under the present experimental conditions ($\theta = 45°$, $\lambda \sim 5{,}000$ Å) the intensity in the light scattering decreases linearly with the increase in length of the rod-shaped particle,

*) In a previous paper (12) three-forth of myosin B was assumed to be myosin. In this paper the value recently estimated by us is adopted and the unit weight of myosin is recalculated.

when its length ranges from 5,000 to 18,000 Å (44). TSAO (45) investigated rabbit myosin to be decomposed by concentrated urea and alkali (pH 10.7) and found that the most part of the decomposition products consisted of cyclic polypeptides whose molecular weight were about 1.7×10^5 g. This value coincides with the value of the functional unit weight of rabbit myosin calculated in the present experiment. This suggests that the functional unit is nothing but a cyclic polypeptide of this order, and the particles of this cyclic peptide are considered to be independent of each other. Recently, ELLENBOGEN and OLSON (46) have obtained a purified myosin from dog heart, of which they measured the viscosity and sedimentation constant. The data have suggested that dog cardiac myosin may be of molecules much smaller than those of rabbit skeletal myosin, having a molecular weight of the order of 2×10^5. BLUM (47) has found that the combinding weight per Hg^{++} required to eliminate ATPase activity is of the order of 10^5. These results support also our deduction that, in myosin, the site active for deformation and that active for ATPase are identical and the constituent units of myosin are independent on each other.

Recently, GERGELY (48) showed by means of the ZIMM method that the weight-average molecular weights of his actomyosin preparations became smaller following the addition of ATP. However, whether this was due to the complete dissociation of actomyosin into myosin and actin, as he postulated, is not clear. If the dissociation is to take place, the average molecular weight of "actin" in the dissociated state must be calculated from his data and the known molecular weight of myosin. Thus, the present author has found that the molecular weight of the assumed actin thus calculated is almost equal to or sometimes even larger than that of actomyosin. Therefore, it may be concluded that ATP swells the actomyosin particle and under certain experimental conditions its disaggregation also occurs following the addition of ATP, but ATP does not dissociate actomyosin completely into myosin and actin. Though the results of the present experiments can not exclude the possibility of a small degree of disaggregation *), they support the conclusion of BLUM and MORALES that the molecular weight of

*) For example, when a rod-shaped particle 18,000 Å long is disaggregated into two rod-shaped particles 12,000 and 6,000 Å long, the intensity of the scattered light will be reduced to about 93% of the original value (cf. 49). So our data obtained from the present analysis, which is probably a first approximation for the actual molecular change, can not exclude the existance of such a disaggregation though to a small extent.

myosin B remains essentially unchanged following the addition of ATP.

Summary

In the present study, many experimental results obtained on the change in the light scattering and in the viscosity of myosin B solution in the presence of ATP on the one hand, and ATPase action of myosin B on the other hand were analyzed. It has been found that there exists an intimate relation between these two reactions. A consistent reaction formula for the interaction between myosin B and ATP, which is surmised to be a complicated cyclic process, was presented and the effects of divalent ions for each elementary reaction were described. Furthermore, the functional unit weights of myosin Bs from various muscles were determined and the relation between the light scattering drop and the amount of ATP added was analyzed quantitatively.

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